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Mapping quantitative trait loci for seminal root angle in a selected durum wheat population

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Abstract

Seminal root angle (SRA) is an important root architectural trait associated with drought adaptation in cereal crops. To date, all attempts to dissect the genetic architecture of SRA in durum wheat (*Triticum durum* Desf.) have used large association panels or structured mapping populations. Identifying changes in allele frequency generated by selection provides an alternative genetic mapping approach that can increase the power and precision of QTL detection. This study aimed to map quantitative trait loci (QTL) for SRA by genotyping durum lines created through divergent selection using a combination of marker-assisted selection (MAS) for the major SRA QTL (*qSRA-6A*) and phenotypic selection for SRA over multiple generations. The created 11 lines ($BC_1F_{2:5}$) were genotyped with genome-wide single-nucleotide polymorphism (SNP) markers to map QTL by identifying markers that displayed segregation distortion significantly different from the Mendelian expectation. QTL regions were further assessed in an independent validation population to confirm their associations with SRA. The experiment revealed 14 genomic regions under selection, 12 of which have not previously been reported for SRA. Five regions, including *qSRA-6A*, were confirmed in the validation population. The genomic

Abbreviations: GWAS, genome-wide association study; ICARDA, International Centre for Agricultural Research in the Dry Areas; KASP, kompetitive allele-specific PCR; MAS, marker-assisted selection; NAM, nested association mapping; QTL, quantitative trait locus; RIL, recombinant inbred line; RSA, root system architecture; SNP, single-nucleotide polymorphism; SRA, seminal root angle.

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regions identified in this study indicate that the genetic control of SRA is more complex than previously anticipated. Our study demonstrates that selection mapping is a powerful approach to complement genome-wide association studies for QTL detection. Moreover, the verification of *qSRA-6A* in an elite genetic background highlights the potential for MAS, although it is necessary to combine additional QTL to develop new cultivars with extreme SRA phenotypes.

Plain Language Summary

Seminal root angle (SRA), expressed at the seedling stage, is a key component of root system architecture that influences the root's capacity to explore the soil volume. While SRA has been recognized as an important drought-adaptive trait, its genetic makeup is less understood, which limits its value in breeding programs. Here, a total of 14 genomic regions were identified in durum wheat lines that were created in an elite genetic background via backcrossing, with divergent selection for SRA based on phenotype and haplotypes for the major SRA quantitative trait loci (QTL), *qSRA-6A*. Twelve of the regions were novel, some of which overlapped with previously reported QTL for other root traits. The identified regions may be crucial for root development. Further study of them could assist breeders to improve the root system of durum wheat.

1 | INTRODUCTION

A major contributing factor to the current yield gap for key cereal crops such as rice, wheat, and maize is scarcity and variability in water and/or nutrient resources (Mueller et al., 2012). Thus, the development of crop cultivars with enhanced resource-use efficiency is likely to lead to improved agricultural productivity and sustainability. Despite the recognition that root system architecture (RSA) is an important target for crop breeding to help close the yield gap and meet the future demand for food (Langridge et al., 2022; Maqbool et al., 2022; Ober et al., 2021), the ability to apply direct selection for RSA is challenging. For instance, phenotyping RSA in the field involves excavating root systems (Trachsel et al., 2011) or sampling of mature root systems by soil coring (Rezzouk et al., 2023; Wasson et al., 2014).

RSA is a complex and integral phenotype made up of multiple component traits. An alternative approach to direct selection is to instead target selection for components of RSA, which are correlated with field performance and can be measured at early growth stages using high-throughput methods under controlled conditions. A good example is narrow seminal root angle (SRA), which is considered a proxy for deep rooting that could improve water and nitrogen uptake from deep soil layers (Lynch, 2013; Manschadi et al., 2008; Singh et al., 2012; Wasson et al., 2012).

Since the objective of targeting component traits is to select optimal RSA for improving grain yield, it is important to understand the genetic control of the component root traits, so that information about potential linkage or pleiotropy with other traits can be considered. The genetic dissection of quantitative traits is commonly achieved by detecting quantitative trait locus (QTL) using various genetic mapping methods such as linkage mapping or genome-wide association study (GWAS). Linkage mapping is typically carried out using biparental populations. It offers high power to detect QTL, but relatively low precision due to limited recombination events. In contrast, GWAS can often provide higher mapping resolution due to historical linkage disequilibrium, but its power to detect QTL is comparatively lower. Both methods rely on phenotyping and genotyping large numbers of individuals, which is not always feasible.

Studying a small to medium-sized population may fail to detect QTL, particularly those with small effect. One avenue to improve such power is to develop a segregating population subjected to multiple cycles of recurrent phenotypic selection (Coque & Gallais, 2006). If selection is effective, the marker alleles associated with the target trait will change in the direction of the phenotype. Hence, studying the differences in allele frequencies in highly selected or divergent subpopulations may identify markers linked to QTL. This approach is known as selection mapping (Wisser et al., 2008) and offers several advantages for identifying QTL. For example, multiple segregating generations can increase mapping precision via more recombination. Moreover, by studying divergent subpopulations, there is the potential to identify individuals

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with minimal linkage drag around the loci of interest, thereby increasing precision and reducing the impacts of linkage with other traits. These make selection mapping an efficient and effective alternative method for QTL mapping.

The allele frequencies of markers linked to a QTL in the selected genotypes will differ from those in the unselected base population. For a line-crossing type of population, the allele frequencies in an unselected population are theoretically known, which follow the Mendelian segregation ratio. Therefore, detecting markers that display segregation distortion in divergent lines can provide information on the loci under selection, which are presumably linked to QTL for the target trait (Cui et al., 2015). In this scenario, marker alleles showing a change in frequency or segregation distortion can be identified as those that deviate from an expected frequency in a random unselected population. Depending on the crossing strategy that is employed, this method may identify materials that are highly valuable for evaluating a trait in elite genetic backgrounds, such as recombinant inbred lines (RILs) or near-isogenic lines.

To date, there have been few studies on genetic mapping for RSA in durum wheat. In a previous GWAS, we identified a major QTL for SRA, *qSRA-6A* on chromosome 6A (Alahmad et al., 2019), using a subset of a durum nested association mapping (NAM) population (Alahmad et al., 2022). Through haplotype analysis, it was observed that the variation in SRA between the two major contrasting haplotypes for *qSRA-6A* was only 7.7°, while the SRA variation within this subset was ∼53°. This suggests that SRA is influenced by multiple loci with relatively small effects, which were not detected by our GWAS due to limited power. This indicated that additional investigation into the genetic control of SRA in durum wheat is required. The objectives of this study were therefore to (1) identify additional genomic regions that are important for modulating SRA and (2) assess the expression of *qSRA-6A* in a new durum genetic background.

Core Ideas

- Selection mapping identified 14 genomic regions for seminal root angle in durum wheat, of which 12 were novel.
- A major quantitative trait locus, *qSRA-6A*, previously detected via genome-wide association study, was verified.
- Stacking multiple QTL is necessary to create extreme seminal root angle phenotypes.
- The elite durum lines developed in this study provide valuable genetic resources for studying root genetics.

2 | MATERIALS AND METHODS

2.1 | Donor line for the narrow haplotype of *qSRA-6A*

Alahmad et al. (2022) previously developed a durum NAM population consisting of 920 RILs derived from 10 families by crossing eight elite lines from the International Centre for Agricultural Research in the Dry Areas (ICARDA) as "founders" (i.e., Fastoz2, Fastoz3, Fastoz6, Fastoz7, Fastoz8, Fastoz10, Outrob4, and Fadda98) with two Australian durum wheat cultivars Jandaroi and DBA Aurora as "reference" cultivars.

The GWAS study on SRA that used a subset of the NAM population ($n = 393$ RILs from 10 families) detected only a single QTL designated as *qSRA-6A* (Alahmad et al., 2019). Seven SNP markers were significantly associated with *qSRA-6A*, all of which showed high levels of pair-wise linkage disequilibrium ($r^2 > 0.6$). Haplotype analysis identified two major contrasting haplotypes for *qSRA-6A* (i.e., hap1 = narrow, hap2 = wide). RIL 6_21 derived from the cross between Jandaroi and Fastoz8 (NAM family 6), carrying hap1 and displaying a narrow SRA of 51°, was selected as the donor parent for introgressing the narrow haplotype of *qSRA-6A* into DBA Aurora (hap 2 with a wide SRA of 81°).

2.2 | Development of intro-selection lines with narrow and wide SRA

To create durum wheat lines with divergent SRAs in a common genetic background, a backcrossing scheme was conducted in the speed breeding facility at the University of Queensland (Watson et al., 2018). A single-plant selection approach was adopted that combined nondestructive phenotypic screening for SRA with marker-assisted selection (MAS) using five kompetitive allele-specific PCR (KASP) markers (Rambla et al., 2022), similar to the approach employed to develop KASP markers for root traits in bread wheat (Makhoul et al., 2020). Results from initial screening of the newly developed KASP markers in a subset of durum NAM lines, revealed that all five KASP marker assays showed conclusive segregation of the lines based on allele type and SRA phenotype (Figure S1). Hence, the robust KASP marker derived from the SNP (1004240) at the QTL peak position (Figure 1), named "KASP-1004240" hereafter, was used for MAS in this study. The KASP primers and protocol are outlined in Figure S2; Tables S1–S3.

The resulting $BC_1F_{2:5}$ population was developed via the following steps (summary provided in Figure 2): (1) three crosses were made between 6_21 and DBA Aurora to generate F_1 seeds; (2) a backcross was made between F1 plants and the

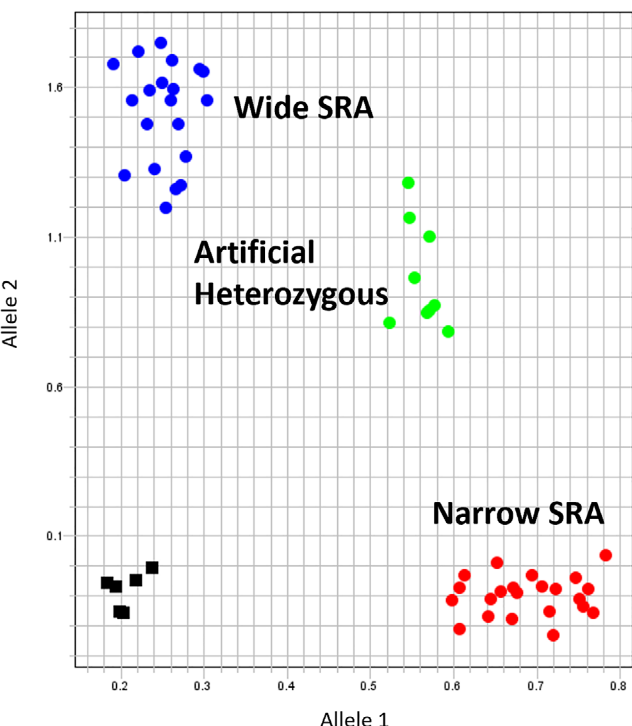

FIGURE 1 Allelic discrimination plot showing results from kompetitive allele-specific PCR (KASP) assay for the single-nucleotide polymorphism (SNP) (1004240) associated with *qSRA-6A* in a subset of durum nested association mapping (NAM) lines contrasting for seminal root angle (SRA) (44 lines total: 22 wide and 22 narrow). Blue dots indicate homozygous allele group corresponding with hap1, red dots indicate homozygous allele group corresponding with hap2; green dots indicate artificial heterozygous alleles; and black dots indicate undetermined (no call). Allele1 was reported by fluorescein amidite (FAM) fluorescence value; and Allele 2 was reported by hexachloro-fluorescein (HEX) fluorescence value.

recurrent parent DBA Aurora to generate BC_1F_1 seeds; (3) the BC_1F_1 plants were selfed to generate BC_1F_2 seeds; (4) the BC_1F_2 population was subject to glasshouse screening for SRA to select individual plants showing extreme SRA phenotypes (both wide and narrow); (5) the selected BC_1F_2 plants were selfed to generate $BC_1F_{2:3}$ seeds, which were planted for further screening for SRA, and $BC_1F_{2:3}$ individuals were selected based on the extreme SRA phenotypes; and (6) the selected $BC_1F_{2:3}$ plants were screened using KASP-1004240, and were selfed to generate $BC_1F_{2:4}$ lines, which were sown into the field in 2020 where the lines were evaluated for key agronomic traits, including flowering time and plant height. A total of 60 $BC_1F_{2:4}$ lines that displayed flowering time and plant height similar to DBA Aurora were selected, and genotyped with KASP-1004240 to verify the QTL status; (7) the 60 $BC_1F_{2:5}$ lines were then characterized for SRA under controlled conditions (described below), and based on the phenotype and KASP genotyping output, a subset of 11 lines were selected showing extreme and contrasting SRAs, as well as contrasting alleles of KASP-1004240. Overall, the final set of 11 $BC_1F_{2:5}$ lines was developed using a divergent selection approach, combining MAS and phenotypic selection. As they differ from the classical definition of near-isogenic lines, the lines are referred to as "intro-selection" lines.

In 2020, a total of 240 $BC_1F_{2:4}$ lines were planted at the Hermitage Research Facility, Warwick, QLD (28.12° S, 152.06° E). Selfed seed produced by the selected single plants was sown into 6 m rows. Weeds and diseases were controlled as required to ensure that high-quality seed was obtained. The lines were subject to evaluation for key phenology traits, including flowering time and plant height in the field. The preferred lines were then selected using phenology trait notes and genotyping results using KASP-1004240 from the previous generation ($BC_1F_{2:3}$). This enables root trait variation to be assessed without the potential confounding effects of phenology and facilitated development of lines with desirable alleles of *qSRA-6A*. A subset of 60 homozygous $BC_1F_{2:4}$ lines was then selected (26 and 34 lines carrying alleles GG and AA, respectively), and sampled at flowering time for KASP-1004240 screening to validate the presence/absence of the alleles. Each row was hand-harvested at maturity to provide $BC_1F_{2:5}$ seed.

Considering the original donor line was relatively elite (i.e., a RIL derived from a cross between an elite ICARDA breeding line × Australian commercial cultivar), fewer backcrosses, combined with selection for appropriate phenology, were required to recover agronomically sound lines with a high proportion of elite genetic background.

2.3 | Characterizing the intro-selection lines for SRA

The panel of 60 $BC_1F_{2:5}$ intro-selection lines and two parental lines (i.e., donor line 6_21 and recurrent parent DBA Aurora) were characterized for SRA in a "clear pot" experiment, as described by C. A. Richard et al. (2015), which was conducted in March 2021 in a temperature-controlled glasshouse (17 ± 2°C) under diurnal natural light conditions at The University of Queensland, Australia (27.50° S, 153.01° E). Clear (transparent) pots (ANOVApot, 4 L, 200-mm diameter, 190 mm height) were filled with UQ23 potting mix (70% composted pine bark 0–5 mm, 30% cocoa peat, mineral fertilizer). All pots were thoroughly watered before sowing, and no additional water was supplied afterwards. The experiment adopted a randomized complete block design, with 10 replicates per genotype. To avoid confounding genetic effects, uniformly sized seeds were selected at sowing that were closest to the

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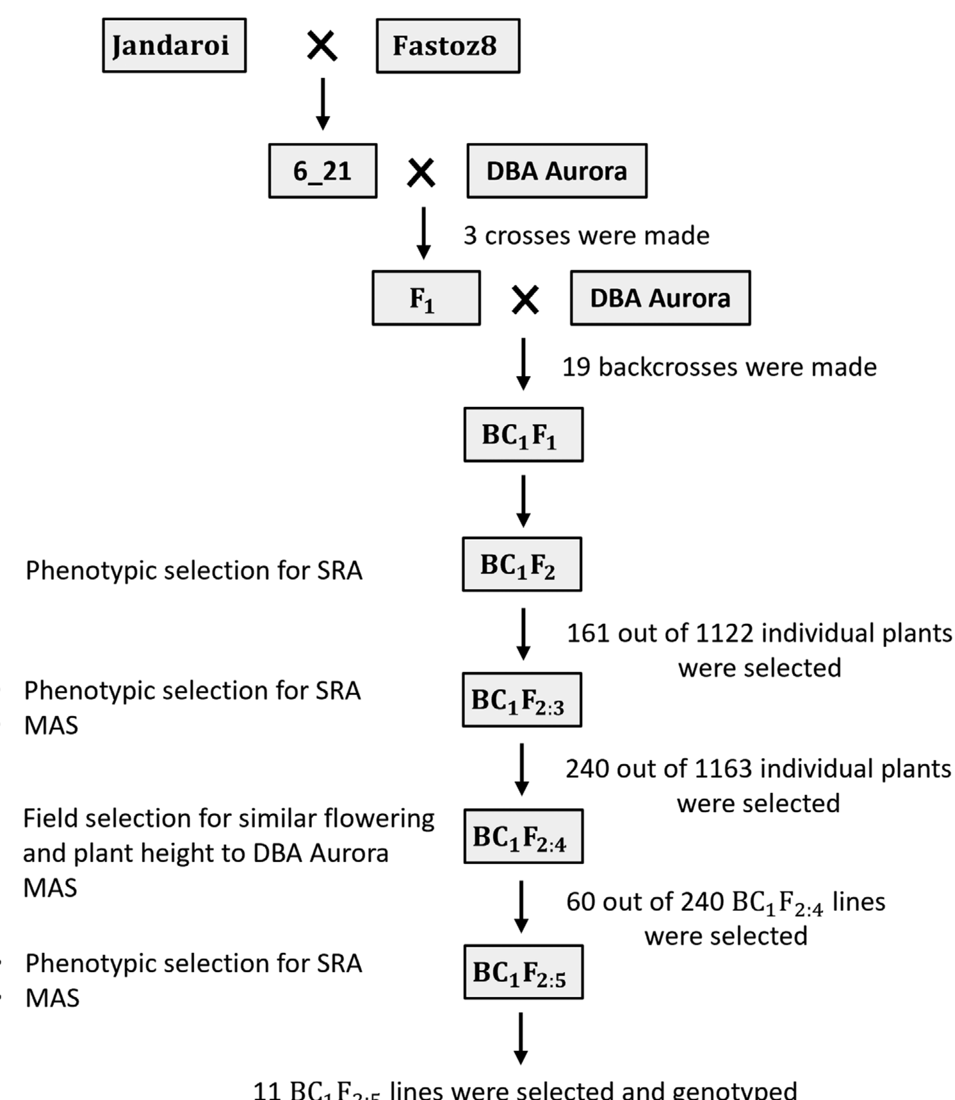

FIGURE 2 Development of elite durum intro-selection lines in the DBA Aurora genetic background using a divergent selection strategy for seminal root angle (SRA) incorporating both marker-assisted selection for *qSRA-6A* and phenotypic screening. The mean (standard deviation) days to flowering for 60 $BC_1F_{2:4}$ lines and 11 $BC_1F_{2:5}$ lines was 80 (2) days and 80 (2) days, respectively. Similarly, the mean (standard deviation) plant height for these lines was 74.6 (3.9) and 74.7 (3.9) cm, respectively.

median size for each genotype. Seeds were sown at a depth of 2 cm, with embryo facing downwards and towards the wall of the pot. After sowing, the clear pots were placed inside black pots to avoid light, thereby protecting the development of roots. Root images were taken at 5 days after sowing using a high-quality smartphone camera (Apple iPhone11). The SRA, which is the angle between the first pair of seminal roots, was measured from the images using ImageJ software (Schneider et al., 2012). Spatial analysis was conducted for SRA to correct for spatial heterogeneity across the experiment. A restricted maximum likelihood-based linear mixed model was fitted in ASReml-R to estimate adjusted genotype means (best linear unbiased estimates) for SRA (Butler et al., 2009). In the model, genotype was fitted as a fixed effect, and replicate and pot were fitted as random effects. For the residual effect, individual measurements were assumed to be independent and have a common error variance structure.

2.4 | Genotyping the intro-selection lines with whole-genome SNP markers

Leaf tissue was sampled at flowering time from each intro-selection line and recurrent parent. Genomic DNA was extracted from leaf tissue following the protocol provided by Diversity Arrays Technology Pty Ltd. Genotyping identified 2,603 DArTseq single-nucleotide polymorphism (SNP) markers with mapped chromosome positions, aligned to the durum

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TABLE 1 Descriptive statistics for seminal root angle in the 60 $BC_1F_{2:5}$ lines, including number of samples (N), mean, standard deviation (SD), median, minimum (Min) and maximum (Max).

KASP-1004240	N	Mean (°)	SD (°)	Median (°)	Min (°)	Max (°)
AA	34	83.6	8	85.3	57.5	92.7
GG	26	64.1	15.1	59.3	45.5	98.6
Total	60	75.2	15.1	81.8	45.5	98.6

Abbreviation: KASP, kompetitive allele-specific PCR.

wheat (cv. Svevo) reference genome, showing polymorphism between the donor parent 6_21 and the recurrent parent DBA Aurora, all of which were used in the subsequent genome analysis of intro-selection lines (Supporting Information S2).

2.5 | Mapping genomic regions under selection in the intro-selection lines

Without selection, each marker in the intro-selection lines has an expected 3:1 segregation of the DBA Aurora alleles and 6_21 alleles, respectively. The selection for "narrow" and "wide" SRA phenotypes during line development was expected to change the ratio of marker alleles linked to genomic regions containing QTL for SRA. Thus, χ^2 tests on the observed allele frequencies in the selected intro-selection lines against the expected allele frequencies provide valid statistical tests to identify putative QTL for SRA. For each marker, χ^2 test accounting for missing data was performed to detect the segregation distortion with three degrees of freedom.

To facilitate the classification of genomic regions under selection, the centiMorgans (cM) marker locations were predicted based on the genetic map published by Maccaferri et al. (2019). Putative regions under selection were defined as having at least three adjacent markers spanning ≤5 cM showing (1) significant segregation distortion ($p < 0.05$; critical χ^2 value of 7.815) and (2) a similar pattern that contrasts between wide and narrow intro-selection lines. For each identified genomic region under selection, the parental allele most represented in the set of narrow intro-selection lines was considered the donor for narrow SRA, whereas the parental allele most represented in the wide tail population was considered the donor for wide SRA.

2.6 | Validation of putative genomic regions associated with SRA identified by selection genotyping

Segregation distortion observed in the 11 selected intro-selection lines may be due to either the presence of SRA QTL, random chance, or selection for other traits that occurred unknowingly during line development (e.g., fertility during self-fertilization or crossing). To validate the putative QTL detected by selective genotyping, the markers linked to each region were tested for significant associations with SRA in a unique validation population from NAM family 3 (DBA Aurora × Fastoz8; $n = 92$), of which the "founder" line (i.e., Fastoz8) passed the narrow alleles of *qSRA-6A* onto the donor parent 6_21 (Figure 2). The two homozygous allele groups at each marker locus were compared for SRA using a *t*-test. Since the number of effective tests for such QTL validation was much smaller compared to the original GWAS, we applied a less stringent *p*-value threshold of 0.05 to declare significant markers.

2.7 | Comparison with previously reported QTL

To compare our results with previously reported QTL associated with root traits in durum wheat, we first uploaded the regions under selection to the Svevo durum reference genome browser in GrainGenes (Blake et al., 2019; Maccaferri et al., 2019). Notably, the genome browser has not yet integrated the QTL recently identified. Hence, QTL for root traits in durum identified through a search of the literature were also cross-referenced whenever possible by projecting the associated markers onto the reference genome (Alemu et al., 2021; Cane et al., 2014; Iannucci et al., 2017; Maccaferri et al., 2016). The relevant root QTLs were then compiled using information collected from both the browser and an updated search of previous studies. If the region under selection did not match any of the reported QTL for SRA, it was reported as a potentially novel QTL for SRA.

3 | RESULTS

3.1 | Phenotypic and genomic evaluation of intro-selection lines

The 60 $BC_1F_{2:5}$ lines grouped according to the screening results for KASP-1004240 (Supporting Information S3) showed a highly significant difference in mean SRA (almost 20°), where lines carrying the AA allele showed a mean SRA of 83.6° whereas lines carrying the GG allele showed a mean SRA of 64.1° (p = 7.7E-07) (Table 1). The bimodal

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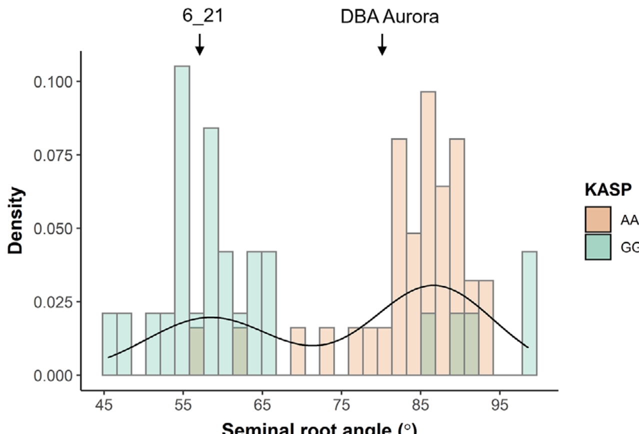

FIGURE 3 Distribution for seminal root angle in the 60 $BC_1F_{2:5}$ lines. Different colors represent the results from kompetitive allele-specific PCR-1004240 (KASP-1004240). Parental values are indicated by arrows.

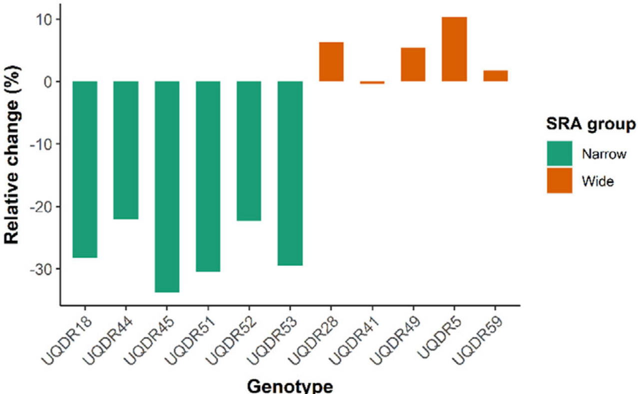

FIGURE 4 Relative change in seminal root angle (SRA) for the selected 11 intro-selection lines compared to the recurrent parent DBA Aurora.

distribution suggested a consistency of ∼88% between the homozygous KASP-1004240 allele grouping and the SRA phenotype (Figure 3). Notably, there were still a few lines ($n = 7$) that showed phenotypes that did not correspond with the KASP allele. Transgressive segregation was observed in both directions for SRA, indicating that both parents contributed alleles with both narrow and wide effects, thus making it possible to obtain intro-selection lines with a SRA narrower than parent 6_21 or wider than DBA Aurora.

The KASP genotyping results of the 11 selected intro-selection lines showed a perfect match with their SRA phenotypes (Table 2). The phenotypic evaluation of these lines showed that the introgression of narrow alleles of *qSRA-6A* reduced the SRA in narrow lines by 22%–34% in comparison to the recurrent parent DBA Aurora (SRA = 81.1°) (Figure 4). The SRA for wide lines ranged from 83.5° to 92.4°, representing an increase up to 10% in comparison to the recurrent parent.

Of the 2,603 SNP markers that were polymorphic between DBA Aurora and 6_21, between 2,151 and 2,517 SNPs had genotyping results across the 11 intro-selection lines (Table 2). These marker profiles were used to calculate the percentage of recurrent parent genome recovery (RPGR) for each line. With co-dominant markers such as SNPs, heterozygotes can be differentiated from homozygotes, which is vital in backcross breeding to accurately assess the recovery of the

TABLE 2 Phenotypic and genotypic information for the selected 11 intro-selection lines. The values of seminal root angle (SRA) are listed as best linear unbiased estimates. Results from kompetitive allele-specific PCR (KASP) assays were based on the KASP-1004240 associated with *qSRA-6A*. The number of polymorphic single-nucleotide polymorphisms (SNPs) without missing data was summarized using a set of 2 (603 polymorphic SNP markers between the recurrent parent DBA Aurora and donor line 6_21). For each line, the markers were categorized accordingly to whether the marker alleles were homozygous for the recurrent parent (R), homozygous for the donor (D), or heterozygous (H). The percentage of recurrent parent genome recovery (RPGR) was calculated as the ratio of the number of homozygous SNPs for the recurrent parent (R) to the number of polymorphic SNPs without missing data.

Genotype	SRA phenotype	SRA (°)	KASP allele	Polymorphic SNPs	R	H	D	RPGR (%)
UQDR18	Narrow	60.1	GG	2319	1752	106	461	77.8
UQDR44	Narrow	65.3	GG	2491	1883	215	393	79.9
UQDR45	Narrow	55.5	GG	2517	1822	227	468	76.9
UQDR51	Narrow	58.2	GG	2453	1874	125	454	78.9
UQDR52	Narrow	65.1	GG	2437	1909	85	443	80.1
UQDR53	Narrow	59.1	GG	2417	1784	115	518	76.2
UQDR28	Wide	89.1	AA	2435	1869	136	430	79.5
UQDR41	Wide	83.5	AA	2151	1503	137	511	73.1
UQDR49	Wide	88.3	AA	2489	1845	175	469	77.6
UQDR5	Wide	92.4	AA	2205	1561	87	557	72.8
UQDR59	Wide	85.3	AA	2430	1782	193	455	77.3

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recurrent parent genome in the progeny. Analysis revealed that the RPGR ranged from 72.8% to 80.1%, with the highest proportion recovered in UQDR52 and the lowest recovered in UQDR5. On average, the RPGR for the 11 lines was 77.3%. The greater RPGR in most lines ($n = 9$) than the expected mean of 75% for BC_1-derived lines was likely due to the selection for aboveground traits in the seed increase trial, which ensured high similarity to the recurrent parent. In contrast, two wide-angle intro-selection lines (UQDR41 and UQDR5) showed 73% RPGR, which was slightly lower than expected.

3.2 | Genomic regions under selection

A total of 2,603 polymorphic SNPs were distributed unevenly across 14 chromosomes of durum wheat, with the lowest number found on chromosome 4B ($n = 114$) and the highest number found on chromosome 7A ($n = 282$). Marker segregation distortion analysis was performed using these polymorphic markers to identify the genomic regions under selection. A total of 126 SNP markers showed segregation distortion according to χ^2 tests, which represented only 4.8% of the total number of polymorphic markers. Markers were grouped into 14 genomic regions across six chromosomes, including 2B, 3B, 4A, 6A, 7A, and 7B (Table 3). Notably, 5 of the 14 regions were located on chromosome 6A.

Of the five SNPs associated with *qSRA-6A* that were used to design KASP markers, four of them (i.e., 2256226, 1038214, 3023468, and 2258245) were found to be polymorphic between the parents (6_21 and DBA Aurora). Only SNPs 2258245 and 1004240 were located in a region under selection (interval between SNPs 1690605 and 1091873) as identified in the intro-selection lines (Figure 5). Based on the original QTL mapping and set of markers, the *qSRA-6A* region spanned 6.7 cM, as indicated by the interval between SNPs 2256226 and 2258245/1004240 (Figure 5). However, through analysis of intro-selection lines, the critical region was narrowed down to a smaller interval of 1.4 cM between SNPs 1690605 and 1385174 (*qSRA-6A-5*, Figure 5). Notably, in this region, narrow and wide intro-selection lines showed contrasting segregation patterns, where five out of six narrow lines (except for UQDR53) carried narrow SRA alleles introduced from the narrow parent 6_21. This indicated that backcrossing 6_21 to DBA Aurora successfully introgressed the narrow allele for *qSRA-6A* into the elite genetic background.

Additional genomic regions under selection in the intro-selection lines were identified. Interestingly, many putative QTLs ($n = 10$) for narrow SRA at regions under selection were contributed by the wide parent DBA Aurora, indicating the possibility to reduce SRA beyond the phenotype displayed by the narrow parent 6_21 (Table 3).

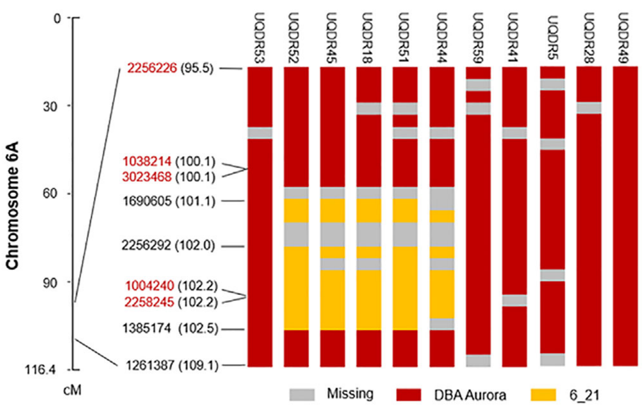

FIGURE 5 Chromosomal segments of the *qSRA-6A* region in the 11 selected intro-selection lines. The segments are colored according to the polymorphic single-nucleotide polymorphism (SNP) markers contributed by parental lines 6_21 and DBA Aurora. SNP names in red font are those linked to *qSRA-6A* as identified in the durum nested association mapping (NAM) population (Alahmad et al., 2019). The six lines on the left represent narrow intro-selection lines (i.e., UQDR53, UQDR52, UQDR45, UQDR18, UQDR51 and UQDR44), whereas the five lines on the right represent wide intro-selection lines (i.e., UQDR59, UQDR41, UQDR5, UQDR28 and UQDR49).

3.3 | Validation of five genomic regions showing consistent effects on SRA

To further explore the 14 genomic regions under selection in the intro-selection lines, association analysis was performed using a validation population (family 3 durum NAM, DBA Aurora × Fastoz8), which tested each SNP marker within each region under selection for association with SRA. The mean SRA for NAM lines grouped according to different homozygous alleles at each marker locus was statistically compared with *t*-tests. At the 5% significance level, significant marker-trait associations were detected at 5 of the 14 genomic regions, including *qSRA-2B-1*, *qSRA-4A-1*, *qSRA-6A-1*, *qSRA-6A-2*, and *qSRA-6A-5* (Table 3). This provided further evidence of the importance of additional genomic regions influencing SRA in durum wheat.

The number of significant SNP markers within each of the validated regions ranged from 1 to 13 (data not shown), and the tag SNP showing the strongest association was selected to test the effect of stacking multiple QTLs on SRA. A linear regression analysis was conducted between SRA and the number of narrow-angle alleles based on the tag SNPs. The total number of narrow-angle alleles in each NAM line from family 3 ranged from 0 to 5. Regression analysis revealed that for every additional narrow SRA allele, it decreased SRA by 3.4° on average ($p = 1.9\text{E-}06$) (Figure 6).

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TABLE 3 Summary of the 14 genomic regions under selection for seminal root angle (SRA) in the 11 intro-selection lines, and their associations with other traits and seminal SRA in family 3 of the durum nested association mapping (NAM) population.

Chr.[a]	Interval (cM)	Region[b]	No. of SNPs[c]	Narrow allele donor	Association with other traits[d]		SRA validation	
					DTF	PH	Tag SNP[e]	Difference in SRA (°)[f]
2B	151.5-156.3	***qSRA-2B-1***	6	DBA Aurora			3023243	10
	158.6-162.6	*qSRA-2B-2*	12	DBA Aurora				
	163.8-165.5	*qSRA-2B-3*	5	DBA Aurora				
3B	87.9-91.2	*qSRA-3B-1*	16	6_21				
4A	114.9-115.7	***qSRA-4A-1***	15	6_21	x	x	986964	7.4
6A	19.2-21.7	***qSRA-6A-1***	7	DBA Aurora			1012529	5.1
	24.5-25.1	***qSRA-6A-2***	3	DBA Aurora			4003894	5
	39.9-44.9	*qSRA-6A-3*	9	DBA Aurora				
	45-45.5	*qSRA-6A-4*	5	DBA Aurora				
	101.1-102.5	***qSRA-6A-5***	10	6_21		x	2322437	8.2
7A	64.6-69.2	*qSRA-7A-1*	3	DBA Aurora				
	70.3-72.2	*qSRA-7A-2*	8	DBA Aurora				
	76.1-80.2	*qSRA-7A-3*	21	DBA Aurora				
7B	12.7-17.6	*qSRA-7B-1*	6	6_21				

[a]Chr.: Chromosome.
[b]The names of genomic regions consisted of a “q” for quantitative trait locus, trait name SRA, followed by chromosome and serial number. For example, *qSRA-2B-2* corresponds to the second putative region for SRA on chromosome 2B. Regions in bold are the ones being confirmed in the validation population (family 3 durum NAM).
[c]No. of SNPs: Number of SNP markers contained in the genomic region under selection that showed not only significant segregation distortion but also similar segregation patterns between narrow- and wide-angle intro-selection lines.
[d]The two homozygous allele groups at each marker locus in the genomic region under selection were compared for days to flowering (DTF) and plant height (PH) using a *t*-test. The association between a genomic region under selection and a phenology trait was indicated by “x” when a marker showed a statistical significance (p-value < 0.05).
[e]Within each validated genomic region under selection, the tag SNP was defined based on its strongest association with SRA in the family 3 durum NAM.
[f]Difference in SRA between the two homozygous allele groups of the tag SNP.

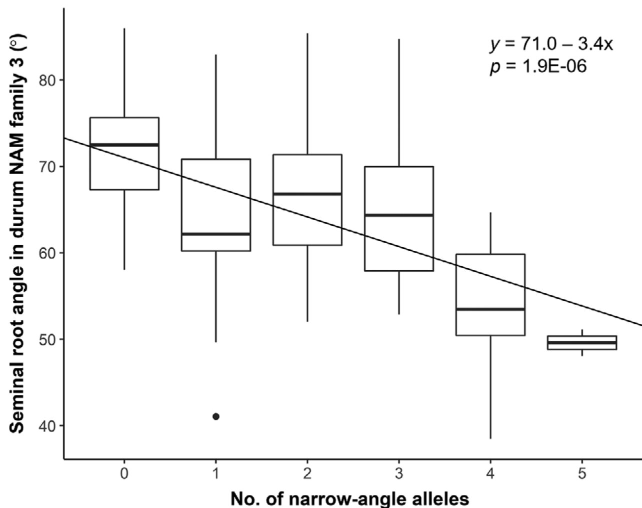

FIGURE 6 The linear relationship between the number of narrow-angle alleles of tag single-nucleotide polymorphisms (SNPs) and seminal root angle in the validation population (family 3 durum nested association mapping [NAM]). The number of narrow-angle alleles per genotype was determined using the most significant SNP marker (i.e., tag SNP) in the validated regions under selection (Table 3).

3.4 | Novel QTL for SRA on multiple chromosomes

Comparative analysis with previous mapping studies revealed that 6 of the 14 genomic regions under selection for SRA were found to be co-located with previously reported QTL for root traits (Table 4). Two regions *qSRA-2B-1* and *qSRA-6A-5* overlapped with QTL for root growth angle reported by Maccaferri et al. (2016). In the case of the *qSRA-6A-5* region, it was not only detected in different durum mapping populations for SRA but was also found to be associated with other root traits, such as root length, number, and surface area. While *qSRA-3B-1*, *qSRA-4A-1*, *qSRA-6A-3*, and *qSRA-6A-4* overlapped with QTL associated with root traits, previous studies have not reported their associations with SRA. Additionally, comparison revealed eight genomic regions that have not been previously associated with any root traits. Therefore, 12 genomic regions identified by selective genotyping were associated with the SRA trait for the first time, including the three validated genomic regions (*qSRA-4A-1*, *qSRA-6A-1*, and *qSRA-6A-2*).

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TABLE 4 Alignment of 14 genomic regions under selection for seminal root angle (SRA) with previously reported root trait quantitative trait locus (QTL) in the literature. Candidate genes contained in each genomic region are listed in Supporting Information S4.

Chr.	Region	Physical position (bp)[a]	Root trait	Root QTL	Reference
2B	*qSRA-2B-1*	772946978-778393823	Root growth angle	*QRga.MrxCl-2B*	(Maccaferri et al., 2016)
	qSRA-2B-2	781033713-787578626	–	–	
	qSRA-2B-3	788476525-789707949	–	–	
3B	*qSRA-3B-1*	586080335-631721288	Total root number	*QTrn.UniboDP-3B.2*	(Maccaferri et al., 2016)
4A	*qSRA-4A-1*	646833659-654581896	Primary root length	*QPrl.UniboDP-4A.3*	(Maccaferri et al., 2016)
6A	*qSRA-6A-1*	15625032-17584079	–	–	
	qSRA-6A-2	19364943-19647935	–	–	
	qSRA-6A-3	34114771-51884884	Presence of the sixth seminal root	*QRt6.MrxCl-6A*	(Maccaferri et al., 2016)
	qSRA-6A-4	52235454-61889379	Average root length	*QArl.UniboDP-6A.1*	(Maccaferri et al., 2016)
	qSRA-6A-5	596198717-599658969	Total root length, presence of the sixth seminal root average root length, root growth angle, total root number, root length, root surface area	*QTrl.MrxCl-6A, QArl.CoxLd-6A, QTrl.CoxLd-6A, QRga.CoxLd-6A, QRga.UniboDP-6A.2, QTrn.SMxMC-6A, QRl.SMxMC-6A, QTrs.SMxMC-6A, EPdwRGA-6A, QSRA4-6A*	(Alemu et al., 2021; Cane et al., 2014; Iannucci et al., 2017; Maccaferri et al., 2016)
7A	*qSRA-7A-1*	79495340-85270676	–	–	
	qSRA-7A-2	86939815-92348418	–	–	
	qSRA-7A-3	107612969-140055702	–	–	
7B	*qSRA-7B-1*	4865014-5924811	–	–	

[a]The base pair (bp) position for the genomic region determined by its start and end SNP positions on the Svevo durum reference genome.

4 | DISCUSSION

4.1 | Selective genotyping complements previous GWAS for SRA

SRA in durum wheat is a quantitative trait that is controlled by multiple loci. However, our previous GWAS on SRA in the durum NAM population only identified a single QTL *qSRA-6A* (*qSRA-6A-5* in the current study) (Alahmad et al., 2019). As presented in this study, our follow-up research to perform backcrossing and divergent selection for SRA revealed 13 additional putative QTLs, of which four (*qSRA-2B-1*, *qSRA-4A-1*, *qSRA-6A-1*, and *qSRA-6A-2*) were validated in an independent population and/or co-located with previously reported QTL for SRA. The nine other regions could still harbor important loci influencing SRA, but our ability to verify their effects may have been limited by the size of the validation population ($n = 92$), or alternatively, effects may be specific to the elite genetic background. The power of QTL detection is affected by multiple factors, including minor allele frequency. A low minor allele frequency can decrease the likelihood of detecting QTL. However, selection can cause allele frequencies to diverge in the high and low tails and therefore increases the minor allele frequency. Thus, cycles of divergent selection applied during development of the intro-selection lines likely improved our ability to detect critical regions through selective genotyping even though a small number of lines were genotyped.

Based on the tag SNP in each validated QTL, the difference in SRA between lines carrying the respective narrow and wide

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alleles ranged from 5 to 10° (Table 3). Yet, lines that combined narrow alleles at all five QTLs only showed an average reduction in SRA of 17°. This suggests there may be epistatic interaction among some QTLs, where specific combinations could provide larger effects, and if so, this could enable more targeted MAS of fewer QTLs to achieve moderate to large changes in SRA. Interestingly, both parental lines donated narrow-angle alleles at the 14 putative QTLs in intro-selection lines. This explains the transgressive segregation for SRA in the $BC_1F_{2:5}$ lines, where the narrow intro-selection lines combined narrow alleles from both parents. Similar results have been reported in bread wheat, where alleles for narrow SRA in backcross-derived progeny were also inherited from the parent displaying wide SRA (C. Richard et al., 2018). Surveying 14 putative QTLs for SRA across narrow intro-selection lines showed that most narrow-angle alleles (n = 10) were contributed from the wide parent DBA Aurora. The presence of narrow alleles in the wide parent likely indicates the dominance of wide SRA at these loci or epistasis among these loci. Taking these observations together, it appears that the SRA is controlled by major QTL in combination with other QTLs of minor or inhibitory effects.

4.2 | QTL for SRA in durum wheat

The consistent identification of *qSRA-6A* across different mapping populations (i.e., diverse panels, bi-parental populations, NAM) and studies conducted across countries (Alahmad et al., 2019; Alemu et al., 2021; Cane et al., 2014; Maccaferri et al., 2016) provides strong evidence this is a key locus contributing to RSA in durum wheat. This hotspot region, also known as *QRga.ubo-6A.2*, is considered a promising candidate for MAS and positional cloning (Maccaferri et al., 2016). Using an association mapping panel, QTLs for 1000-kernel weight and grain yield were also co-located with *QRga.ubo-6A.2* (Maccaferri et al., 2016). Alleles at this region showed effects on both yield traits under various water availability scenarios in the Mediterranean Basin (Maccaferri et al., 2011, 2016). Thus, narrow SRA may serve as a valuable drought-adaptive trait that could enhance grain yield in water-limited environments. To determine the value of *qSRA-6A* to enhance access to stored soil moisture under drought, it is critical to validate its effect on root distribution in the field.

The two validated genomic regions on chromosomes 2B (*qSRA-2B-1*) and 4A (*qSRA-4A-1*) showed associations with root traits reported by Maccaferri et al. (2016): *QRga.MrxCl-2B* and *QPrl.UniboDP-4A.3*. Interestingly, *QPrl.UniboDP-4A.3* was associated with primary root length, whereas *QRga.MrxCl-2B* was associated with root growth angle. Thus, we consider the 4A region detected in this study to be a novel QTL for SRA. By comparing results across studies, *QPrl.UniboDP-4A.3* could be associated with a deep rooting mechanism similar to *Dro1* in rice, which confers both narrower and deeper root growth (Uga et al., 2011, 2013), although this hypothesis needs to be tested. In contrast, *QRga.MrxCl-2B* appears to be consistently associated with root angle across both studies. The utility of these root QTLs for breeding should also be explored to determine the extent of pleiotropic effects on other traits (Salvi & Tuberosa, 2015). For example, QTLs for heading date have also been reported in the region of *QRga.MrxCl-2B* (Maccaferri et al., 2011). Similarly, the *QPrl.UniboDP-4A.3* region has been reported to influence days from booting to anthesis (Soriano et al., 2017) and a range of other traits including aboveground biomass (Mengistu et al., 2016), disease resistance (Aoun et al., 2016), grain protein (Peleg, Cakmak, et al., 2009), plant height (Soriano et al., 2017), yield component traits (Peleg, Fahima, et al., 2009; Peleg et al., 2011; Tzarfati et al., 2014), and, importantly, grain yield (Mengistu et al., 2016; Peleg, Fahima, et al., 2009). Our study also validated two genomic regions on chromosome 6A (*qSRA-6A-1* and *qSRA-6A-2*) that appeared to be novel QTL for SRA. Again, both regions overlapped with QTL for other traits, including yield component traits (Mangini et al., 2018; Patil et al., 2013), disease resistance (Liu et al., 2017; Marone et al., 2013), days from sowing to booting (Soriano et al., 2017), and/or quality traits (Giraldo et al., 2016).

The link between SRA QTL and phenology traits may be problematic. If this is due to pleiotropy, manipulating SRA could potentially shift flowering time. QTL expression for quantitative traits can be influenced by genetic background and the environment. In the validation population, only two (*qSRA-4A-1* and *qSRA-6A-5*) of the 14 identified genomic regions under selection showed significant associations with days to flowering and/or plant height (Table 3). Thus, taken together with the narrow range in flowering and plant height displayed by intro-selection lines, it suggests it is possible for durum breeders to deploy specific allelic combinations to achieve desirable SRA and aboveground agronomic traits.

4.3 | MAS for SRA: Opportunities and challenges

MAS can enable selection for traits that are challenging to phenotype, including root architectural traits. During backcrossing, molecular markers associated with the target QTL can also be used to track and select the desirable allele from the donor parent (Hospital, 2001). For quantitative traits, the effectiveness of MAS is often challenged by how to identify marker-QTL associations that are relevant and can be used in a particular genetic background context. The selection response of MAS is affected by many factors, in particular the complexity of genetic control of the trait, the number and

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position of markers (i.e., single or flanking), and the linkage disequilibrium between the marker and the QTL (Edwards & Page, 1994). MAS is generally viewed as the preferred approach when a small number of major QTLs (each with a large effect) underpin the trait (Davies et al., 2006; Edwards & Page, 1994). While *qSRA-6A* is considered a major QTL (Alahmad et al., 2019), it seems evident that additional QTLs are necessary to develop durum lines with extreme SRA phenotypes.

The KASP marker screening for KASP-1004240 across the 60 $BC_1F_{2:5}$ lines supported the differentiation of genotypes with wide and narrow SRA (88% accuracy). However, this result should be interpreted with caution. Phenotypic selection was made on the $BC_1F_{2:5}$ lines in order to match their genotypes and phenotypes. Therefore, the accuracy of KASP-1004240 could be partly attributed to the response from divergent selection for SRA, which may have shifted allele frequency at the QTL. The lack of perfect consistency between the KASP-1004240 results and SRA phenotypes points to either additional QTL modulating SRA or the possibility of recombination between the marker and *qSRA-6A*, which can result from the decay in linkage disequilibrium over generations (Edwards & Page, 1994). This may explain the absence of the narrow-angle allele for *qSRA-6A* in the narrow intro-selection line UQDR53. To decrease costs associated with molecular marker assays, in this study we only used a single KASP marker within the critical region of *qSRA-6A*. To identify additional recombination events and potentially improve the selection response, multiple KASP markers spanning the critical region from 101.1 to 102.5 cM (Figure 5) could be used to track and select the narrow or wide haplotype for *qSRA-6A*.

There have been few empirical studies that have compared the effectiveness of MAS with phenotypic selection for quantitative traits in plant breeding (Davies et al., 2006; Robbins & Staub, 2009; Zeng et al., 2023). The effectiveness of each selection method is very context-dependent, where each has its own case-specific advantages and disadvantages. For SRA in durum wheat, adopting both selection methods may be practical. On one hand, the QTLs identified in this study hold promise for the successful implementation of MAS. However, the moderate to high heritability (0.65–0.90) of SRA in durum wheat (Alemu et al., 2021; Cane et al., 2014; Maccaferri et al., 2016) and the ability to measure the trait at the seedling stage in a high-throughput manner supports the utility of phenotypic selection. In this study, integrating both approaches improved efficiency because each round of phenotypic selection reduced the number of plants or lines to be genotyped for MAS. Therefore, we suggest that combining phenotypic selection with MAS offers significant advantages over individual selection strategies for SRA. The efficiency of this integrated approach has been demonstrated in other studies, resulting in the successful development of lines with improved disease resistance and drought tolerance (Kamal et al., 2021; Zhang et al., 2022; Zhou et al., 2003).

5 | CONCLUSION

The intensive divergent selection achieved through a combination of MAS and phenotypic screening resulted in the development of elite intro-selection lines with divergent SRA. As a result, the intro-selection lines provided ideal genetic materials to identify QTL associated with SRA through selective genotyping. While 14 genomic regions showed segregation distortion and could be associated with SRA, the effects associated with five of the regions were confirmed through a validation population. Results confirmed the key role of *qSRA-6A*, which makes this a promising target for MAS. However, if the goal is to select and develop new cultivars with extreme SRA phenotypes, it will be necessary to stack a number of additional QTLs, such as those identified in this study. The elite intro-selection lines developed in this study provide valuable genetic resources for future research to explore the relationship between SRA, mature RSA, and durum yield in a range of production environments.

AUTHOR CONTRIBUTIONS

Yichen Kang: Conceptualization; data curation; formal analysis; methodology; visualization; writing—original draft. **Samir Alahmad**: Resources; writing—review and editing. **Shanice V. Haeften**: Writing—review and editing. **Oluwaseun Akinlade**: Writing—review and editing. **Jingyang Tong**: Writing—review and editing. **Eric Dinglasan**: Writing—review and editing. **Kai P. Voss-Fels**: Writing—review and editing. **Andries B. Potgieter**: Writing—review and editing. **Andrew K. Borrell**: Writing—review and editing. **Manar Makhoul**: Methodology. **Christian Obermeier**: Methodology. **Rod Snowdon**: Writing—review and editing. **Emma Mace**: Methodology; writing—review and editing. **David R. Jordan**: Methodology; writing—review and editing. **Lee T. Hickey**: Conceptualization; funding acquisition; project administration; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data supporting the findings of this study are available within the paper and within its supplemental materials published online.

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