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Laboratory phenomics predicts field performance and identifies superior *indica* haplotypes for early seedling vigour in dry direct-seeded rice

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ABSTRACT

Seedling vigour is an important agronomic trait and is gaining attention in Asian rice (*Oryza sativa*) as cultivation practices shift from transplanting to forms of direct seeding. To understand the genetic control of rice seedling vigour in dry direct seeded (aerobic) conditions we measured multiple seedling traits in 684 accessions from the 3000 Rice Genomes (3K-RG) population in both the laboratory and field at three planting depths. Our data show that phenotyping of mesocotyl length in laboratory conditions is a good predictor of field performance. By performing a genome wide association study, we found that the main QTL for mesocotyl length, percentage seedling emergence and shoot biomass are co-located on the short arm of chromosome 7. We show that haplotypes in the indica subgroup from this region can be used to predict the seedling vigour of 3K-RG accessions. The selected accessions may serve as potential donors in genomics-assisted breeding programs.

1. Introduction

Asian rice (*Oryza sativa*) is a major cereal crop grown worldwide and an essential food source for over half of the world's population. It is commonly grown by transplanting seedlings into puddled soil. Although puddled transplanted rice (PTR) has several benefits, particularly in terms of weed control, it is also highly intensive in its requirements of labour, water and energy [1,2]. PTR is also a major greenhouse gas emitter, contributing up to 20% of total global methane emissions [3]. Direct seeded rice (DSR) is a more sustainable alternative to PTR, particularly where dry seeding is used in combination with low or zero tillage. To obtain good yields using dry DSR, fast and uniform seedling establishment is considered paramount to counter the higher weed pressure and adverse physical conditions that are encountered in the soil [1,4]. Surface seeding or broadcasting may lead to poor establishment and uneven crop stand due to rain splashing, drought, high temperature, greater vapour pressure gradient, and predation [1,5]. Deep sowing is an effective method ensuring the seeds are protected and can access moisture. The availability of moisture is more consistent at greater soil depths and seed are less vulnerable to pests. In general, however, deep sowing is not recommended as it can be challenging for the seedlings to push through the soil. Deep sowing can lead to slow seedling emergence, poor seedling establishment, low dry mass accumulation and deeper crown placement [6]. Historically, there has been little selection for seed vigour among rice varieties which have been bred for transplanting prevalent in PTR, and agronomists have highlighted a need for improvement in this trait for DSR [4]. One limiting factor is the elongation of the mesocotyl (first internode of the stem), which is critical to allow emergence from deeper soil. In particular, high-yielding semi-dwarf rice varieties tend to have a short mesocotyl, which limits their potential for emergence at greater sowing depths [4,7,8]. Mesocotyl length, along with seedling emergence and establishment are three important traits for determining high rice yields in dry DSR systems [9].

A handful of studies have utilised Genome Wide Association Studies (GWAS) to investigate natural variation in mesocotyl length in rice. Wu et al. [10] screened 270 rice accessions and 16 loci were identified to be

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associated with mesocotyl elongation. In another study, a total of 469 *indica* accessions were used to measure mesocotyl length, and 23 loci were found to be significantly associated with mesocotyl length [11]. Zhao et al. [12] evaluated the mesocotyl length of 621 rice accessions and detected 13 QTLs whereas Sun et al. [13] identified three QTL for mesocotyl length from 510 rice accessions and furthermore reported that OsGSK2, a conserved glycogen synthase kinase 3 - like kinase involved in brassinosteroid signalling, contributes to variation in rice mesocotyl length. More recently, a study focusing on mesocotyl elongation at 5 cm planting depth was performed using 54 chromosome segment substitution lines and was successful in identifying 3 potential QTLs contributing to mesocotyl length [9]. Not surprisingly, due to the unique experimental characteristics of each study (soil, water, sand), there is little overlap between the identified QTLs. Field studies are also subject to environmental variations further adding to the complexity of separating genetic and environmental effects.

Plant phenomics has received increasing interest in recent years as a tool to help bridge the genotype-to-phenotype knowledge gap. However, accurate high-throughput phenotyping is critical to exploit this resource by linking important agronomic traits to specific loci and this has emerged as a major bottleneck in crop improvement. In the case of rice seed vigour, there have been many studies where manual measurements have been performed on a few selected parameters in small diversity sets and mapping populations [4]. However, no systematic analysis of multiple traits has so far been performed in both laboratory and field conditions using a large and diverse panel of rice accessions such as those re-sequenced in the 3000 Rice Genomes (3K-RG) Project [14]. Mesocotyl length is known to be affected by the environment and numerous QTL and GWAS experiments have been performed on this trait in either aerobic and anaerobic conditions, at different depths and on different media such as agar, water, filter paper, soil, and soil mixtures [15].

Here we report the development of a phenotyping platform for rice skotomorphogenic growth allowing multiple seedling trait analyses. To the best of our knowledge, this is the first study to compare traits in a laboratory setting directly with those acquired under natural field growing conditions involving a large number of accessions at different sowing depths. Moreover, this laboratory-based system accurately predicts seedling performance of the germplasm in the field. The present study aimed i) to dissect the genotypic variation existing in a diverse rice germplasm from the 3K-RG for component traits governing germination, emergence and establishment from deep soil, grain yield and related traits ii) to uncover the correlation among the measured traits across laboratory and field conditions iii) perform GWAS to detect significant marker trait associations (MTAs), which can be used further in molecular breeding efforts iv) to identify haplotypes that accurately predict performance in the field. By harnessing the power of the laboratory, we can help accelerate advances in the breeding of DSR.

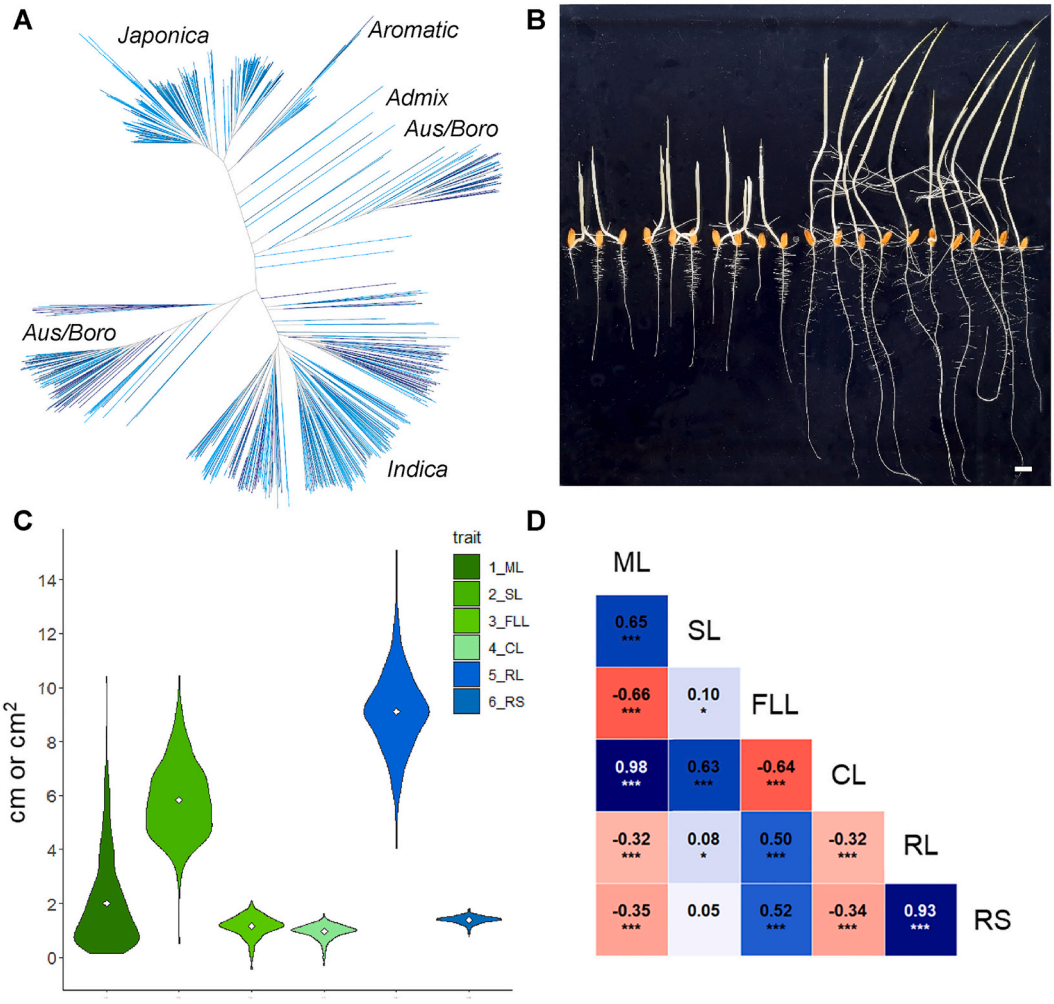

Fig. 1. Laboratory phenotyping of skotomorphogenic seedling growth and morphology. (A) Phylogenetic neighbour-joining tree of the 684 accessions indicating the genetic relatedness at subpopulation level and relationship among the different accessions. (B) Representative image of rice seedlings on gellan gum plate after 5 days of growth. Bar = 1 cm (C) Violin graph showing phenotypic range for traits measured in the laboratory. Shoot length (SL), 1st leaf length (LL), coleoptile length (CL), mesocotyl length (ML), root length (RL) and root surface area (RS). (D) Pearson's correlations between traits using accessions means.

2. Results

2.1. Laboratory phenotyping

In our study, we used a sub-set of 684 re-sequenced accessions from the 3K Rice Genomes (3K-RG) selected from tropical and temperate *japonica*, *indica*, *aromatic*, *aus/boro* and *admix* subgroups that were collected from 66 countries (Fig. 1A). All seed lots were produced in the field at the International Rice Research Institute (IRRI) and the same lots were used for parallel laboratory and field experiments to aid comparison. A high throughput lab phenotyping pipeline was established to obtain accurate quantitative data for skotomorphogenic seedling growth and morphology under aerobic conditions (Fig. S1). Images were captured after 5 days and shoot length (SL), 1st leaf length (LL), coleoptile length (CL), mesocotyl length (ML), root length (RL) and root surface area (RS) were measured using an adapted version of SmartRoot (Lobet et al., 2011) (Fig. 1B).

Our data was approximately normally distributed for all traits, except for mesocotyl length which exhibited a skewed distribution (Fig. 1C). This is consistent with previous studies that show that most rice accessions have short mesocotyls [12]. Broad sense heritability for shoot traits varied between 0.8 and 0.9, while for roots the value was 0.7. Shoot length ranged from 2.7 to 10.4 cm, while the length of its component tissues the first leaf, coleoptile and mesocotyl ranged from <0.1 to 2.0 cm, from 0.4 to 3.1 cm and from <0.1 to 9.4 cm, respectively. Root length and surface area also ranged from 4.0 to 15.1 cm and 0.8 to 1.8 cm^2, respectively (Fig. 1C). We calculated Pearson's correlation coefficients between all traits means (Fig. 1D). Mesocotyl length was positively correlated with shoot and coleoptile length ($r = 0.65$ and 0.98 respectively,). Whereas mesocotyl length was negatively correlated with first leaf and root length ($r = -0.66$ and -0.32 respectively), shoot length was positively correlated with coleoptile length ($r = 0.63$). First leaf length was positively correlated with root length ($r = 0.50$) and with root surface area ($r = 0.52$). Leaf and root length were negatively correlated with coleoptile length ($r = -0.64$ and -0.32 respectively). Root length and surface area were also positively correlated ($r = 0.93$). Taken together, these data suggest that shoot length is primarily explained by variation in mesocotyl length and that root length and surface area are only weakly negatively related to shoot length. Among the sub-groups, the range in mesocotyl and shoot length were greatest in *aus/boro* followed by *indica* and *admix* (Fig. S4A). *Aromatic* and *japonica* accessions had the shortest mesocotyl and shoot length (Fig. S4B).

2.2. Field phenotyping

We also evaluated the performance of the 684 3K-RG accessions in parallel experiments in the field at three sowing depths (4, 8 and 10 cm) (Fig. S6). We measured mesocotyl length, percentage seedling emergence (SE), shoot dry weight (SW), root dry weight (RW), nodal root number (NR) after 30 days and plant height (PH), flowering time (FT) and grain yield (GY). Summary statistics for the trial data is presented in Table S1. Broad sense heritability for most traits measured in the field was lower than for seedling phenotypes measured in laboratory conditions and values ranged between 0.1 and 0.8 (Table S1). Substantial variation was observed in all traits (Table S1). For example, mesocotyl length ranged from 0.5 to 3.5 cm at 4 cm sowing depth, from 0.6 to 8 cm at 8 cm sowing depth and from 2 to 10 cm at 10 cm sowing depth (Fig. S6). We also calculated Pearson's correlation coefficients between all field traits and included laboratory mesocotyl length (Fig. S7). Mesocotyl length measured in the laboratory showed a moderate correlation with mesocotyl length measured in the field across all three planting depths ($r = 0.36$, 0.29 and 0.19; $P < 0.01$) and a stronger correlation with percentage emergence at all three planting depths ($r = 0.47$, 0.43 and 0.51; $P < 0.01$).

Among the sub-groups, *aus/boro* accessions exhibited the longest mesocotyls at the greatest soil depth as well as the largest root dry weight and nodal root number, followed by *indica* and *japonica* accessions. This pattern was consistent across two seasons, with accessions IRGC 128442, IRGC 127184, IRGC 127189, IRGC 125810, IRGC 125853, and IRGC 125657 consistently exhibiting the greatest mesocotyl length. The *aus/boro* accessions flowered earlier than *japonica* and *indica* accessions at all depths (Fig. S6). In general, accessions also flowered earlier at 8 and 10 cm depth compared to 4 cm depth (Fig. S6). Grain yield was higher in *indica* accessions than *aus/boro* and *japonica* accessions at 4 cm depth but *aus/boro* accessions exhibited better grain yield compared to *indica* and *japonica* accessions when sown at 8 and 10 cm depth (Fig. S6). These data suggest that mesocotyl length measured in both the laboratory and field is a good predictor of percentage emergence when seeds are sown at depth, and that *aus/boro* accessions exhibit the largest variation in these traits, although substantial variation is also present within *indica*.

2.3. Genome-wide association study (GWAS)

To verify the population structure, principal components (PCs) and kinship matrix were calculated in GAPIT [16] and linkage disequilibrium (LD) decay in PopLDDecay using the 684 accessions together with 795,745 SNPs distributed across the 12 rice chromosomes with minor allele frequency (MAF) of >0.05 and the missing rates <0.2. We performed GWAS for all traits implemented with GAPIT CMLM [16]. Using a significance threshold of $-\log_{10}(P) \geq 7.2$ (5% after the Bonferroni multiple test correction), we found significant marker trait associations (MTAs) for each trait (all experiments taken together): 338 MTAs with ML, 31 with SE, 335 with SW, 39 with RW, 31 with FT, 49 with GY, 32 with PH and 392 with NR (Fig. 2 and Fig. S2, S3). 281 MTAs had $-\log_{10}(P)$ values ≥10 and 46 MTAs had $-\log_{10}(P)$ values ≥15, indicating that some associations between traits of interest and SNPs were highly significant (Fig. 2, Fig. S2,S3, Table S2).

The most striking finding from our GWAS is that there is a convergence of MTAs for mesocotyl length in laboratory and field experiments, and with percentage seedling emergence and shoot dry weight in the field, that all lie in the same region on the short arm of chromosome 7 (Fig. 2). 10 MTAs on chromosome 7 with $-\log_{10}(P)$ values of ≥7.2 showed association with percentage seedling emergence at least two of the three depths, while 5 MTAs common at all three depths crossed a -log (P) ≥7.2 significance threshold value. A ~ 4.3 Mb region on chromosome 7 constituting 62 MTAs was significantly associated with mesocotyl length. A total of 16 MTAs (Table S3) were common to the three different sowing depths in the field and the laboratory with a $-\log_{10}(P)$ value ≥7.2 in 50% or more of the four experiments. These data suggest that one or more major QTL situated on the short arm of chr. 7 control mesocotyl length, seedling emergence and shoot dry weight across all planting depths and these are known to be key traits for rice seed vigour in dry DSR [9].

QTL were also identified for other important agronomic traits in the field such as flowering time, plant height and grain yield. MTAs within a 126 kb region on the short arm of chr. 6 showed a significant ($-\log_{10}(P) \geq 7.2$) association with flowering time at all three sowing depths. MTAs within a 73 kb region on the long arm of chr. 1 showed a significant ($-\log_{10}(P) \geq 7.2$) association with plant height at all three sowing depths. MTAs in a 41 kb interval region on the long arm of chr. 2 also showed a significant ($-\log_{10}(P) \geq 7.2$) association with grain yield at all three sowing depths. Multiple MTA were also identified for root dry weight and number of nodal roots that lie above the $-\log_{10}(P) \geq 7.2$ significance threshold (Table S2). These MTAs were observed less consistently between sowing depths. Three MTAs on the long arm of chr. 4 ($-\log_{10}(P) \geq 7.2$) associate with number of nodal roots and root dry weight. MTAs are also collocated on the long arm of chr. 2 for number of nodal roots, root dry weight and grain yield at different sowing depths (Table S2).

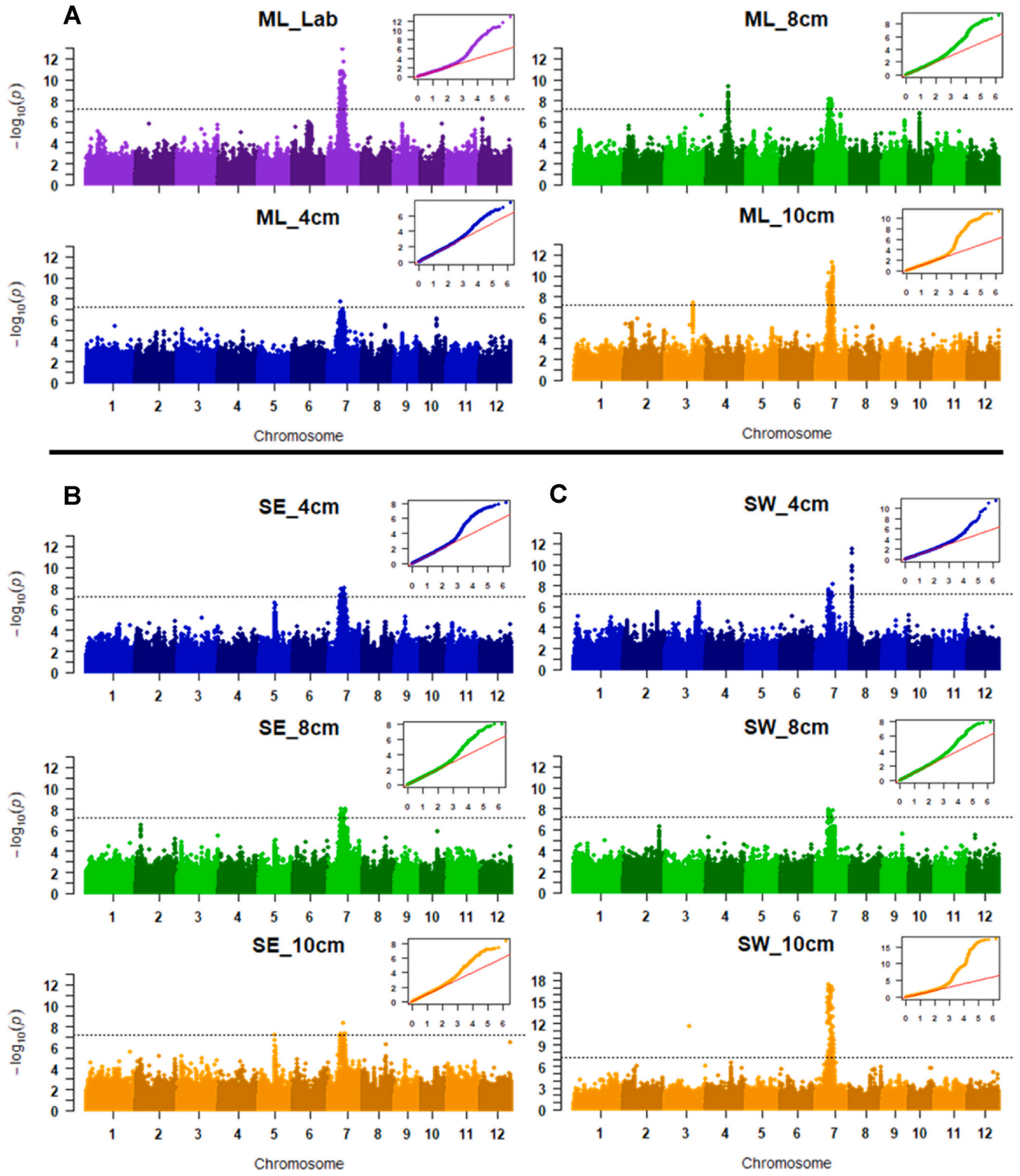

Fig. 2. Manhattan plot and QQ plot for the various seedling establishment traits under dry direct seeded conditions. (A) Mesocotyl length (ML) at 4 cm, 8 cm, 10 cm, and in laboratory conditions (Lab). (B) Percentage seedling emergence (SE) at 4 cm, 8 cm, and 10 cm. (C) Shoot dry weight 30 days after sowing (SW) at 4 cm, 8 cm, and 10 cm.

2.4. Haplotype analysis for mesocotyl length in both laboratory and field

Given that there is a convergence of MTAs for mesocotyl length in laboratory and field within a single region on chr. 7, we decided to perform a combined haplotype analysis. We identified 30, 86, 303 and 196 MTAs in this region for 4 cm, 8 cm, 10 cm field and laboratory measurements, respectively that were above the -$\log_{10}$(P) 7.2 threshold. Venn diagrams showed that 16 MTAs are common to all four data sets

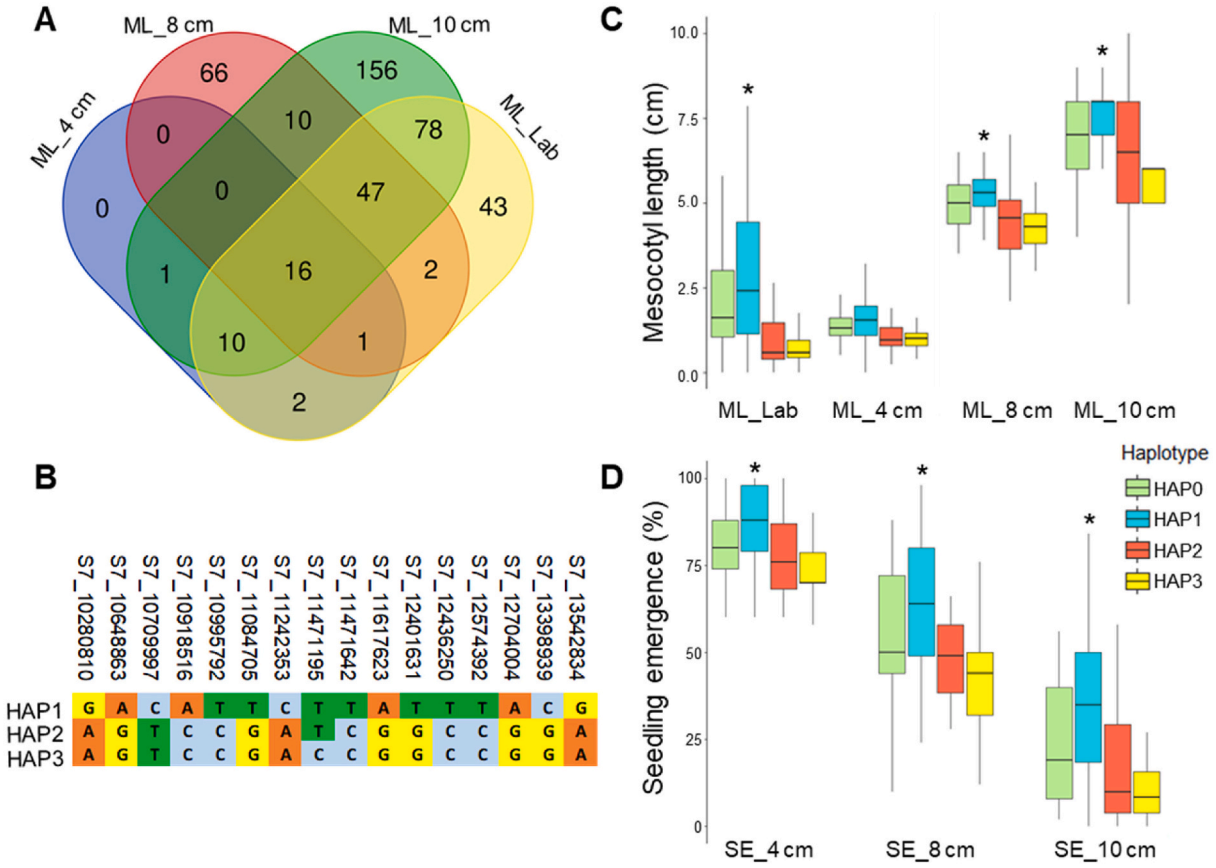

Fig. 3. Haplotype analyses for mesocotyl length in laboratory and field. (A) Venn Diagram analysis to identify common MTAs across all four data sets for mesocotyl length in laboratory (ML), 4, 8 and 10 cm. (B) Haplotype analysis of 16 core set of SNPs with a logP>6 reveals 3 groups, HAP1, HAP2 and HAP3. (C) Variation in mesocotyl length (ML) in *indica* sub-group for each haplotype across the 4 datasets, laboratory, 4, 8 and 10 cm. (D) Variation in seedling emergence (SE) as a percentage in *indica* sub-group for each haplotype across the 3 datasets, 4, 8 and 10 cm soil depth. HAP0 refers to the absence of HAP1, 2 or 3. *$p < 0.05$ (One-way p-value with pairwise comparisons).

(Fig. 3A). Among those MTAs that are shared by three of the four data sets, the largest number (36 MTAs) are common to 8 cm, 10 cm and laboratory (Fig. 3A), while 36 MTAs were common across both 8 and 10 cm depth and the laboratory with a -$\log_{10}$(P) value ≥7.2 in at least two of the three experiments (Table S4) The allelic variations for each of the 16 MTA that were common to 4, 8, 10 cm and laboratory were extracted from the hapmap that was previously used to perform the GWAS. The bulk of the panel of 684 accessions used in our study carry either HAP1: "GACATTCTTATTTACG", HAP2: "AGTCCGATCGGCCGGA" or HAP3 "ACTCCGACCGGCCGGA" (Fig. 3B).

The frequency of HAP1, HAP2 and HAP3 are 0.19, 0.55 and 0.08, respectively in 684 accession panel. We also determined haplotype frequency within the *indica*, *aus/boro* and *japonica* subgroups. This analysis revealed that HAP1 is common in *indica* (0.44) but it is absent from both *aus/boro* and *japonica* subgroups. HAP3 is rarer in *indica* (0.19) and is also absent from both *aus/boro* and *japonica*. HAP2 is also rarer in *indica* (0.11) but it is common in *aus/boro* (0.81) and *japonica* (0.82). Box plots of both the laboratory and field mesocotyl length data show that HAP1 is associated with a longer mesocotyl in *indica*, which is represented by a group of 266 accessions in the panel (Fig. 3C). This positive association of HAP1 also extends to percentage emergence in the field (Fig. 3D). These data suggest that HAP1 represents a superior haplotype for tolerance to deep seeding in dry direct seeded conditions and that HAP1 is present within the *indica* subgroup. However, HAP1 is absent from the 131 *aus/boro* and 280 *japonica* accessions that we screened in this study and it is only present at a frequency of ~0.04 in all *aus/boro* and *japonica* accessions from the 3K-RG.

2.5. Candidate genes for mesocotyl length in field and laboratory

Given that HAP1 appears to be a good predictor of tolerance to deep seeding within the *indica* subgroup we searched further for candidate genes associated with this haplotype. We extracted gene models neighbouring both the 16 MTAs that are fully conserved between the mesocotyl length data for laboratory and 4 cm, 8 cm and 10 cm planting depth in the field (Table S3) and the 35 MTAs conserved between laboratory, 8 cm and 10 cm. For the core set of 16 MTAs the neighbouring gene models include genes encoding three proteins of unknown function, and ankyrin domain containing protein, two F-box containing proteins, a putative glycosyl hydrolase, a protease inhibitor protein and six retrotransposons. Gene models neighbouring the additional set of 35 MTAs are listed in Table S4. The expression levels of the 16 neighbouring genes, based on public data sets (IC4R Information Commons for Rice), are listed in Table S5.

3. Discussion

In this study we screened 684 accessions from the 3K-RG population for variation in multiple traits associated with seedling vigour in both the laboratory and in the field at three planting depths. GWAS performed on these data sets revealed that the strongest MTAs for mesocotyl length measured in the laboratory and the field at 4, 8 and 10 cm planting depth, as well as percentage seedling emergence and shoot dry weight at all depths, were co-located in the same region on the short arm of chr. 7. These data suggest that one or more QTL that are important for seedling vigour under dry direct deep seeded conditions in the field are

present in this region, and that mesocotyl length measured in the laboratory is a good marker for this trait. The Pearson's correlation coefficient between laboratory mesocotyl length and percentage emergence in the field at multiple planting depths was around 0.5 in our study. The high throughput laboratory phenotyping pipeline that we have developed is advantageous because it is more time and cost effective than field phenotyping. Field performance is also influenced by environmental variables such as soil type and climate. Previous studies also suggest that mesocotyl length is an important trait for deep seeding [15]. However, our study is unique in that we have screened a large diversity set at multiple planting depths in field conditions and compared this data to laboratory measurements performed simultaneously on seed from the same batches.

In a common region on the lower arm of chr. 7, we identified 16 SNPs that are strongly associated with mesocotyl length in the lab and with all seedling vigour traits at all planting depths in the field. These SNPs constitute a haplotype (HAP1) for superior vigour in dry direct seeded conditions that is only present in accessions from the *indica* subgroup in our population, and not those of *aus/boro* or *japonica*. Even within the complete 3K-RG set HAP1 rarely occurs outside of *indica*. This finding is surprising given that accessions with both the longest mesocotyl in the laboratory and best vigour in field experiments tended to be *aus/boro* and accessions from this subgroup are often considered to be good donors of resilience alleles for breeding purposes. Nevertheless, *indica* accessions within our population also exhibited wide variation in mesocotyl length and percentage emergence in the field. Given that most modern cultivated semi-dwarf varieties are *indica* it may be advantageous to use HAP1 for breeding DSR, since this strategy is less likely to be prone to linkage drag than utilising *aus/boro* donors.

More than 40 mesocotyl length QTL have also been reported across all 12 chr. (Fig. 4). Despite this, only a few genes that control mesocotyl length have been cloned and in only the case of GSK3 (a glycogen synthase kinase-like kinase) was the candidate gene initially identified using natural variation [13]. Other examples are GLY1 (a phospholipaseA1) and PAO5 (a polyamine oxidase), which were identified by mutagenesis and transcriptomics, respectively [17,18]. In all three cases these genes are associated with phytohormone metabolism or signalling, and none locate in the same position as the QTL we have detected on chr 7. In contrast, both Zhao et al., [12] and Liu et al., [19] recently reported QTL for mesocotyl length at 6 cm depth in soil that are also located between 10 and 14 MB on chr. 7. We detected a 4.3 Mb region on chromosome 7 associated with mesocotyl length constituting 16 common SNPs under field and laboratory conditions. We identified a strong association of nine of these SNPs (Table S2) with three seedling traits, emergence, mesocotyl length and SDW at different depths across different environments (lab and field) signifying a high correlation among these traits. The accessions possessing longer mesocotyl length showed better germination from deep sowing depths and more vigorous growth as exemplified by SDW. The earlier reported QTLs, QTL for mesocotyl and coleoptile length [20], shoot dry weight and length [21] were observed to be located in the 3.99 Mb hotspot region on chr. 7, indicating the role of mesocotyl length in improving vegetative vigour. Interestingly the SNPs associated with *qFML7–1* (12552125) and *qFML7–2* (13602658–13,746,039, 13,729,329) as reported for mesocotyl length by Zhao et al. [12] were common with the present study.

In addition to seedling vigour QTL, we also found major QTL for days to flowering, plant height and grain yield on chr. 6, chr. 1 and chr. 2 respectively, that are conserved across all three planting depths. The QTL for flowering time co-locates with previously reported QTLs, *Lhd1 (t)* for flowering [22,23], *En-Se1* for days to heading [24] and *qGYD-6-1* for grain yield [25]. The 73 kb interval associated with plant height on chromosome 1 colocalized with the gibberellin 20-oxidase encoding semi-dwarfing gene (*Sd1* [26]) and with another QTL region, *ph 1* [27] *Fh1–2* [28]; *qPHT-1*, *qPHT1–1* [29]; *ph 1.1* [30]; QHB [31] (Supplementary Table S4). The colocation of identified genomic regions/significant marker-trait associations for GY and root traits on chromosome

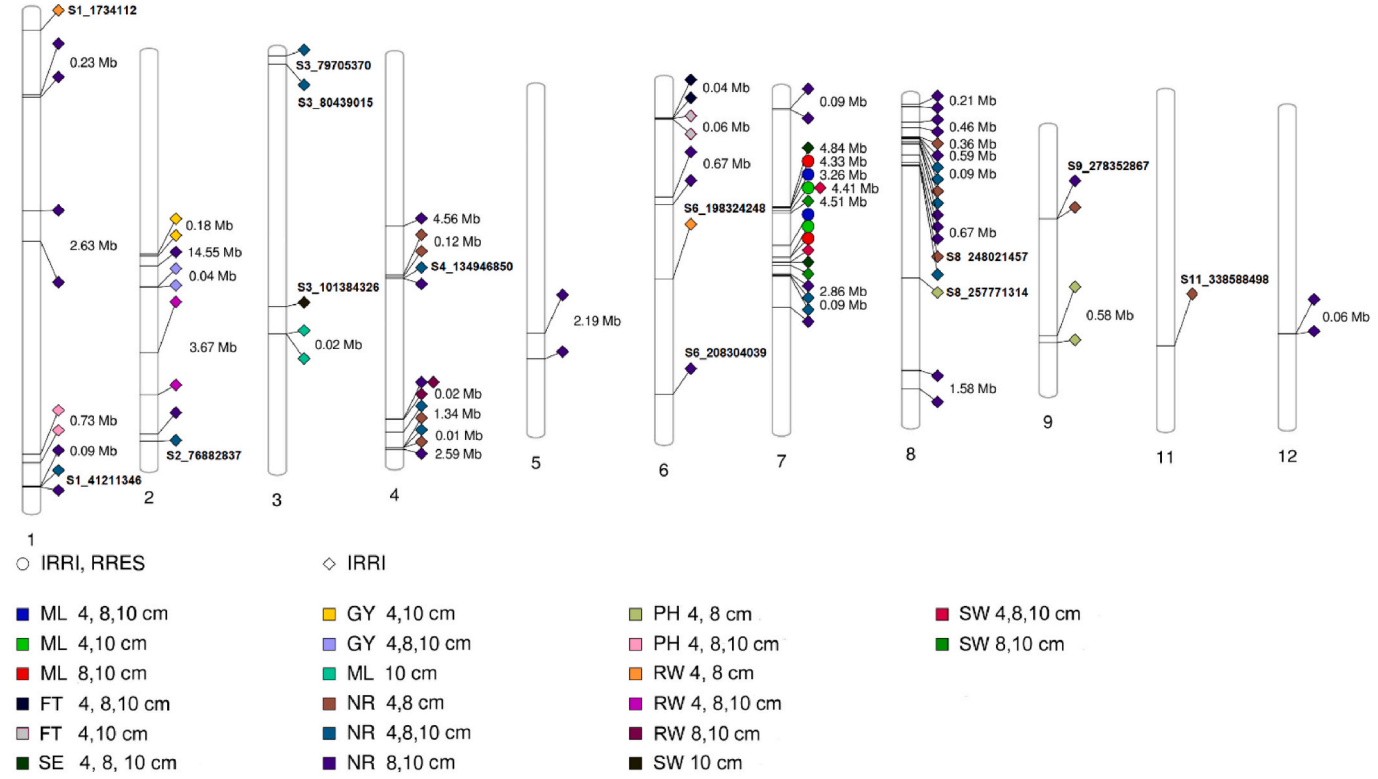

Fig. 4. A schematic chromosome map showing genomic regions where MTAs for seedling and agronomic traits have been identified in rice. The numbers below each chromosome indicate chromosome number. ML = mesocotyl length, FT = flowering time, SE = percentage seedling emergence, GY = grain yield, NR = nodal root number after 30 days, PH = plant height, RW = root dry weight after 30 days, SW = shoot dry weight after 30 days.

2 with the earlier reported QTLs/genes for eating quality [32]; source activity [33]; tillering and flowering [34]; pollen development (MTR1)/ spikelet fertility [35,36], grain related traits [37,38], and root development and nutrient uptake/translocation [39–52] further confirms the role of these correlated traits in improving grain yield and quality, nutrient uptake and adaptability under DSR (Table S2). The colocation of genomic regions associated with the root traits and GY signifies the relatedness of genetic regions associated with source and sink capacities under DSR (Table S2). These novel or previously reported MTAs/ candidate genes may help rice researchers in harnessing their potential to be further used in genomics-assisted selection.

The prevalence of the PTR cultivation system may have resulted in the loss of the long mesocotyl character as observed for many accessions in our study that showed poor seedling emergence and establishment in the field, when sown at a soil depth of 8 and 10 cm. Previous studies have also reported a low emergence rate in semi-dwarf rice varieties when sown deep [53–57]. Nevertheless, significant differences among the genotypes were observed for most of the measured traits indicating that considerable genetic variation exists in the population that can be harnessed. The improved emergence from deeper sowing depth, improved seed vigour and haplotypes that accurately predict field performance explored in the present study can help accelerate breeding for dry direct seeded rice.

4. Material and methods

4.1. Plant material

A sub-set of 684 re-sequenced accessions from the 3K Rice Genomes (3K-RG) representing the extent of both genetic and phenotypic diversity in the core collection was selected from tropical and temperate *japonica (n = 266)*, *indica (n = 266)*, *aromatic (n = 7)*, *aus/boro (n = 131)* and *admix (n = 14)* subgroups that were collected from 66 countries. All seed lots were produced in the field at the International Rice Research Institute (IRRI).

4.2. Laboratory phenotyping of rice seedlings

All laboratory experiments were performed at Rothamsted Research, Harpenden, UK.

Rice seeds from each accession were subjected to a 48-h period of incubation at 50 °C to break dormancy, subsequently sterilised using 10% sodium hypochlorite using a bespoke high throughput sterilisation system, incubated in water at 28 °C in the dark for 48 h.

Seed were then placed on a 25 cm square plate (Corning) containing sterile Gellan gum gel (FUJIFILM Wako Pure Chemical Corporation) at 2 g/L in H_2O, (pH 5.0) with the addition of $CaCl_2$ to a final concentration of 4 mM. Barcodes were used to track the accessions. Plating was performed using a defined grid ensuring consistent positioning of the seed across the plate between plates and experiments. Plates were sealed using micropore tape and placed in a light tight bag (BLK1215HEAVY 300 mm × 375 mm x 100mu/ Polybags.co.uk) sealed to ensure complete darkness. Plates in batches of 20 were arranged upright (80^o) and kept in complete darkness in black boxes and placed on shelves in a randomized design in a Sanyo growth cabinet at constant 28 °C and 80% relative humidity. After 5 days, the plates were unwrapped and photographed using a high-resolution camera (Nikon D5300 + 50 mm Nikkor) mounted on a photobench equipped with additional light for better exposure. The camera was controlled by opensource software DigiCamControl 2.0. Images were stored in TIFF format.

All 684 accessions were replicated at least 3 times with some accessions replicated 4 times. Allocation of the 4-rep lines was balanced across the blocks to enable a more efficient estimate of batch-to-batch variation. Batches were randomly allocated to cabinet run. Randomizations were obtained from CycDesigN v5.0 [58].

The platform allowed two people to screen >240 accessions per week. 2–3 days were needed to extract and exploit the data, depending on user experience. We calculated that the whole pipeline for growth and image analysis (below) increases the throughput by ~5-fold as compared to fully manual screening. Using the data from our platform, we calculated the generalized estimate of heritability to be 0.963 for ML_Lab. The mesocotyl length measured for IR64–21 across our screen was 1.12 ± 0.06 cm (Mean ± SD, $N = 38$).

4.3. Image analyses

Images were automatically pre-processed using an in-house FIJI (ImageJ) [59] script. In brief, the barcode of each accession was read and used to rename the segmented image corresponding to the accession name. Individual images were then transformed in binary mode and segmented further to allow analysis of root and shoot tissues of the seedling separately. The SmartRoot plugin [60] in the opensource software ImageJ modified for rice, was used to achieve semi-automatic measurement for all traits.

4.4. Analysis of laboratory data

Any measurement below 1.44 mm was considered spurious and replaced as 'missing' implying the seed did not germinate. Predicted values of each trait for each germplasm were obtained from linear mixed models estimated via REML [61] to then be used in the GWAS. Random effects included the structure: Cabinet/Run/Box/Position. Line was included as a fixed effect and assessed via Kenward-Roger approximate F-tests. To estimate heritability, Line was included as a random effect and calculated by the method of Cullis, Smith & Coombes [62]. Where necessary, traits were first transformed to ensure homogeneity of variance. Analysis was done using asreml-R v3.0 and Genstat 19th Edition.

4.5. Field preparation and growth conditions

The field phenotyping experiment was conducted at IRRI during the 2017 wet season (WS) and 2018 dry season (DS). The soil type in the experimental field was Maahas clay loam; isohyperthermic mixed typic tropudalf. The experiment was conducted in α-lattice design (8 × 119) in 1.8-m single row plot with 2 replications maintain 20 × 20 cm plant spacing in 2017WS. A total of 160 accessions with good germination at 4 (100%), 8 (>80%) and 10 cm (>60%) with good mesocotyl length, number of nodal roots and root length were selected for screening in 2018DS. The experiment was planted in two replications, randomized complete block design in 3 m of 3 rows plot maintaining 20 × 20 cm plant spacing.

Land preparations involved ploughing using terra disc plough followed by 3 rotovations at weekly intervals. The field was laser levelled and allowed for the first-flush of weeds to emerge and grow for 3 weeks, then controlled with the application of glyphosate (1.0 kg ai ha^{-1}). Furthermore, a combination of pre-emergence (Oxadiazon at 0.5 kg ai ha^{-1} at 6 days after seeding (DAS)), early post emergence (Bispyribac sodium 0.03 kg ai ha^{-1} (9.7%, nominee) at 11 and 22 DAS) and spot weeding at 35 and 55 DAS was used to control weeds. Integrated pest management practices involving rat baiting using ditrac (0.05 g kg^{-1} = 0.005% brodifacoum) bait to control rats, pre-seeding application of Fipronil (0.075 kg ai ha^{-1}) along the bunds and at 7 DAS along the plot edges was followed. The seeds were sown at three different seeding depth; 4 cm, 8 cm, and 10 cm using Duncan Seeder (Fig. S5). The experiment was sprinkler irrigated during the seedling establishment stage and thereafter flooded as required.

4.6. Phenotyping of field grown plants

The number of emerged seedlings (% Emergence) per plot was recorded daily starting from 4 DAS until 15 DAS. Destructive sampling of six-eight plants per plot was performed at 30 DAS to evaluate early

root and shoot traits (Fig. S5). Shoots were separated from the roots and subsequently oven dried at 70 °C until a constant shoot dry weight (in grams) was observed and recorded, while the roots were cleaned thoroughly for root trait evaluation. Total number of nodal roots (NR), total root length (RL), and mesocotyl length (ML) was recorded. Nodal roots were counted manually, total root length and mesocotyl length was measured with a centimetre scale (cm) for six random plants sampled per plot. The roots were then oven dried at 70 °C until a constant root dry weight observed. Flowering time (FT) was recorded when 50% of the plants in the plot exerted their panicles. Plant height (PH) was measured as the mean height of five random plants for each plot measured from the base of the plant to the tip of the panicle during maturity stage. The plants were harvested at physiological maturity or when 80–85% of the panicles turned colour to golden yellow and the panicles at the base were already at the hard dough stage; harvested grains were threshed and oven dried for 3 days at 50 °C. Moisture content was measured using a grain moisture meter, and grain weight data were normalized to a moisture content of 14% to determine grain yield (GY).

4.7. Analysis of field data

Trial and trait-wise mean for each season was calculated using mixed model analysis in PBTools V 1.4.0. [61]. The replications and block within replication were considered as random effect and genotypes as fixed effect. Considering genotypes as random effect heritability (H), least square difference ($LSD_{0.05}$) and F-test was calculated in PBtools 1.4.0. The broad sense heritability (H) was calculated using the formula below:

$$H = \frac{\sigma^2 G}{\sigma^2 G + \sigma^2 E/r}$$

where H is the broad sense heritability, $\sigma^2 G$ is the genetic variance, $\sigma^2 E$ is the error variance and r the number of replications. The correlation analysis among traits was performed in R.

4.8. Population structure, linkage disequilibrium and genome wise analysis (GWAS)

From the available 1 million SNPs dataset (http://snp-seek.irri.org), a total of 795,745 SNPs with MAF (minor allele frequency) of >5% and missing rates <0.2 were used to estimate the genetic relationship, construction of Neighbour Joining (NJ) tree to uncover the significant marker-trait association for different component traits under dry direct seeded conditions. The PCA was carried out to detect and correct for population structure.

Model-based program in STRUCTURE V. 2.3.4 [63] software with *K* value 1 to 10, burn-in period to 50,000 and running length 10,000 was used to access the population structure of 944 accessions using a total of 7,95,745 SNPs dataset. The consistency and accuracy of the results over each was validated by a total of 10 runs for each *K*. The *K* value with maximum likelihood over the 10 runs was used to estimate the most appropriate number of clusters [64]. Principal components analysis (PCA) was performed in GAPIT V2 [16] and added iteratively to the fixed part of the model, ranging from PC1 to PC10. To calculate the distance matrix and to construct an unweighted neighbour-joining tree TASSEL5 [65] and R was used respectively.

The significant marker-trait associations (MTA) with the trait of interest were identified using CMLM (compressed mixed linear model)/P3D (population parameters previously defined) in GAPIT V2 [16] executed by R package. Identical by state (IBS) values and the relatedness matrix were used to estimate the random effect and genetic similarity of the accessions respectively. The statistical power of the association studies was further improved considering the population structure (*Q* value) and kinship matrix (*K*) estimated from the genotyping data.

The most stringent "Bonferroni Correction" method was used to correct the false positive in the genome wide association analysis even keeping the stringent *p*-value benchmark. The Bonferroni multiple test correction was performed (0.05/7,95,745, significance level of 5%/total number of markers used in analysis) and the threshold obtained was 6.28×10^{-8}.

4.9. Candidate gene identification

Significant MTAs were uploaded on either RAPD (www.rice.plantbiology.msu.edu/) or SNP-seek (www.snp-seek.irri.org/_snp.zul) and gene identities were extracted from the online analysis to predict putative candidate genes mapping to the identified genomic region.

4.10. Haplotype analyses

Haplotype analysis was performed for mesocotyl length (ML), percentage seedling emergence (SE) traits. For each experiment (Lab, 4,8,10 cm), the ML measurement were sorted by highest value and the 100 longest and the 100 shortest germplasm IRIS number were used for Venn Diagram analysis using the webtool develop by Gent University (http://bioinformatics.psb.ugent.be/webtools/Venn/). For each experiment (Lab, 4,8,10 cm), the ML GWAS results were extracted and all the SNP with a logP>6 were selected and use for Venn Diagram analysis. 16 common SNP were found to be common between the 4 depths and to have a $-\log_{10}(P) \geq 7.2$ in at least 2 of the 4 experiments. For each set of common germplasm obtained from the Venn Diagram analysis, hapmaps formed of the 16 common SNPs haplotypes were extracted using TASSEL5 [65] and exported as a tabular file. The haplotypes were then clustered in group forming HAP1, 2 and 3 and the frequency of occurrence of each group was calculated within the whole collection screened. The range of variation of ML and SE for each haplotype was then extracted from each experiment (lab, 4,8,10 cm) and visualised using the R package ggplot2. Each trait was analysed through ANOVA to test i) for differences between the three subgroups and ii) within each subgroup is there a difference in the haplotypes. Analysis has assumed no design or blocking structure in the data.

Author contributions

P.E. and S.K., with input from A.K. conceived the study. N.S. performed the field experiments and related GWAS analysis. G.M. and D.A. performed the laboratory phenotyping, trait measurements and data extraction. G.M. performed related laboratory data related GWAS and haplotype analysis. K.H. performed the data processing prior to GWAS and the downstream statistical analysis of laboratory and haplotype data. N.S., G.M., P.E., S.K., and A.K were involved in the interpretation of the results. N.S., G.M., P.E. and S.K. wrote and reviewed the manuscript. All authors have read and approved the final manuscript.

Declaration of Competing Interest

The authors declare no competing interests.

Authors statement

No human or animal experiments have been performed for this study; hence no consent was necessary.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2021.11.006.

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