



Linkage mapping and quantitative trait loci detection for seedling vigor and grain size in advanced backcross introgression lines from wild accessions of *Oryza nivara*

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Abstract Detection of beneficial genes from wild accessions of cultivated crops is becoming increasingly important for crop improvement, especially for traits lost during domestication. A set of wild introgression lines derived from advanced back cross breeding of Swarna with *Oryza nivara* species were characterized for grain size and seedling vigor related traits. Grain length in the population varied between 7.23 and 8.22 mm, while width varied from 2.66 to 3.13 mm. The length-to-width ratio (LWR) ranged from 2.41 to 3.06 mm, showing various grain shapes in the population. Simultaneously, seedling vigor-related traits were studied, and a significant correlation was observed between seedling vigor traits and grain size parameters. Further mapping of quantitative trait loci (QTL) using simple sequence repeat (SSR) and single nucleotide polymorphism

(SNP) genotyping identified 18 QTL for seedling vigor- and grain size-related traits. Among these QTL, three major QTL (*qAS7.1*, *qPL7.1* and *qL7.1*) were detected for grain size-related traits on chromosome 7, explaining 21.44%, 19.11% and 22.60% of phenotypic variation, respectively. Similarly, for seedling vigor traits, seven major QTL, viz., *qSDW3.1*, *qTDW3.1*, *qSVI2-3.1*, *qSL3.1*, *qTL3.1*, *qSVI1-6.1*, and *qRDW8.1*, were detected, explaining 18.3–26.38% of phenotypic variation. These QTL were on chromosomes 6 and 7 for seedling vigor and grain size traits and were collocated with common flanking markers. Hence, the results are useful for marker assisted breeding programmes for introgression and simultaneous improvement of seedling vigor and grain size, and further fine mapping of the related genes.

Keywords Seedling vigor · Grain size · Wild introgression lines · SNP markers · QTL mapping

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Introduction

Rice is a widely consumed cereal crop, cultivated in 145 million hectares of area across different ecosystems in the world and feeds more than half of the world's population. With the advent of changing climatic conditions, unpredictable average temperature and rainfall patterns affected the global food production adversely (Wing et al. 2021; Abbas et al. 2022; Chandio et al. 2022). Along with these, diminishing

water resources and increased labor cost is leading to a major shift in rice cultivation from puddled transplanted conditions to direct seeded rice cultivation (Mahender et al. 2015). Compared to conventional transplanted cultivation methods, direct seeded rice has the advantage of reduced labor, inputs and cost. However, mostly the cultivation system is affected with non-uniform seed germination, weed damage, poor establishment and growth, resulting in low productivity (Wu et al. 2006). In this scenario, seedling vigor is an important agronomic trait for consideration (Kaldate et al. 2023) as it has a direct impact on rapid uniform growth and establishment of seedlings especially in water limited conditions. In addition, vigorous plants have the advantage under adverse conditions due to their smothering effect due to better vegetative growth and weed competitiveness. Along with early establishment and survival at vegetative stage, special focus is also need to be given to the yield contributing traits under the changing cultivation practices. Rice yield is primarily determined by grain characteristics, especially grain size and weight. Grain parameters are of much importance as they not only govern yield variation but also determine quality, consumer preference and market value. The grain size preference varies across the rice-consuming regions of the globe. Knowledge on the genetic basis for grain size variation can help breeders develop high-yielding rice cultivars (Duan et al. 2017). High-throughput phenotyping is needed for accurate quantitative evaluation of the variation in rice grain shape and size. Both these component traits significantly contribute towards better productivity both in direct seeded as well as irrigated transplanted conditions. Grain size is also an economically important critical trait that determines high yield as well as desirable quality meeting farmer's requirements and consumer's preferences respectively. Grain yield and seedling vigour are complex quantitative traits with several contributing and correlated traits and highly influenced by environment (Balakrishnan et al. 2016). Grain size is more important in breeding point of view as it is a highly stable and heritable common component trait in both yield and seedling vigour improvement.

There are several reports on various field and vegetable crops about the influence of grain size to seedling vigour contributing for better crop establishment which ultimately result in high yield (Ellis 1992; Elliott et al. 2008; Zaidman et al. 2010; Magagula and

Ossom 2011; Jung et al. 2012; Adebisi et al. 2013; Ankaiah et al. 2013; Amin and Brinis 2013; Tabakovic et al. 2020; Rathinavel et al. 2021; Andrade et al. 2023; Kristo et al. 2023). Previous research stated a significant increase in seed size associated with high germination percentage and seedling vigor in rice (Pandey et al. 1994; Roy et al. 1996; Ambika et al. 2014; Kaldate et al. 2023). Jung et al. (2012) reported that seed size influenced mean emergence time and maximum emergence rate index and percentage of emergence of seedlings. However genetic regulation and relation between seed parameters and seedling vigor for crop establishment and yield improvement is not well studied. Kaldate et al. (2023) studied seed traits and seedling parameters in 28 accessions of wild and cultivated species and a substantial relationship between seed size with seedling vigour and grain yield was observed indicating selection pressure during evolution for suitable seed size for various agronomic conditions.

Further exploration and identification of novel genetic variation for seed and seedling traits and incorporation into cultivated germplasm is essential to meet the increasing demands for rice production and for its sustainability in changing cultivation practices. The existence of high genetic diversity in rice has contributed to a major advance in rice production and productivity in recent decades (Nachimuthu et al. 2015). Wild accessions are the potential genetic pool of various traits for genetic enhancement and an excellent source for novel alleles. Wild introgression lines are important genetic stocks for specific trait improvement and have been utilized in the past for biotic and abiotic stress tolerance/resistance in rice (Zhang et al. 2006; Rahman et al. 2007; Hu et al. 2015; Ma et al. 2016; Hamaoka et al. 2017; Xing et al. 2021; Manohara et al. 2021; Balakrishnan et al. 2022). The wild and weedy related species tend to propagate naturally thus providing a diverse adaptation to adverse conditions and weed competition. However, they are not much well characterized or explored in breeding programs. Although wild species are known for better seedling vigor and growth, only a few investigations have been conducted for the evaluation of wild species and wild introgression lines for seedling vigor traits (Addanki et al. 2019; Beerelli et al. 2019; Eizenga et al. 2016; Najeeb et al. 2020; Kaldate et al. 2023). Previously QTL research have been carried out for seedling vigor and

associated traits such as germination percentage and germination index in rice (Fujino et al. 2004, 2008; Liu et al. 2014; Jiang et al. 2017; Zeng et al. 2021). However, the effects of major quantitative trait loci (QTL) were not studied, evaluated or introgressed in breeding programmes.

The major objective of this study is to identify the variability and novel genetic loci associated for the traits seedling vigour and grain size in wild introgression lines. These are the major component traits which contribute to crop establishment and high yield both in direct seeded as well as irrigated transplanted conditions. The advanced backcross introgression lines derived from the cross of Swarna, a popular *Oryza sativa* cultivar with a wild progenitor *O. nivara* accession IRGC81832, were evaluated and further, an attempt was made to find the QTL regions associated with these traits.

Materials and methods

Experimental materials used in the study

A set of 90 advanced backcross introgression lines (ABLs) derived from a cross between the widely cultivated *indica* rice cultivar Swarna (*Oryza sativa*) and the wild accession *O. nivara* (IRGC81832) (Kaladhar et al. 2008; Balakrishnan et al. 2020) were used to study seedling vigor and grain size-related traits at the BC₂F₁₀ stage. The selected ABLs were maintained by single panicle selection and named NPK (1–90) lines. The seedling vigor research were carried out at the Indian Institute of Rice Research (IIRR), and Hyderabad and grain size-related investigations were carried out at the Japan International Centre for Agricultural Sciences (JIRCAS), Japan.

Phenotyping for seedling vigor traits

Freshly harvested seeds of NPK lines were used for the study of seedling vigor-related traits such as germination (%), shoot length (cm), root length (cm), plant length (cm), shoot fresh weight (g), root fresh weight (g), plant fresh weight (g), shoot dry weight (g), root dry weight (g), plant dry weight (g), seedling vigor index 1 and seedling vigor index 2. Fifty completely matured seeds from each line and parents were used for the seedling vigor trait study as described in

Addanki et al. (2019). The seedling vigor indexes 1 and 2 were calculated by adopting the formula suggested by Kharb et al. (1994) using parameters such as germination %, seedling length and seedling dry weight.

The seedling vigor index-1 (SVI1) was estimated using seedling growth parameters at 7 days and 14 days after sowing and germination percentage using the formula:

Seedling Vigor Index-1

$$= \frac{\text{Seedling length} \times \text{Germination percentage}}{100}$$

Seedling Vigor Index-2 (SVI2) was assessed using seedling dry weights at 14 days after sowing by the formula

Seedling Vigor Index-2

$$= \frac{\text{Seedling dry weight} \times \text{Germination percentage}}{100}$$

Phenotyping for grain size-related traits

Smart Grain software developed by Tanabata et al. (2012) was used for high-throughput measurement of grain shape, grain length, width, area, and perimeter length using image analysis methods at TARF, JIRCAS, Japan. It measures length (L), width (W), seed area (AS), perimeter length (PL), circularity (CS), length-to-width ratio (LWR), intersection of length and width (IS), center of gravity (CG), and distance between IS and CG (DS). One hundred mature seeds from each line and parents were used for determining grain size-related traits such as grain length (L) (mm), grain width (W) (mm), seed area (AS), perimeter length (PL), circularity (CS), length-to-width ratio (LWR), intersection of length and width (IS), center of gravity (CG), and distance between IS and CG (DS).

Phenotyping for root-related traits

Phenotyping for root traits carried out at ICAR-IIRR and the lines were directly sown in black polythene covers of 80 cm length containing 15 kg soil in two replications (5 seeds per bag). To maintain the aerobic condition, need-based irrigation was given at the rate of measured volume of 150 ml water. The soil

macro, micronutrients, pH were maintained throughout the experiment, and recommended dose of fertilizer was provided. A single and healthy seedling was maintained till panicle initiation stage based on germination and vigour and extra seedlings were thinned out to maintain the single plant per replication. Based on the panicle initiation stage/booting stage of each line, the whole plant in each polythene bag was carefully removed by cutting the polythene cover without damaging the roots. The roots were washed carefully using a high-pressure water pump (Barbadikar et al. 2016), and the same samples were phenotyped for traits like shoot length (SL), root length (RL), tiller number (TN), shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW) and root dry weight (RDW). The root average diameter (RAD), root length per volume (RLPV) and root volume (RV) were analysed and recorded in WinRHIZO Pro software (ver. 4) (Arsenault et al. 1995).

Genotyping of ABLs for grain size and seedling vigor traits using SSR and SNP markers

The genomic DNA from fresh healthy leaves was extracted using the CTAB method (Doyle and Doyle 1987), and the DNA quality and quantity were estimated at 260/280 nm and 260/230 nm using a UV spectrophotometer. Genotyping data using 1394 single nucleotide polymorphisms (SNPs) from 1 K RiCA-based SNPs (Arbelaz et al. 2019) and core set of simple sequence repeats (SSRs) (Orjuela et al. 2010) were used for mapping the ABLs. Polymerase chain reaction (PCR) was performed with a final volume of 10 µl per reaction, including template DNA (15 ng), 1×PCR assay buffer, 1.5 mM MgCl₂, 200 µl of dNTPs, 10 pmol of primer (F/R), Taq polymerase (1 U) and nuclease-free water. The PCR cycle followed the initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final extension of 72 °C for 10 min. Amplified PCR products were resolved in 3% agarose gel with 0.5×TAE buffer, and gel electrophoresis was carried out at 120 V for 2 h. The PCR fragments were visualized by staining the gel with ethidium bromide (10 mg/ml) and illuminated under UV light in a gel documentation unit. Additionally, genotyping data using 1 K RiCA (*1 K- Rice Custom Amplicon*) based SNP markers were also included (Arbelaz

et al. 2019) for QTL mapping. It is a sequence-based amplicon panel consisting of ~1000 SNP distributed uniformly across the genome of *indica* sub population for genomic prediction.

Statistical analysis of morphological traits

The data obtained from seedling vigor and grain size traits were used for statistical analysis. Frequency analysis was performed, and a histogram was made using RStudio (R core team 2018). Variability and correlation research were performed in RStudio, and a correlogram was constructed using the “*metan*” package (Olivoto and Col Lucio 2019).

QTL mapping for grain size and seedling vigor traits

Molecular screening and QTL mapping were performed on the 90 ABLs from Swarna/*O. nivara* using 718 polymorphic SSR and SNP markers together, covering all 12 chromosomes, and were utilized to generate a molecular linkage map. To determine the association between specific marker loci and phenotypic data, QTL analysis was conducted using single marker analysis (SMA), inclusive composite interval mapping (ICIM-ADD) of additive QTL and inclusive composite interval mapping of epistatic QTL (ICIM-EPI) functions by QTL IciMapping v4.2 (www.isbreeding.net) in a stepwise regression model. The minimum LOD value expected to identify a significant QTL using 1000 permutation runs. Phenotypic variation explained by the QTL value was computed as the fraction of total variation described by each QTL (Maniruzzaman et al. 2022).

Results

Variation in key phenotypic traits related to seedling vigor and grain size

Significant phenotypic variation was observed for seedling vigor- and grain size-related traits (Fig. 1). Grain size traits such as grain length ranged between 7.23 mm (NPK24, NPK37) and 8.22 mm (NPK 81), grain width was highest at 3.13 mm (NPK73, NPK1) and lowest at 2.665 mm (Swarna), and the length–width ratio ranged between 2.41 mm (NPK47) and 3.065 mm (Swarna). Based on the grain length,

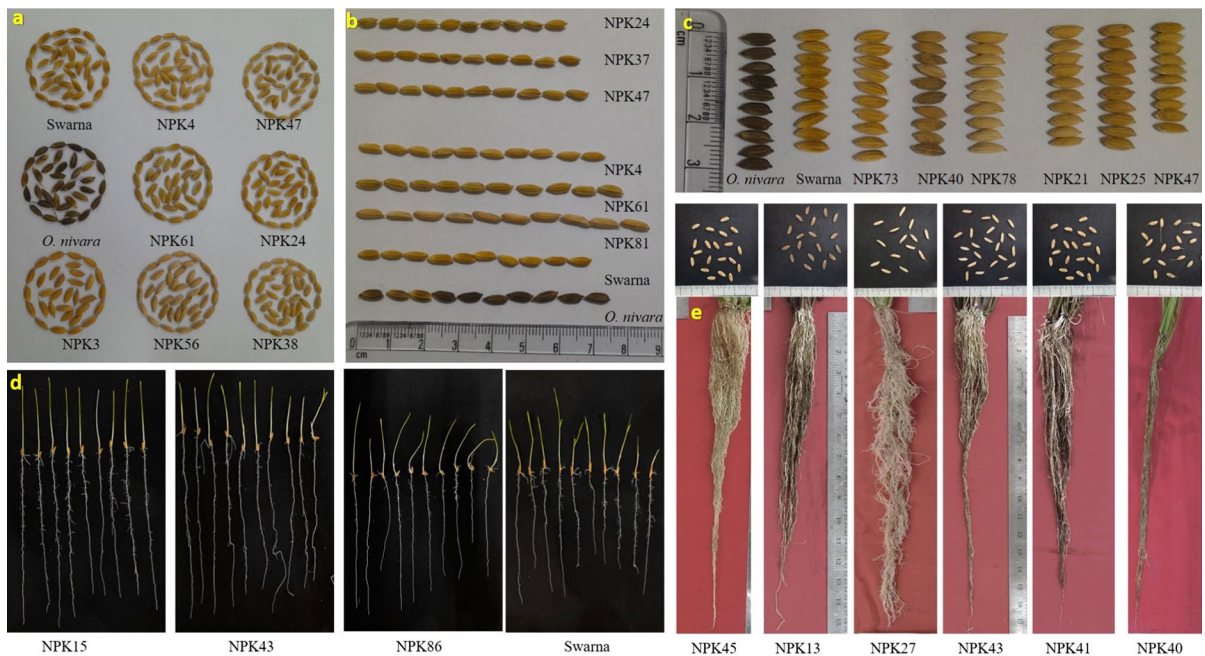


Fig. 1 Morphological variation for grain size, seedling vigour and root traits in the introgression lines. **a** Comparative view of genotypes with varying grain parameters along with parents; **b**

variation in grain length; **c** variation in grain width; **d** variation in seedling vigour; **e** variation in root traits

the lines were grouped into three groups, i.e., short (<5.5 mm), medium (5.51–6.6 mm), and long (6.6–7.5 mm) grain types, and all the ABLs in the population fell into long grain sizes. The area of seeds ranged between 15.26 (NPK47) and 18.95 (NPK90), and the perimeter length ranged between 18.74 (NPK24) and 21.03 mm (NPK18). The circularity of the seed ranged from 0.49 (NPK56) to 0.59 (NPK36, NPK38), and the diameter ranged between 0.45 (NPK11) and 1.34 mm (NPK81).

Similarly, seedling vigor-related morphological traits also showed significant variation for all traits. The percentage of germination ranged between 80 (NPK90, NPK7, NPK49, NPK 27) and 100% (NPK15, NPK23, NPK24, NPK21, NPK1, NPK74), the length of shoots ranged between 5.93 (NPK86) and 18.73 cm (NPK15), the root length was between 5.23 (NPK83) and 22.6 cm (NPK43), and the total plant length ranged from 13.67 (NPK86) to 38.07 cm (NPK26). The fresh weights of roots, shoots and total plants ranged from 1.44 g (NPK71, NPK56) to 1.92 g (NPK30), 1.56 g (NPK30) to 2.21 g (NPK82) and 3.16 g (NPK71) to 3.89 g (NPK82), respectively. The root dry weight ranged between 1.40 (NPK8) and

1.76 g (NPK9), the shoot dry weight ranged between 1.4 (NPK44) and 1.54 g (NPK81), and the total plant dry weight ranged between 2.83 (NPK86) and 3.26 g (NPK9). Seedling vigor indexes 1 and 2 were calculated, and SVI1 ranged between 13.67 (NPK86) and 38.07 (NPK26), and SVI2 ranged between 2.31 (NPK49) and 3.07 (NPK44). Positive skewness was observed for root fresh weight (2.73), total fresh weight (1.19), shoot fresh weight (0.6), root dry weight (6.25), total dry weight (3.95), grain width (0.17), length width ratio (0.39) and seed area (0.04). The highest positive kurtosis was observed for root dry weight (50.75), total dry weight (27.9), root fresh weight (13.03), and seedling vigor index 2 (5.66), and the remaining traits showed either the lowest or negative kurtosis values (Table 1).

Correlation on seedling vigor and grain size traits

The correlation between grain size traits and seedling vigor-related traits was calculated, and the correlogram is presented in Fig. 2. A positive significant correlation was observed for grain length with the length width ratio ($r=0.68$), seed area ($r=0.81$)

Table 1 Morphological variation and descriptive statistics data for seedling vigour and grain size related traits

Variable	Minimum	Maximum	Mean	Median	Variance	Standard deviation	Standard ErrorMean	Coefficient of variation	Skewness	Kurtosis
G%	80.00	100.00	98.22	100.00	23.77	4.88	0.51	4.96	-2.80	7.07
SL	5.93	18.73	11.70	12.12	7.86	2.80	0.30	23.97	-0.18	-0.13
RL	5.23	22.60	15.32	16.10	13.14	3.62	0.38	23.66	-0.55	-0.04
TL	13.67	38.07	27.02	27.86	25.55	5.05	0.53	18.70	-0.31	-0.05
SW	1.56	2.21	1.87	1.87	0.01	0.11	0.01	6.08	0.60	0.95
RW	1.44	1.92	1.52	1.50	0.00	0.07	0.01	4.44	2.73	13.03
TW	3.16	3.89	3.39	3.38	0.02	0.15	0.02	4.32	1.19	1.89
SDW	1.40	1.54	1.48	1.48	0.00	0.02	0.00	1.64	-0.66	2.04
RDW	1.38	1.76	1.44	1.43	0.00	0.04	0.00	2.73	6.25	50.75
TDW	2.83	3.26	2.92	2.92	0.00	0.05	0.01	1.64	3.95	27.90
SVI1	13.67	38.07	26.58	27.25	27.64	5.26	0.55	19.78	-0.37	0.01
SVI2	2.32	3.26	2.87	2.91	0.02	0.15	0.02	5.22	-2.15	5.66
AS	15.26	18.95	17.02	16.98	0.48	0.69	0.07	4.06	0.04	0.26
PL	18.74	21.03	19.88	19.87	0.26	0.51	0.05	2.57	-0.02	-0.56
L	7.23	8.22	7.76	7.77	0.06	0.23	0.02	3.02	-0.09	-0.71
W	2.81	3.13	2.95	2.95	0.01	0.07	0.01	2.43	0.17	-0.45
LWR	2.41	2.95	2.64	2.64	0.01	0.12	0.01	4.44	0.39	-0.14
CS	0.49	0.59	0.55	0.55	0.00	0.02	0.00	3.92	-0.11	-0.38
DS	0.45	1.34	0.92	0.92	0.03	0.17	0.02	18.80	-0.02	-0.02

PL, perimeter length; AS, Seed area; L, grain length; W, grain width; LWR, Length width ratio; CS, circularity; DS, distance between IS & CG; G, Germination percentage; SL, Shoot length; RL, rot length; TL, Total length; SW, shoot fresh weight; RW, root fresh weight; TW, total plant fresh weight; SDW, shoot dry weight; RDW, Root dry weight; TDW, total plant dry weight; SVI1, seedling vigour index1; SVI2 seedling vigour index 2

and plant shoot length ($r=0.89$). Plant shoot length showed a positive significant association with the length width ratio ($r=0.51$) and seed area ($r=0.79$). Seed diameter had a significant positive correlation with seed area ($r=0.22$) and length-width ratio ($r=0.28$). Grain width was positively correlated with seed area ($r=0.53$) and plant shoot length ($r=0.25$). Seed circularity had a significant negative association with the length width ratio ($r=-0.6$), perimeter length ($r=-0.48$) and grain length ($r=-0.37$). Seedling vigor index 1 showed a positive significant correlation with germination percentage ($r=0.37$), seedling vigor index 2 ($r=0.36$), root length ($r=0.83$) and total shoot length ($r=0.97$). Similarly, seedling vigor index 2 showed a significant association with shoot dry weight ($r=0.38$), root dry weight ($r=0.52$), total dry weight ($r=0.52$) and germination percentage ($r=0.86$). Shoot fresh weight and total fresh weight showed a significant association with grain width

($r=0.3$, 0.21), shoot dry weight ($r=0.61$, 0.44), root dry weight ($r=0.34$, 0.22), total dry weight ($r=0.5$, 0.35), total shoot length ($r=0.33$, 0.37), seedling vigor index 1 ($r=0.31$, 0.36), and shoot length ($r=0.35$, 0.41). Grain width showed a positive significant association with shoot dry weight ($r=0.37$), root dry weight ($r=0.32$), total dry weight ($r=0.38$), shoot fresh weight ($r=0.3$) and root fresh weight ($r=0.21$). Grain length was negatively associated with seed circularity ($r=-0.37$).

QTL mapping for seedling vigor and grain size traits

Single marker analysis (SMA)

The genotypic data and the phenotypic data obtained for grain size traits and seedling vigor traits were used for mapping the quantitative trait loci (QTL) in advanced backcross introgression lines. Single

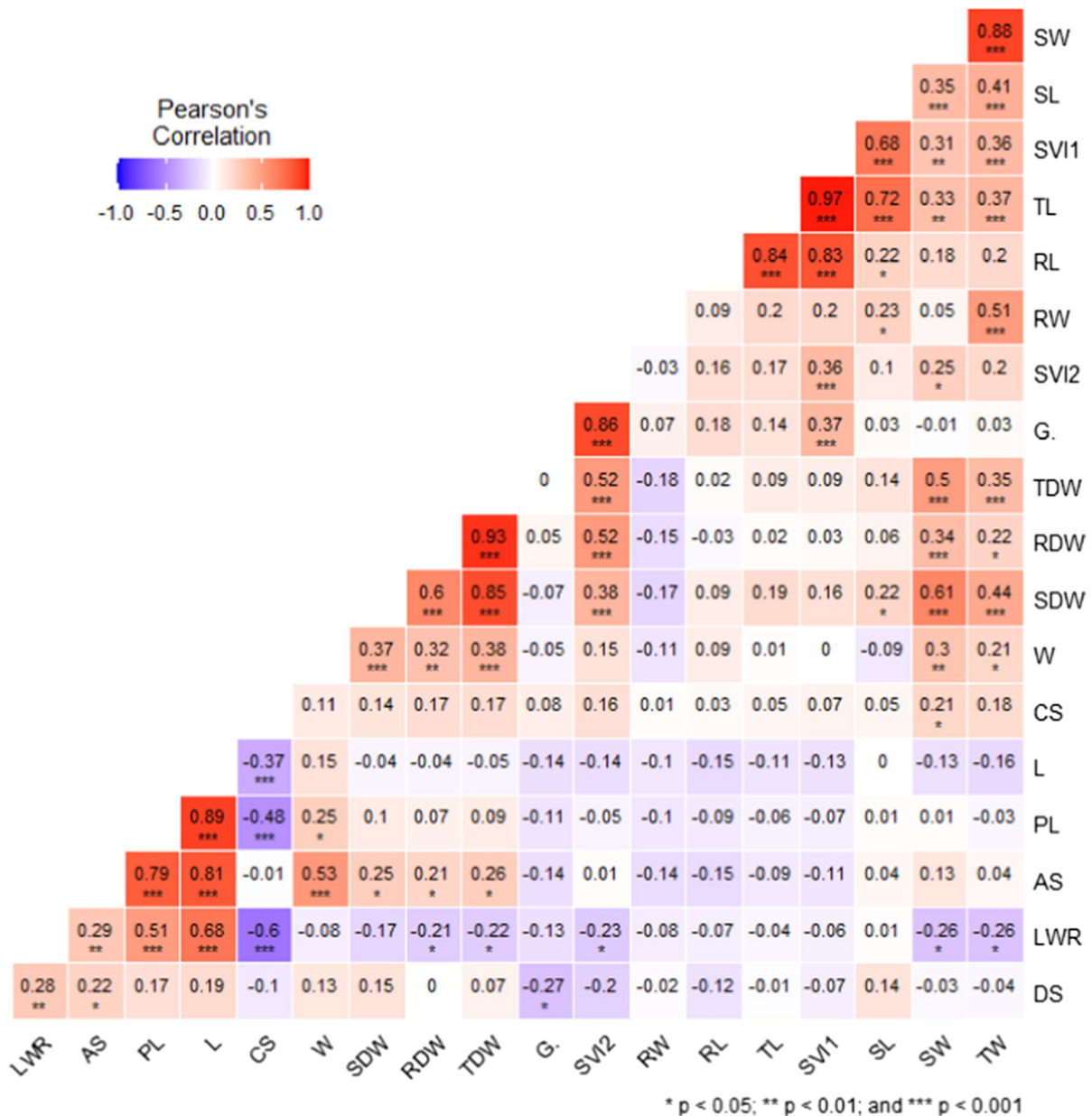


Fig. 2 Correlation analysis between seedling vigour and grain size related traits in Swarna/*O. nivara* introgression lines. *PL*—perimeter length; *AS*—Seed area; *L*—grain length; *W*—grain width; *LWR*—Length width ratio; *CS*—circularity; *DS*—distance between IS & CG; *G*—Germination percentage; *SL*—

Shoot length; *RL*—rot length; *TL*—Total length; *SW*—shoot fresh weight; *RW*—root fresh weight; *TW*—total plant fresh weight; *SDW*—shoot dry weight; *RDW*—Root dry weight; *TDW*—total plant dry weight; *SVI1*—seedling vigour index1; *SVI2*—seedling vigour index 2

marker analysis was performed to identify the marker trait associations that clarify the presence of trait-linked QTL or genes in the population. A total of 107 marker trait associations ($\text{LOD} > 3$) were identified through single marker analysis on chromosomes

1, 2, 3, 5, 6, 7, 8, 9, 11 and 12 for seedling vigor traits such as shoot dry weight, total dry weight, root dry weight, shoot length, total length, perimeter length, seedling vigor index1, seedling vigor index2 and grain size traits such as grain length and

area of seed (Supplementary Table 1). Significant marker trait associations were observed for seedling vigor traits, and only two significant associations were observed for grain size-related traits. Among them, shoot dry weight, total dry weight, and seedling vigor index 2 were associated with the same SNP marker chr12_24705450 with an LOD score of 3.29. Similarly, on chromosome 7, a significant association was observed with the SNP at chr07_18503646 (LOD=3.80), and on chromosome 9, the marker chr09_3934768 was associated with the same traits with an LOD value of 3.46. Grain length and area of seed were associated with marker chr07_5729056 (LOD score 3.51 and 4.129). Seed width, circularity of seed, length width ratio and diameter of seed and root length of seedlings were not associated with any of the markers in this study.

Inclusive composite interval mapping (ICIM)

QTL detection was carried out using ICIM mapping, and 16 QTL (8 QTL for seedling vigor and 8 QTL for grain size) were detected on chromosomes 1, 2, 3, 4, 6, 7, 8, 11 and 12 (Fig. 3). Among the identified QTL, 11 were observed with LOD scores > 3 and explained 7.92–26.38% of the phenotypic variation (Table 2).

QTL identified for grain size-related traits

For grain size-related traits, eight QTL were detected, and only three QTL (*qAS7.1*, *qPL7.1* and *qL7.1*) were observed above 3 LOD scores (LOD > 3). Among the eight QTL, two QTL were detected for grain length (*qL1.1*, *qL7.1*), one QTL each was detected for DS (*qDS12.1*), circularity of seed (*qCS4.1*), length width ratio (*qLWR11.1*), grain width (*qW2.1*), perimeter length (*qPL7.1*) and area of seed (*qAS7.1*). Seven QTLs (*qCS4.1*, *qDS12.1*, *qLWR11.1*, *qL7.1*, *qW2.1*, *qAS7.1* and *qPL7.1*) were detected, explaining 14.86%, 14.74%, 15.48%, 22.60%, 13.85%, 21.44% and 19.11% of phenotypic variation, respectively. Among the identified QTL, three major QTL (*qAS7.1*, *qPL7.1* and *qL7.1*) were detected within the same marker interval on chromosome 7. The major QTL were identified between the marker interval of chr07_5729056 to chr07_514293 (*qAS7.1*, *qPL7.1* and *qL7.1*), chr02_2819095 to chr02_494373

(*qW2.1*), chr11_15850522 to chr11_162848 (*qLWR11.1*), chr04_14229856 to RM16742 (*qCS4.1*) and chr12_15342492 to chr12_19161731 (*qDS12.1*). The traits explained additive gene effect, and the desirable allele was derived from Swarna for circularity of seed, grain length, area of seed and perimeter length, whereas grain width, length width ratio and diameter of seed were derived from the wild parent *O. nivara*.

QTL identified for seedling vigor-related traits

A total of eight QTL were detected for seedling vigor traits, and among them, one QTL each was detected for shoot dry weight (*qSDW3.1*), root dry weight (*qRDW8.1*), total dry weight (*qTDW3.1*), shoot length (*qSL6.1*), root length (*qRL7.1*), total length (*qTL6.1*), SVI1 (*qSVI1-6.1*) and SVI2 (*qSVI2-3.1*). A threshold LOD score > 3 was observed in eight QTL, explaining 9.62% to 26.69% of the phenotypic variation. The major QTL were detected for seven traits, i.e., *qSDW3.1*, *qTDW3.1*, *qSVI2-3.1*, *qSL3.1*, *qTL3.1*, *qSVI1-6.1*, and *qRDW8.1*, explaining 24.71%, 26.38%, 26.38%, 18.3%, 19.68%, 19.68% and 20.69%, respectively. The desirable allele for seedling vigor traits was derived from *O. nivara*, except for root length. The major QTL for seedling vigor are collocated in the marker interval of chr03_30604896 to chr03_32430702 (*qSDW3.1*, *qTDW3.1*, *qSVI2-3.1*) and chr06_677288 to chr06_3721346 (*qSL6.1*, *qTL6.1*, *qSVI1-6.1*).

Collocated QTL for seedling vigor and grain size traits

Among the 16 QTL identified, three seedling vigor-related QTL (*qSDW3.1*, *qTDW3.1* and *qSVI2-3.1*) were collocated on chromosome 3 between the same marker interval of chr03_30604896 to chr03_32430702. Similarly, on chromosome 6, three seedling vigor QTL (*qSL6.1*, *qTL6.1* and *qSVI1-6.1*) were present within the same marker interval of chr06_677288 to chr06_3721346. For the grain size trait, two QTL (*qL7.1* and *qAS7.1*) were collocated between the interval of chr07_5729056 to chr07_514293. For grain size and seedling vigor traits, one seedling vigor QTL (*qRL7.1*) and three grain size QTL (*qL7.1*, *qPL7.1* and *qAS7.1*) were

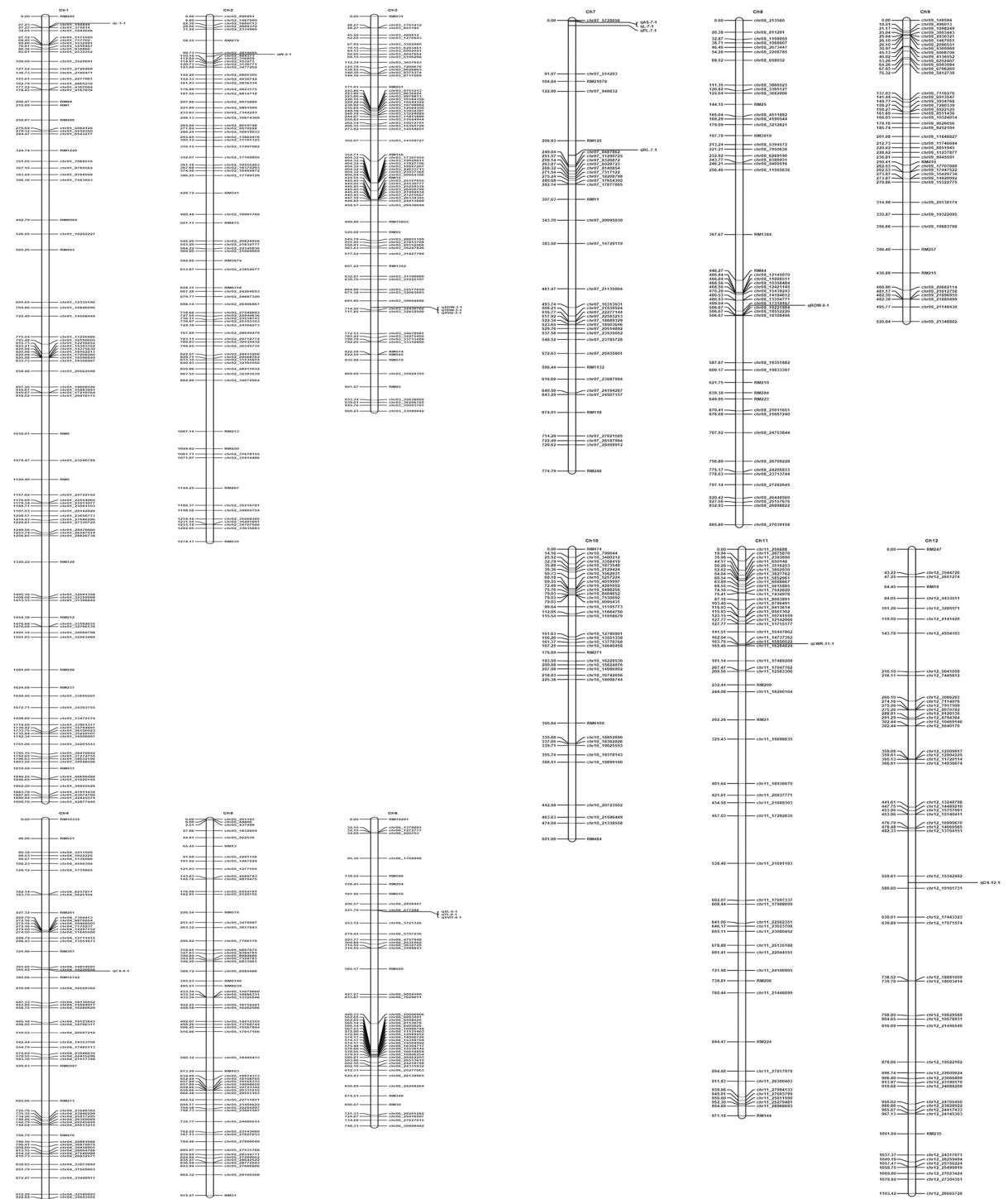


Fig. 3 Molecular linkage map for seedling vigour and grain size related traits in Swarna/*O. nivara* introgression lines using simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) markers. *PL*—perimeter length; *AS*—Seed area; *L*—grain length; *W*—grain width; *LWR*—Length width ratio; *CS*—circularity; *DS*—distance between IS and CG; *G*—Ger-

mination percentage; *SL*—Shoot length; *RL*—root length; *TL*—Total length; *SW*—shoot fresh weight; *RW*—root fresh weight; *TW*—total plant fresh weight; *SDW*—shoot dry weight; *RDW*—Root dry weight; *TDW*—total plant dry weight; *SVI1*—seedling vigour index 1; *SVI2*—seedling vigour index 2

Table 2 Quantitative trait loci (QTL) identified through confidence interval mapping (CIM) on wild introgression lines for seedling vigour and grain size related traits

QTL name	Trait name	Chromosome	Position	Left marker	Right marker	LOD	PVE (%)	Add
<i>qSL6.1</i>	SL	6	222	chr06_677288	chr06_3721346	3.5088	18.299	− 1.4745
<i>qRL7.1</i>	RL	7	232	RM125	chr07_8487862	3.6209	9.6173	3.3795
<i>qTL6.1</i>	TL	6	222	chr06_677288	chr06_3721346	3.7614	19.6782	− 2.8043
<i>qSDW3.1</i>	SDW	3	708	chr03_30604896	chr03_32430702	5.2367	24.7134	− 0.0986
<i>qRDW8.1</i>	RDW	8	506	chr08_17319167	chr08_18221884	4.0666	20.6927	− 0.0189
<i>qTDW3.1</i>	TDW	3	708	chr03_30604896	chr03_32430702	5.636	26.3799	− 0.1096
<i>qSVI16.1</i>	SVI1	6	222	chr06_677288	chr06_3721346	3.7614	19.6782	− 2.8043
<i>qSVI23.1</i>	SVI2	3	708	chr03_30604896	chr03_32430702	5.636	26.3799	− 0.1096
<i>qAS7.1</i>	AS	7	0	chr07_5729056	chr07_514293	4.1344	21.4438	0.346
<i>qPL7.1</i>	PL	7	9	chr07_5729056	chr07_514293	3.874	19.1149	0.3433
<i>qL1.1</i>	L	1	27	RM495	chr01_194844	2.6139	7.9191	0.0808
<i>qL7.1</i>	L	7	2	chr07_5729056	chr07_514293	5.5467	22.6045	0.1398
<i>qW2.1</i>	W	2	97	chr02_2819095	chr02_494373	2.5899	13.8522	− 0.0487
<i>qLWR11.1</i>	LWR	11	164	chr11_15850522	chr11_16284824	2.8814	15.488	− 0.094
<i>qCS4.1</i>	CS	4	366	chr04_14229856	RM16742	2.8151	14.8651	0.0142
<i>qDS12.1</i>	DS	12	571	chr12_15342492	chr12_19161731	2.6787	14.7456	− 0.1183

PL, perimeter length; AS, Seed area; L, grain length; W, grain width; LWR, Length width ratio; CS, circularity; DS, distance between IS & CG; G, Germination percentage; SL, Shoot length; RL, rot length; TL, Total length; SW, shoot fresh weight; RW, root fresh weight; TW, total plant fresh weight; SDW, shoot dry weight; RDW, Root dry weight; TDW, total plant dry weight; SVI1, seedling vigour index1; SVI2, seedling vigour index 2

located close to the marker interval of chr07_5729056 to chr07_8487862 on chromosome 7.

The QTLs hot spot regions on chromosomes 3, 6 and 7 with multiple QTL and major QTL were scanned for candidate genes in RAP-DB database and the search was made within the QTL region and found related candidate genes and their trait ontology are presented in Supplementary Table 2. Among the regions, search was performed in a 1 Mb region upstream and downstream from the flanking markers for loci on chromosome 7 at 5729056–5829056 bp and chromosome 3 at 30604896–31604896, and related candidate genes and their trait ontology were found. The locus linked to chr07_5729056 was associated with grain size QTL, closely located with seedling vigor QTL and showed a significant single marker association with grain length, plant length and area of seed.

Scanning on chromosome 7 resulted in several genes (Supplementary Fig. 1), most of which were associated with abiotic stress tolerance, such as cold tolerance (*Os07g0105700*, *Os07g0119400*), drought tolerance (*Os07g0101400*, *Os07g0119400*), salt tolerance (*Os07g0100800*, *Os07g0119400*), submergence

tolerance, phosphorous sensitivity (*Os07g0137800*), potassium uptake (*Os07g0150700*) and blast disease (*Os07g0124600*). Interestingly, the region also harbors genes for grain size (*Os07g0155600*), grain length (*Os07g0129700*), grain length to width ratio (*Os07g0603300*), root length (*Os07g0155600*), coleoptile length (*Os07g0105700*, *Os07g0155600*), mesocotyl length (*Os07g0155600*), seedling vigor (*Os07g0604700*), germination rate (*Os07g0139300*, *Os07g0633100*), relative root dry weight (*Os07g0678600*), relative root fresh weight (*Os07g0678600*), and gibberellic acid content (*Os07g0103500*), showing the potential of these loci in further genomics and crop improvement research.

Discussion

Seedling vigor is considered an important agronomic trait in direct seeded rice cultivation. Germination percentage, early seedling establishment and growth are the key traits associated with seedling vigor in rice (Abe et al. 2012; Reed et al. 2022). Earlier research noted a significant positive relationship between seed

morpho-physiological characteristics and seedling vigor (Ambika et al. 2014). Fu et al. (2017) and Wang et al. (2018) reported a significant positive correlation between starch content and seedling vigor and a negative correlation between soluble sugar and protein content in rice. Since physiological traits are highly sensitive to environmental conditions, only seed morphological traits were studied to determine their relationship with seedling vigor traits. Significant variation was observed for all the traits studied, and the frequency curve showed a normal distribution for seed area, grain length, grain width, grain circularity, distance between IS and CG, shoot length, root length, total length, total fresh weight, and seedling vigor index 1 (Supplementary Fig. 2). The remaining traits exhibited discontinuous variation. Selection for slender grain size in the population led to changes in the frequency, which might be a reason for the discontinuous distribution of some traits. Furthermore, a significant positive correlation was observed between grain length and the length–width ratio, seed area and shoot length. Similarly, a positive significant association was observed between grain width and seed area, shoot length, shoot fresh weight, total fresh weight, shoot dry weight, root dry weight and total dry weight. However, seed circularity showed a negative association with grain length, length–width ratio and perimeter length.

Based on the phenotypic data, extreme ABL lines were compared for the abovementioned traits, and the lines NPK15, NPK81, NPK82 and NPK84 had the highest shoot fresh weight, total fresh weight, shoot dry weight, total dry weight, seedling vigor index 2, seed area, grain length, length–width ratio, perimeter length and distance between the IS and CG. Similarly, NPK90 had the highest shoot dry weight, total dry weight, area of seed, perimeter length, grain length, grain width, circularity of seed and distance between IS and CG and the lowest root length, seedling vigor index1, and seedling vigor index 2. NPK24 had the highest seedling vigor index1, shoot, root, total lengths, fresh weights, and circularity of seed, and the lowest was observed in the area of seed, grain length and grain length width ratio. The recurrent parent Swarna was reported to have the highest root weight, grain length, and length width ratio and the lowest circularity of seed, grain width, SVI2, shoot fresh weight, shoot, and root dry weight. Earlier research indicated the differential effect of grain size on

seedling vigor traits in maize, barley, wheat, rapeseed and rice (Bicer 2009; Royo et al. 2006; Stougaard and Xue 2004; Mian and Nafziger 1992; Kandasamy et al. 2020; Ambika et al. 2014). Kaydan and Yagmur (2008) reported the effect of seed size on germination percentage, seedling vigor, competition to weed and grain yield in triticale. Durum and spring wheat with larger seed sizes had higher seedling vigor traits and significantly increased the grain yield up to 16% (Royo et al. 2006; Stougaard and Xue 2004). Similarly, Demirlicakmak et al. (1963) reported a positive association of seed size with seedling vigor traits in barley. Contradict to this report, Mian and Nafziger (1992) and Adebisi et al. (2013) reported very little or no effect of seed size on yield and vigor in maize and soybean.

The lines with greater root length (NPK43, NPK33, NPK26 and NPK15) in our study performed well under low P conditions in the northeastern (NE) region of India (Debnath et al. 2021). Earlier research indicated the active elongation of root length associated with phosphorous deficiency tolerance in the *aus*-type rice cultivar Kasalath (Shimizu et al. 2004). Further reduced seed P content significantly affects seedling vigor and germination percentage in rice, as reported by Rose et al. (2012) and Pariasca-Tanaka et al. (2015), especially in weed competitive areas. White and Venekloas (2012) reasoned that greater seed size and seed P reserve enable more rapid early root growth and greater seedling vigor. In contrast, Wissuwa and Ae (2001) reported that the *aus* cultivar Kasalath has very small seeds and contains a lower seed P reserve than Nipponbare with large seed size and higher seed P content and has greater initial root growth and seedling vigor. The introgression line NPK43 had the highest root length, total plant length, shoot dry weight and distance between IS and GC.

Selected NPK lines were also studied under irrigated and aerobic conditions for root and yield-related traits (Padmashree et al. 2023). The introgression lines NPK13, NPK45, and NPK43 showed promising seedling vigor, root traits and yield under aerobic conditions (Fig. 1). Similarly, the introgression line NPK13 (IET22624) was reported to have high seed circularity, seedling vigor index2, root fresh weight and total dry weight in the present study and was also observed to have a high photosynthetic rate (Haritha et al. 2019), low P deficiency tolerance (Debnath et al. 2021) and, high zinc content and was

promoted in the All India Coordinated Rice Project (AICRP)-Initial Evaluation Trial. The introgression line NPK 61 recorded the highest perimeter length, grain length, and length width ratio in our study out performed Swarna for grain yield, significantly higher per day productivity, reduced DFF than Swarna and contained 26.51% of the allele from *O. nivara* (Balakrishnan et al. 2020).

Mapping QTL for seedling vigor traits detected seven major QTL on chromosomes 3, 6, 7 and 8 for shoot dry weight, root dry weight, total dry weight, seedling vigor index2, shoot length, total length and seedling vigor index1. Many QTL controlling seedling/total dry weight and seedling vigor index 1, 2 have been detected in previous research (Zhang et al. 2005a, b; Zhang et al. 2005a, b; Diwan et al. 2013; Chen et al. 2019; Barik et al. 2022; Anandan et al. 2022). However, the QTL detected in our research were considered novel based on their position compared to earlier research on the same chromosome. The major QTL for shoot dry weight (*qSDW*), total dry weight (*qTDW*), and seedling vigor index2 (*qSVI2*) were localized between the marker interval of chr03_30604896 and chr03_32430702 on chromosome 3 at 708 cM. Two QTL (*qSDW3.1*, *qTDW3.1*) from our investigation were analogous to the previous report by Cui et al. (2002) on chromosome 3, whereas *qSVI2* on chromosome 3 was not reported earlier. Similarly, the major QTL for shoot length, total length and seedling vigor index1 detected on chromosome 6 were collocated between the marker interval chr06_677288–chr06_3721346 at 222 cM. Barik et al. (2022) reported a root growth QTL (*qRRG*) at the same position. A seedling vigor trait QTL (*qRL*) was detected in the nearby region of *qAS*, *qPL* and *qL* at 232 cM on chromosome 7 in our study. In addition, the minor QTL for root length (*qRL7.1*) was detected between the markers RM125- chr07_8487862 on chromosome 7. The desirable allele contribution was derived from the wild accession *O. nivara*, suggesting the availability of wide variation for seedling vigor traits in wild species.

The available variation in seedling vigor traits from natural populations was evaluated and utilized in previous research (Barik et al. 2022; Chen et al. 2019; Anandan et al. 2016). Kaladhar et al. (2008) and Balakrishnan et al. (2020) evaluated NK/NPK lines in previous generations for yield QTL and identified stable QTL on chromosomes 1, 2, 3, 4, 7,

8, 11, and 12, where seedling vigor and grain size QTL were reported. Three QTL detected in our study (*qSDW3.1*, *qTDW3.1*, *qSVI2-3.1*) were located proximally to the previously reported QTL (*qDFF3.1*, *qDM3.1*) for heading date on chromosome 3. Similarly, on chromosome 4, the QTL detected for *qCS4.1* was located closely with the QTL *qPH4.1*. The grain size and heading date QTL *qDS12.1*, *qDFF12.1*, and *qDM12.1* were detected on the same chromosome 12 (Balakrishnan et al. 2020). Although most of the traits showed correlation, no common QTL or genes were identified for those traits, indicating a different mechanism of gene action.

For grain size research, except for grain length, the remaining traits were detected with major QTL. The results were also in conformity with the single marker analysis. The major QTL for grain size (*qW2.1*, *qCS4.1*, *qAS7.1*, *qPL7.1*, *qL7.1*, *qDS12.1*) were reported on chromosomes 2, 4, 7 and 12, respectively, and the alleles for grain width, length width ratio and distance between IS and GC were derived from the wild parent *O. nivara* and other traits were derived from the elite parent Swarna. However, little effort has been made to evaluate wild introgression lines for seedling vigor and seed size-related traits, and no collocated QTLs or strong associations for grain size and seedling vigor traits have been reported, especially in wild introgression lines.

Further, candidate gene search was made on the major QTL region and QTL clustered region for the corresponding traits (Supplementary Fig. 3). The region on chromosome 7 co-located QTLs for grain size traits; i.e., grain length, area of seed and seedling vigour trait; i.e., plant length. The region also showed a significant single marker association with grain length, plant length and area of seed in SMA. A region of 5.21 Mb spanning between chr07_5729056 to chr07_514293 and also exhibited significant MTA in single marker analysis. The candidate genes in this region were mostly associated with mesocotyl length, grain length, germination %, plant height, seedling height, coleoptile length, root length auxin content, gibberellic acid content and also associated with abiotic stress including flood, drought, salt stress tolerance.

On chromosome 6, QTLs co-located for shoot length, total plant length and seedling vigour index1. The region is 3.04 Mb spanning between chr06_677288 to chr06_3721346. A candidate gene

search revealed the region is mostly reported for root length, mesocotyl length, stem length, internode length, seedling length, germination rate, grain length, width and grain size. Of which the genes were not further confirmed for the respective traits. So expression research and further validation help in incorporating these genes in future breeding programmes. 1.97 Mb region on chromosome 3 was detected for shoot dry weight, total dry weight and seedling vigour index 2 in the study. Candidate genes detected for root development trait, relative shoot dry weight, relative root dry weight and relative root length on the region and not validated for the respective traits. These genes can be further studied to be successfully employed in future breeding programmes. In chromosome 4, the region spanning between chr04_14229856- RM16742 was detected for circularity of seed. Candidate genes around the QTL region were mainly detected for insect pest resistance, jasmonic acid activity and grain length and no genes were reported for seed circularity.

Similarly, on chromosome 2, QTL was detected for grain width between a region of chr02_494373- chr02_ 2819095 and candidate genes were reported for grain size, grain shape and grain length in this region but specific gene for grain width was not reported. On chromosome 11, QTL reported for grain length width ratio spanning between chr11_15850522- chr11_16284824. A candidate gene search revealed no genes reported on this region. Thus, it could be a novel region for grain length, width and LWR ratio-related traits. Similarly, on chromosome 12, QTL was detected for seed diameter and candidate genes were reported for gibberellic acid content, auxin content and jasmonic acid content.

Candidate gene search on the QTL clustered region for grain size and seedling vigor-related traits resulted in the narrowing of eighteen genes related to seedling vigor traits on chromosomes 3 and 7. The candidate gene *Os07g0604700 (OsB12D1)* was identified in mitochondrial subcellular localization analysis and enhances electron transport under flooded conditions. He et al. (2011) reported it as a functional gene that contributes to early seedling establishment in rice. Further, He et al., 2014 confirmed that *OsB12D1* is a promising gene that contributes to flooding tolerance and enhances early seedling growth in rice. The gene is primarily expressed in germinating seeds and induced in coleoptile, roots during early seedling

growth (He et al., 2014). *Os07g0604700 B12D*-like protein (*OsB12D1*) has functional domain (InterPro)—NADH-ubiquinone reductase complex 1 MLRQ subunit (IPR010530). According to the in silico expression databases RAPDB RiceXpro, the gene *Os07g0604700 B12D*-like protein, showed flooding tolerance during seed germination and early seedling growth and is highly expressed in the roots at the vegetative stage. With respect to the responses towards phytohormones, the gene displayed immediate upregulation upon cytokinin and jasmonic acid (Supplementary Fig. 4). The gene showed induced induction after 6 h upon treatment with auxin, ABA, brassinosteroid, and gibberellin. This in silico data shows that this gene has the potential of induced after hormonal treatment.

Besides, the region chromosome 7 also has genes for mesocotyl length (*Os07g0155600 (OsEIN2)*) and gibberellic acid content (*Os07g0139300* and *Os07g0169700 (OsGA20ox3)*). The gene *OsGA20ox1* was the first gene reported to be associated with seedling vigour in rice that encodes gibberellic acid (Abe et al. 2012). Further research by Ma et al. (2022) identified GA content genes associated with seedling vigour in rice and mutations in the gene cause non-production of GA and plants eventually showed poor growth and necrosis. The candidate gene *OsGA20ox3* was reported for regulating plant stature and disease resistance in rice (Qin et al. 2013) but it was not reported for enhancing seedling vigour. Mesocotyl length play a crucial role in rapid emergence of seedling. Zhao et al. (2018) reported that an increase in mesocotyl length by 4% enhances the seedling emergence up to 85% in rice. The genes reported in this region were different from the previously validated result and are novel candidate regions.

For grain size traits on chromosome 7; i.e., for grain length *Os07g0505200 (GLW7)* gene was reported by Si et al. (2016) and grain width *Os07g0603300 (GL7/GW7)* was reported by Wang et al. (2014). The genes detected in our study was not reported earlier for grain size traits. Thus, further study in this chromosome 7 region it can help in early seedling establishment, especially in direct seeded rice conditions. Further expression study of these genes in these regions may help in the identification of genes that will give a confirmative result and associated marker can be used in marker assisted breeding programme for introgression involved in seedling

vigour and related QTL/genes traits into advanced cultivars.

Conclusion

Seedling vigor and grain size traits were studied in an advanced backcross population derived from Swarna/*Oryza nivara*. Genotypes with better seedling vigor were identified under various grain type categories. These lines have potential in resource-limited conditions and can be developed as climate smart cultivars after multi-location testing. Significant positive correlations were identified between seedling vigor traits and grain size traits, such as grain length and length/width ratio. Plant shoot length and grain width showed a significant association with shoot fresh weight, root fresh weight, shoot dry weight and total dry weight. Further QTL mapping research identified seven major QTL (*qSDW3.1*, *qTDW3.1*, *qSVI2-3.1*, *qSL3.1*, *qTL3.1*, *qSVII-6.1*, and *qRDW8.1*) for seedling vigor traits and three major QTLs detected for grain size traits (*qAS7.1*, *qPL7.1* and *qL7.1*). The SNP markers chr06_677288–chr06_3721346 linked to major QTLs identified for seedling vigor traits and chr07_5729056–chr07_8487862 for grain size traits in the study can be used in marker-assisted breeding programmes for the simultaneous introgression of multiple seedling vigor and grain size QTL, respectively. The identified novel QTL regions are good candidates for further fine mapping and molecular dissection to detect the causative genes for respective traits.

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Author contributions Conceptualization of research, designing of the experiments (DB, YF, SN); and execution of experiments; grain size parameters (DB, HS, YF), seedling vigor (DB, MP, SD, SP, LVS), root traits (KB, PR) and data collection (DB, PG, SK, LD, JB, PGU); Contribution of experimental materials (DB, SN); Analysis of data and interpretation, Preparation of the manuscript (MP, DB, KB); revision and finalization of the manuscript (DB, RMS).

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Declarations

Conflict of interest The authors declare no conflicts of interest or competing financial interests.

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