

Research Paper

Novel haplotype combinations reveal enhanced seedling vigor traits in rice that can accurately predict dry biomass accumulation in seedlings

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Seedling vigor is of vital importance for the initial plant establishment of direct-seeded rice. Here, 166 recombinant inbred lines were employed to assess eight seedling vigor traits over the first 25 days of germination. Significant correlations were found between most traits, and statistical analysis has revealed, for the first time, a linear relationship that uses seedling height and fresh weight to accurately predict the accumulation of dry biomass. Further, a subset of 20 lines, spanning the phenotypic range of the larger population, were genotyped by using 16 simple sequence repeats (SSR) markers known to be associated with seedling vigor traits. Markers RM317 and RM348 linked with significantly different phenotypes, including seedling height and fresh weight. One combination, haplotype II-1 (RM317 (I/II) and RM348 (1/2)), consistently produced superior values for seedling height, root length, and leaf length. The new prediction tool for seedling dry weight, namely the haplotype of RM317 and RM348, will have a wide range of applications in breeding strategies by using marker-assisted selection to produce elite lines that optimize genetic composition contributing to seedling vigor.

Key Words: rice (*Oryza sativa* L.), seedling vigor, recombinant inbred lines (RILs), haplotype, marker-assisted selection.

Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops, providing staple food for over half of the world's population. The health and fitness of rice seedlings can predict that of the mature plant, with seedling morphology closely linked to the capacity of the mature plant to photosynthesize, absorb nutrients and water from the soil, and resist lodging (Yamane *et al.* 2018), all of which contribute to final crop yield (Balakrishnan *et al.* 2016). Thus, rice cultivars can be assessed early for vigor at the seedling stage, and traits such as seedling height, root and leaf length, leaf width, shoot, and root fresh weight, and seedling dry weight are all important parameters for breeding (Redoña and Mackill 1996a).

Considerable variations exist for these traits across rice varieties, but it is essential to separate the influence of genetic factor, often determined by multiple genes, from that of a complex and changing environment (Li and Rutger 1980, Mahender *et al.* 2015). For example, significant ge-

netic differences for seedling vigor traits were determined by measuring shoot weight, shoot length, and coleoptile length across three environments (Redoña and Mackill 1996b). Genetic determinants have been widely reported in the past several decades as quantitative trait loci (QTLs) based on molecular markers (Addanki *et al.* 2018, Cui *et al.* 2002, Han *et al.* 2007, Kumar *et al.* 2014). This technology has permitted significant improvements in agriculturally important traits with a genetic basis, such as yield, heterosis, tiller number, plant height, heading date, root distribution, and adaptation to cold (Cui *et al.* 2002). To date, QTL analyses have largely been used to assess seedling vigor in combination with suboptimal environmental conditions, such as salinity stress (Chunthaburee *et al.* 2016, He *et al.* 2019), low temperature (Han *et al.* 2007, Zhang *et al.* 2005a), and nutrient deficiency (Yamane *et al.* 2018). However, the mechanisms underlying seedling vigor under normal growth conditions remain largely unclear.

Among the different DNA markers commonly employed in rice breeding, simple sequence repeats (SSR) are used most frequently as they possess many advantageous properties, such as being hyper-variable, co-dominant, robust, and chromosome-specific (Anandan *et al.* 2016). Furthermore, SSR markers have also been widely employed to investigate seedling vigor traits in rice (Table 1). Han *et al.* (2007)

Communicated by Mallikarjuna Swamy

Received June 4, 2019. Accepted August 14, 2019.

First Published Online in J-STAGE on October 24, 2019.

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Table 1. List of SSR markers linked to seedling vigor traits in RILs

SSR	Marker	Traits linked	Chr.	Reference	T _m (°C)	Polymorphism detected in this study
1	RM6	SDW	2	Han <i>et al.</i> (2007)	55	yes
2	RM19	SDW, SH	12	Huang <i>et al.</i> (2004) Han <i>et al.</i> (2007)	55	yes
3	RM26	SDW, RL, SH	5	Cui <i>et al.</i> (2002) Diwan <i>et al.</i> (2013) Xie <i>et al.</i> (2014)	59	yes
4	RM80	SH, SDW	8	Han <i>et al.</i> (2007)	55	no
5	RM148	RL, SH	3	Zhang <i>et al.</i> (2005b) Lu <i>et al.</i> (2007) Zhou <i>et al.</i> (2007)	55	yes
6	RM207	SH	2	Xu <i>et al.</i> (2004)	55	no
7	RM213	SDW, RDW	2	Xu <i>et al.</i> (2004)	55	yes
8	RM214	RL	7	Wang (2013)	55	yes
9	RM240	SDW, RDW	2	Xu <i>et al.</i> (2004)	55	yes
10	RM241	SH	2	Xu <i>et al.</i> (2004)	55	no
11	RM263	SDW	2	Han <i>et al.</i> (2007)	55	no
12	RM270	SDW	12	Han <i>et al.</i> (2007)	55	yes
13	RM284	SH, SDW	8	Han <i>et al.</i> (2007) Huang <i>et al.</i> (2004)	55	no
14	RM287	LL	11	Shen (2005)	55	no
15	RM317	RL	4	Zhao <i>et al.</i> (2013)	55	yes
16	RM348	RL	4	Zhao <i>et al.</i> (2013)	55	yes

SH, seedling height; RL, root length; LL, leaf length; SDW, seedling dry weight; RDW, root dry weight; Chr., chromosome.

reported that QTLs correlated with seedling dry weight were detected on chromosome 2 (RM263, RM6), 8 (RM284, RM80), and 12 (RM19, RM270) under 12°C cold water irrigation, also correlated with shoot height (Huang *et al.* 2004). Xu *et al.* (2004) identified that RM213 and RM240 correlated with seedling dry weight, and with shoot and root fresh weights. Markers RM214, RM317, and RM348 closely associate with root length (Wang 2013, Zhao *et al.* 2013), while RM287 is linked to leaf length. Several other SSRs correlate with multiple traits, such as RM26 (Cui *et al.* 2002, Diwan *et al.* 2013, Xie *et al.* 2014) and RM148 (Lu *et al.* 2007, Zhang *et al.* 2005b, Zhou *et al.* 2007), which link physical seedling traits with germination rate.

In the present study, eight traits determining seedling vigor of 166 recombinant inbred lines (RILs) grown under normal conditions were measured at five time-points in early germination, with an emphasis on the seedling dry weight. Statistical analyses will reveal a relationship between the seedling dry weight and seedling height, fresh weight. Genetic analysis on a subset of the population with SSR markers will provide a theoretical basis for molecular marker-assisted selection.

Materials and Methods

Plant material

A hybrid rice combination was previously generated by using the parents, CG133R (*Oryza sativa* ssp. *indica*, a restorer line) and Javanica22 (a natural mutant from *javanica* rice variety Xiangdali), whose seedling vigor phenotypes have significantly differences (Kang *et al.* 2015). Its proge-

nies were self-pollinated through seven generations in an experimental agricultural field at the Southwest University of Science and Technology (Sichuan, China) and were maintained regularly (Kang *et al.* 2015). Here, 166 F₇ RILs were used to analyze seedling vigor traits.

Determination of the seedling vigor traits

Eight parameters of seedling height (SH), root length (RL), leaf length (LL), leaf width (LW), shoot fresh weight (SFW), root fresh weight (RFW), root-shoot fresh weight ratio (R: S) and seedling dry weight (SDW) were measured to assess seedling vigor. Approximately 100 healthy seeds from each line were sterilized, washed, soaked and germinated for 7 days according to Addanki *et al.* (2018), modified so that germination occurred at 28°C. Germinating seeds of each line were sown hydroponically in large plastic boxes adding enough nutrient solution, detailed nutrient solution information is provided in **Table 2**, cultured in an intelligent light incubator (Rui Hua, HP250G-C, Wuhan, China) under simulated natural conditions (28°C/15°C day/night cycles) (Ma *et al.* 2015). The fresh nutrient solution was added every week. Every five days until the 25-day time-point, twelve replicate, healthy seedlings of each line were selected randomly to evaluate seedling vigor traits. These eight traits were determined according to the approaches used in a previous study (Zhu *et al.* 2016), except for LL and LW, which was measured as the total length of the full-spreading leaf from the base of the leaf blade to its tip; and was taken as the widest dimension of the full-spreading leaf, respectively.

Table 2. Detailed nutrient solution information

Components	Concentration (mg/L)	Components	Concentration (µg/L)
Ca(NO ₃) ₂ ·4H ₂ O	945.00	KI	4.15
NH ₄ NO ₃	80.00	H ₃ BO ₃	31.25
KNO ₃	80.00	ZnSO ₄	43.00
KH ₂ PO ₄	506.00	MnSO ₄	111.50
MgSO ₄ ·7H ₂ O	493.00	Na ₂ MoO ₄ ·2H ₂ O	1.25
FeSO ₄ ·7H ₂ O	1.223	CuSO ₄	0.125
EDTA·Na ₂	18.60	CoCl ₂	0.125

DNA extraction and genotyping

Here, 16 SSR markers (Table 1), which have previously been identified to link to seedling vigor traits (Lu *et al.* 2007, Zhang *et al.* 2005b, Zhao *et al.* 2013, Zhou *et al.* 2007), were used to investigate 20 selected lines that covered the range of seedling vigor phenotypes observed in the larger population (Fig. 1, Supplemental Table 1). About 100 mg of rice young leaf tissue was collected and ground using a Fastprep Sample Rapid Crushing System (MP Biomedicals, Santa Ana, CA, USA). DNA was extracted

using the CTAB method (Murray and Thompson 1980). Polymerase chain reactions (PCR) were carried out by using an Eppendorf Thermal Cycler (Mastercycler[®] nexus GSX1, Germany) with a 10 µL reaction using the Golden Easy PCR System (TIANGEN, Beijing, China), according to manufacturer's instructions. PCRs were conducted with primers and reaction conditions as described by Anandan *et al.* (2016), annealing at 55–59°C (depending on primers, shown in Table 1). All the amplified products were detected on a 3% agarose gel in 0.5 × Tris-Borate EDTA (TBE) buffer using GreenView (Applied BioProbes, Rockville, MD, USA).

Data statistics and analysis

Correlation analysis among seedling vigor traits in RILs, Stepwise regression analysis and Path analysis of dry weight and other seedling vigor traits were conducted using the Statistical Product and Service Solutions software version 19 (SPSS, <https://www.ibm.com/analytics/cn/zh/technology/spss/>). Analysis of variance (ANOVA) was performed using SPSS19 after data were classified according to the genotyping results.

Results

The performance of seedling vigor traits in recombinant inbred lines at different stages

In the present study, 166 rice RILs were grown under laboratory conditions and assessed every 5 days through the first 25 days of germination for various seedling vigor traits. Phenotypes at each time point for seedling height (SH), root length (RL), leaf length (LL), leaf width (LW), shoot fresh weight (SFW), root fresh weight (RFW), root-shoot fresh weight ratio (R:S) and seedling dry weight (SDW) are shown in Fig. 1. The fastest-growing period for all traits was 0–5 days after seed germination. The second-fastest growth phase was the period 5–10 days after germination, except for SFW and RFW, which had a growth spurt at 15–20 days. R:S began to decrease after 5 days when the SFW increased much more quickly than RFW. All traits exhibited a near-normal distribution at every developmental stage (Supplemental Fig. 1), indicating that the seedling vigor traits exhibited quantitative inheritance regulated by multiple genes.

Correlation analysis among seedling vigor traits at different stages in recombinant inbred lines

Significant correlations were found among the eight seedling vigor traits, as analyzed by Pearson's correlation (Fig. 2). Seedling height was significantly and positively correlated with root length, leaf length, shoot fresh weight and seedling dry weight, but negatively correlated with leaf width and root-shoot ratio. Leaf width correlated positively with fresh and dry weights, but negatively with root and shoot lengths and seedling height. Fresh weights of root and shoots correlated significantly with seedling dry weights. Furthermore, seedling dry weight showed a significant

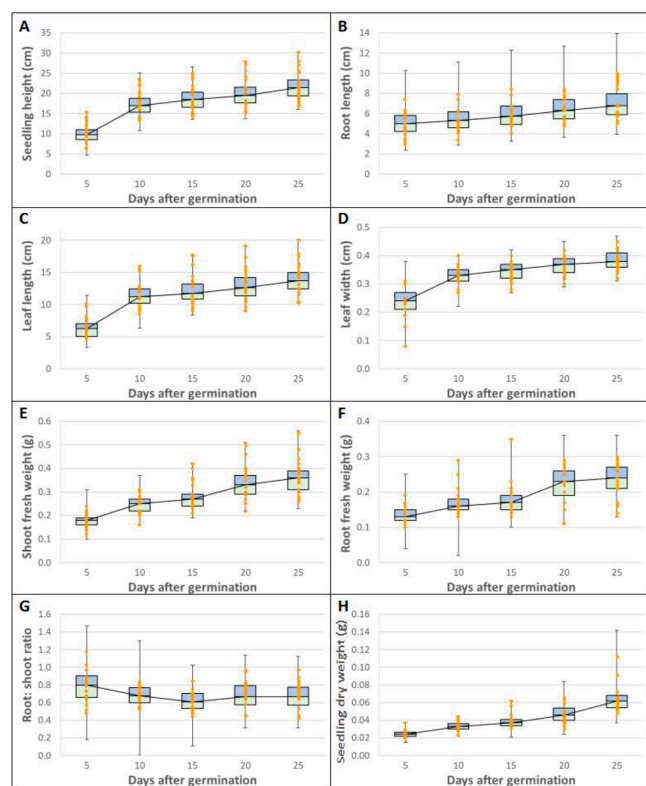


Fig. 1. Box plots showing the quantification of 8 seedling vigor traits for 166 recombinant inbred lines during the first 25 days after germination. The central horizontal line gives the median value, the green box covers values in the second quartile, the blue box covers values in the third quartile, while the whiskers indicate the minimum and maximum values. The 20 orange dots indicate the values for the population subset used for SSR analysis. A) Seedling height; B) Root length; C) Leaf length; D) Leaf width; E) Shoot fresh weight; F) Root fresh weight; G) Root:shoot ratio; H) Seedling dry weight.

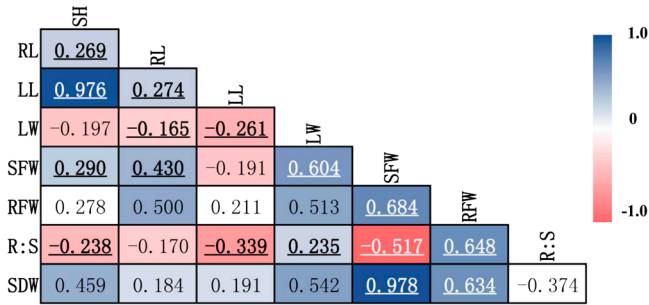


Fig. 2. Phenotypic correlations among eight seedling traits related to seedling vigor in RILs. SH, seedling height; RL, root length; LL, leaf length; LW, leaf width; SFW, shoot fresh weight; RFW, root fresh weight; R:S, root: shoot ratio; SDW, seedling dry weight. Correlations with $P < 0.05$ are in shaded boxes, while values with $P < 0.01$ are in bold and underlined.

positive correlation with seedling height, root length, leaf length, shoot fresh weight and root fresh weight, but no significant correlation with root-shoot ratio.

Stepwise regression analysis and Path analysis of dry weight and other seedling vigor traits

Seedling dry weight is a vital indicator of seedling vigor, which indicates subsequent plant-type establishment, crop growth and yield (Balakrishnan *et al.* 2016). Hence, we assayed the linear relationship among the 166 RILs between seedling dry weight (Y, in cm) and the seven other traits at 25th day after germination: seedling height (X_1 , in cm), root length (X_2 , in cm), leaf length (X_3 , in cm), leaf width (X_4 , in cm), shoot fresh weight (X_5 , in g), root fresh weight (X_6 , in g) and root-shoot ratio(X_7), using Stepwise regression analysis. The optimal regression equation was as follows

$$Y = -0.044 + 0.006X_1 + 0.227X_5 (R^2 = 0.969, P < 0.01)$$

It revealed that SDW could be predicted accurately using only SFW and SH, which could account for 96.9% in the observed variation in SDW. While the influence of SFW was greater than that of SH, the larger numerical values of SFW meant that its contribution to SDW was higher than that of SH.

Path analysis was conducted using the result of Stepwise regression analysis to further understand the influence of SH and SFW on SDW. Both SH and SFW were found to contribute to SDW, and the direct effect of SFW to SDW was higher than that of SH, as indicated by the regression equation (Table 3). However, the indirect effect of SH on SDW through SFW was higher than the indirect effect of SFW on SDW through SH. Taken together, SH and SFW play a key role in promoting the accumulation of dry matter, and the two traits should be selected as major assessment parameters at the early stage of breeding.

Effects of genetic polymorphism on seedling vigor traits

To further investigate the genetic basis for seedling vigor traits at 25 days after germination, a subset of 20 lines ex-

Table 3. Path coefficient of two seedling traits on the seedling dry weight

Independent variable	The total correlation coefficient for SDW	Direct path coefficient	Indirect path coefficient	
			SH	SFW
SH	0.459	0.118		
SFW	0.978	0.935	0.043	0.341

SDW, seedling dry weight; SH, seedling height; SFW, shoot fresh weight.

hibiting the full phenotype range of the broader population were selected for genetic analysis (Fig. 1, Supplemental Table 1). Plant genotype was determined at 16 SSR markers, previously shown to be linked to seedling vigor traits. Polymorphic bands were detected in 10 SSR markers (Table 1), then, the 20 selected RILs were classified as type I or type II at each SSR (Supplemental Fig. 2). Correlation of seedling traits to each SSR marker was assessed using Student’s *t*-test, revealing that genes at markers RM317 and RM348 affected several seedling vigor traits under investigation (Supplemental Table 2). Marker RM19 also showed a significant correlation, but only for one trait (LL), so this marker was not analyzed further. The RM317 linkage affected 6 seedling vigor traits: SH, RL, LL, LW, SFW, and RFW (Fig. 3A). For each of these traits except for LW,

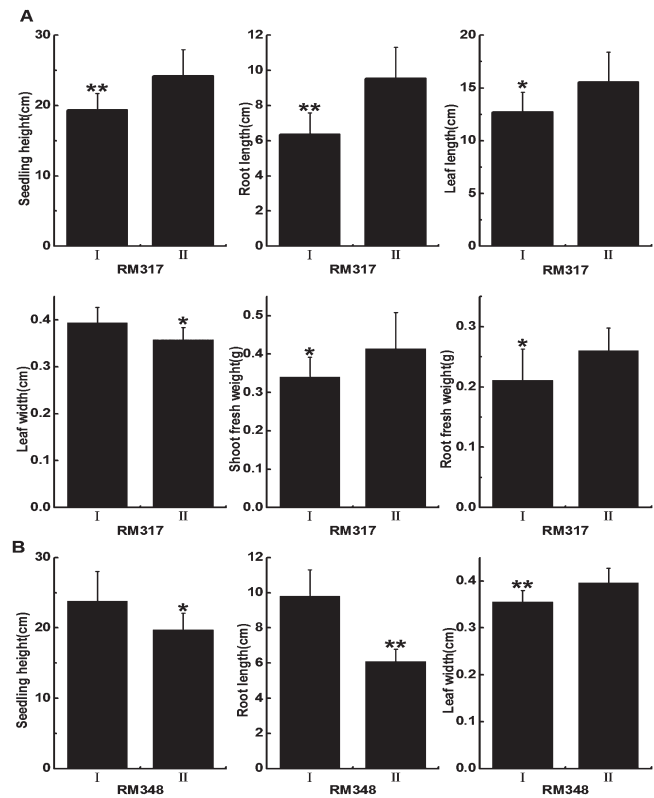


Fig. 3. Comparison of seedling traits between different haplotypes after 25 days of germination in A) Marker RM317, and B) Marker RM348. Each column represents the mean \pm standard deviation. ** indicates $P < 0.01$, according to Student’s *t*-test.

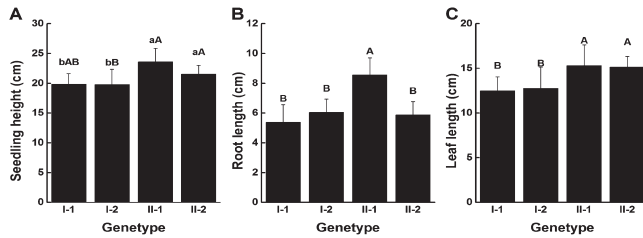


Fig. 4. Comparison of different allelic effects of RM317(I/II) and RM348(1/2) linkages for seedling-vigor traits. A) Seedling height; B) Root length; C) Leaf length. Lowercase and capital letters denote significance at 0.05 and 0.01 levels, respectively.

polymorphic locus 317-II exhibited a significantly higher value, especially, having the best phenotypic effects in SH and LL. Genes linked to RM348 contributed to SH, RL, and LW (Fig. 3B), and again, 348-I that exhibited significantly higher values for SH and RL, but lower value for LW. Because SH was one of important factors that contributed to SDW, taken together, RM317 and RM348 would be extremely useful in marker-assisted selection.

We further investigated the haplotype effects for each of the four RM317 (I/II) and RM348 (1/2) polymorphic loci. Haplotype I-1, I-2, II-1, and II-2 exhibited different phenotypes for the three traits SH, RL, and LL (Fig. 4), in which II-1 displayed the most vigorous traits with the highest mean values of SH, RL, and LL. For RL, while II-1 had the highest value, there were no significant differences among the other three haplotypes with lower RL value. These results reveal that different haplotypes of the polymorphic alleles of RM317 and RM348 linkage have a remarkable influence on seedling vigor traits and beneficial haplotypes can produce superior vigor.

Discussion

Seedling vigor is an extremely complex agronomic trait and can be assessed and characterized by performed many parameters (Milosevic *et al.* 2010). Vigor is controlled by genetic and environmental elements, such as the conditions of seed storage and germination, and the plant growth conditions during late seed maturation and dehydration, and in the early stages of plant development (Adam *et al.* 1989, Sun *et al.* 2007). As a critical objective in rice breeding, seedling vigor has received increasing attention in recent years. However, due to the complexities of multi-genic inheritance, as well as undesirable traits linked with seedling vigor, conventional breeding strategies have not generally been successful (McKenzie *et al.* 1994).

In this study, we evaluated seedling vigor by measuring SH, RL, LL, LW, SFW, RFW, SDW, and R:S, as these traits are considered to be representative indicators of seedling vigor (Redoña and Mackill 1996a, 1996b, Regan *et al.* 1992, Zhang *et al.* 2017). We focused on the first 25 days after germination before independent photosynthesis is fully established; in this time, therefore, main nutrients and ener-

gies for seedling growth and biomass accumulation are still essentially provided by the seed endosperm, so differences in seedling traits are predominantly under genetic control.

Previous studies have shown that seedling vigor traits in rice displayed near normal, continuous distribution in an F_2 population (Chung 1979, Han *et al.* 2007, Qian *et al.* 2000). Our study confirmed these results for the eight seedling traits investigated (Supplemental Fig. 1), providing further evidence that seedling vigor traits are controlled by many loci (Han *et al.* 2007, Regan *et al.* 1992, Redoña and Mackill 1996a, 1996b).

There were strong correlations among these 8 investigated seedling vigor traits (Fig. 2), likely due to pleiotropic effects or close linkage of genes (Aastveit and Aastveit 1993). Of these, SH has been considered the best predictor of seedling and plant vigor in rice (Zhang *et al.* 2005b); while SDW has been reported as one of the most vital factors for contributing to the final yield (Regan *et al.* 1992), and a key indicator for amylase activity in germinated seeds (Cui *et al.* 2002). Our results demonstrate a significant positive correlation between SH and SDW, which was consistent with recently reported by Addanki *et al.* (2018). We also observed significant positive correlations between SH and RL, LL, SFW, and negative correlations with LW and R:S. SDW showed a similar correlation with RL as reported by Cui *et al.* (2002), as well as significantly positively correlated with most other seedling traits (Fig. 2). Intuitively, the correlation between LL and LW was significantly negative, in other word, increases in leaf biomass generally occur either in length or in width, not both (Xia 1983); and plants that tend to grow longer leaves also grow longer roots and shoots. SFW increased along with most other traits except for LL, suggesting that the broader leaves may accumulate more biomass than the longer ones; however, SDW positively correlated with both LL and LW, though more strongly with the LW trait. While several correlation studies between SDW and other agronomic traits exist in the literature (Addanki *et al.* 2018, Cui *et al.* 2002, Zhang *et al.* 2005c), the study of a linear relationship between SDW and other traits has not previously been reported. In this study, stepwise regression analysis revealed a linear relationship between SDW, SH, and SFW, indicating that both SH and SFW contribute to SDW and can predict its value with high accuracy ($R^2 = 0.969$). Along with the result of path analysis for SDW, it can be concluded that SH and SFW are critical indicators of seedling vigor and excellent predictors of SDW; To assess and optimize these phenotypes during the breeding process may lead to substantial improvements in the accumulation of rice biomass throughout plant development.

With the rapid development of sequencing technologies, QTL mapping based on molecular markers has proven to be an effective method for investigating complex traits such as yield (Huang *et al.* 2004, Song *et al.* 2007, Wang *et al.* 2015). In recent years, this method has been used to research seedling vigor traits of rice. Dang *et al.* (2014) detected 27 QTLs related to seed dormancy and established 15

hybrid combinations derived from a cross of elite parents to improve seedling vigor in rice. Lin *et al.* (2010) identified 4 QTLs for SDW on chromosomes 1, 4, 8, 9 by using 71 RILs. Anandan *et al.* (2016) found 2 QTLs associated with SH and 1 QTL for SDW at different stages after germination. Refining the size and attributes of these QTLs will assist breeders to target their strategies to obtain stronger vigor at the early seedling stage. It was reported previously that the two markers, RM317 and RM348, located on chromosome 4 were closely associated with root length (Zhao *et al.* 2013). Here, these markers were found to be significantly associated not only with RL, but also with SH, LW; RM317 also correlated with LL, SFW, and RFW (Fig. 3). For both markers, RM317/II-RM348/1 haplotype directed higher values for all associated seedling traits except for LW, which correlated significantly with the other haplotype. It is possible that genes linked with beneficial root, shoot and leaf length/height associate with the RM317/II-RM348/1 haplotype, and their significant effects on leaf and root size positively affect root and shoot weight.

The effect of the four different haplotypes derived from these two markers played an important role in determining rice seedling vigor, and that combinations of RM317 (I/II) and RM348 (1/2) polymorphic loci significantly differed from each other in phenotypes. In particular, haplotype II-1 exhibited superior SH, RL, and LL compared with the others. Further characterization of major QTLs in the interval of RM317 and RM348 on chromosome 4 may yield novel alleles to substantially boost seedling vigor traits (Fig. 5), independently and in combination with each other.

In summary, we have, for the first time, reported a linear relationship that can accurately predict the dry weight accumulation in rice seedlings by using SH and SFW indicators. Such a predicting tool will be beneficial for breeders to produce lines with improved seedling vigor. This study has also revealed that SH and SFW phenotypes are closely linked to two SSR markers, RM317 and RM348, on chromosome 4. Furthermore, a specific haplotype, II-1, for these markers exhibited consistently superior traits that could be used as a

primary screening tool in breeding populations. These results should facilitate marker-assisted selection to improve seedling vigor and seedling establishment in rice.

Author Contribution Statement

LX and L.Z. G investigated genetic studies. HY and O.L. Z carried out the measure of Seedling vigor related traits. X.C. X designed the overall project. LX analyzed the data and wrote the manuscript. All the authors read and approved the final manuscript.

Acknowledgments

This work was supported by the Key Research & Development program of Sichuan Province, China (Grant No. 2019YFN0019), and the Key Program of Education Bureau of Sichuan Province, China (Grant No. 17ZA0412), and Postgraduate Innovation Fund Project by Southwest University of Science and Technology (Grant No. 19ycx0074). We thank Dr. Natalie for her useful comments and edit on the manuscript.

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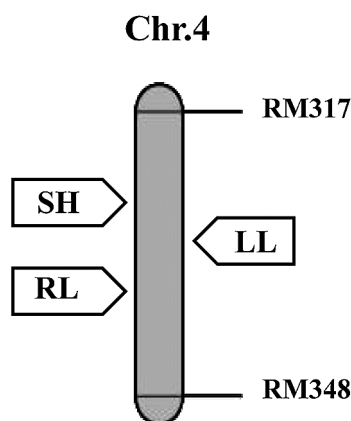


Fig. 5. Genetic linkage map linked to seedling vigor traits on Chromosome 4. SH, seedling height; RL, root length; LL, leaf length.

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