Genotyping-by-sequencing based QTL mapping for rice grain yield under reproductive stage drought stress tolerance

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QTLs for rice grain yield under reproductive stage drought stress (qDTY) identified earlier with low density markers have shown linkage drag and need to be fine mapped before their utilization in breeding programs. In this study, genotyping-by-sequencing (GBS) based high-density linkage map of rice was developed using two BC1F₃ mapping populations namely Swarna*2/Dular (3929 SNPs covering 1454.68 cM) and IR11N121*2/Aus196 (1191 SNPs covering 1399.68 cM) with average marker density of 0.37 cM to 1.18 cM respectively. In total, six qDTY QTLs including three consistent effect QTLs were identified in Swarna*2/Dular while eight qDTY QTLs including two consistent effect QTLs were identified in IR11N121*2/Aus 196 mapping population. Comparative analysis revealed four stable and novel QTLs (qDTY2.4, qDTY3.3, qDTY6.3, and qDTY11.2) which explained 8.62 to 14.92% PVE. However, one of the identified stable grain yield QTL qDTY1.1 in both the populations was located nearly at the same physical position of an earlier mapped major qDTY QTL. Further, the effect of the identified qDTY1.1 was validated in a subset of lines derived from five mapping populations confirming robustness of qDTY1.1 across various genetic backgrounds/seasons. The study successfully identified stable grain yield QTLs free from undesirable linkages of tall plant height/early maturity utilizing high density linkage maps.

Genetic dissection of loci underlying drought tolerance in rice will accelerate the development of new varieties with enhanced grain yield under drought stress conditions. In this context, discovery of grain yield QTLs under drought with large and consistent effect across the genetic backgrounds and environments is the most desirable step for its successful utilization in breeding programs. Rice breeding in the last 10 years at IRRI, Philippines has witnessed the identification of robust grain yield QTLs under drought stress conditions namely, qDTY1.1,2,3, qDTY2.1,3, qDTY3.1, qDTY12.1 using SSR markers. The incorporation of such positive qDTY alleles has been attempted recently into high yielding rice varieties popular in rainfed lowland and rainfed upland ecosystems in Asia. Further, drought tolerant version of IR64, Swarna, Sabitri, and Sambha Mahsuri have been developed for testing and release under national/state trials in different countries⁵.

SSR markers have been successfully applied in mapping and introgression of various QTLs including qDTYs in rice. However, this approach is time consuming and cost ineffective due to laborious gel-based genotyping making it not feasible in the present era of available cheap DNA sequencing technologies⁶. To overcome the limitations of SSR, next generation markers such as SNPs are now available to use. SNPs are more valuable and informative markers over SSR and others due to high abundance and uniform distribution in genomes, high multiplexing and ease of automation⁷. Recent progress in next generation sequencing has developed high-throughput SNP genotyping as a rapid, precise and low-cost genotyping technique to accelerate the process of QTL mapping and gene discovery in breeding populations⁸. In rice, various fixed SNP chips viz., 6 K SNP chip, 44 K SNP chip, 44 K SNP chip, 50 K SNP chip and 700 K SNP arrays⁹¹² have been developed to find association between phenotype and genotype¹¹. Further, a highly efficient and cost-effective sequence based genotyping approach called genotyping-by-sequencing (GBS) has been used for simultaneous genome wide SNP discovery and genotyping¹⁴¹⁶. GBS is now the most widely used genotypic platform for crop genomics studies based on restriction

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Table 1. Mean performances for days to flowering (DTF), plant height (PH) and grain yield (GY) of two rice mapping populations (Swarna*2/Dular and IR11N121*2/Aus 196) under non-stress (NS) and reproductive stage (RS) drought conditions. Note: Env = environment, NS = non-stress, RS = reproductive stage drought stress, P1 = recipient parent, P2 = donor parent, M = population mean, LSD_{0.05} = least significant difference at 5% confidence level, H = heritability, DTF = days to flowering in days, PHT = plant height in cm, GY (kg ha^{-1}) = grain yield in kg per hectare, SS = severe stress, MS = moderate stress.

| Population name | Season    | Env  | Stress level | DTF (Days) | P1  | P2  | M   | LSD_{0.05} | H   | GY (kg ha^{-1}) | P1  | P2  | M   | LSD_{0.05} | H   |
|-----------------|-----------|------|--------------|------------|-----|-----|-----|------------|-----|----------------|-----|-----|-----|------------|-----|
| Swarna*2/Dular  | 2016WS    | NS   | —            | 105        | 75  | 92  | 8   | 0.81       | 112 | 136 | 139 | 28.5       | 0.8 | 4600 | 4065 | 2894      | 3570 | 0.28 |
| Swarna*2/Dular  | 2016WS    | RS   | SS           | 96          | 88  | 86  | 5   | 0.62       | 70  | 88  | 97  | 12.0       | 0.46| 485  | 469  | 735       | 0.30 | 0.30 |
| IR11N121*2/Aus 196 | 2016WS | NS   | —            | 87          | 86  | 85  | 5   | 0.51       | 116 | 129 | 121 | 14.2       | 0.89| 4401 | 4314 | 4095      | 2294 | 0.36 |
| Swarna*2/Dular  | 2017DS    | NS   | —            | 97          | 81  | 90  | 10  | 0.21       | 93  | 132 | 119 | 26.1       | 0.79| 5354 | 4538 | 4740      | 3014 | 0.26 |
| Swarna*2/Dular  | 2017DS    | RS   | MS           | 95          | 89  | 99  | 9   | 0.79       | 68  | 83  | 85  | 12.0       | 0.93| 561  | 1382 | 1471      | 1622 | 0.42 |
| IR11N121*2/Aus 196 | 2016WS | NS   | —            | 85          | 90  | 86  | 5   | 0.62       | 93  | 121 | 104 | 10.9       | 0.92| 6149 | 6003 | 5971      | 2593 | 0.39 |
| IR11N121*2/Aus 196 | 2017DS | NS   | —            | 85          | 95  | 85  | 11  | 0.53       | 76  | 90  | 77  | 13.2       | 0.83| 1331 | 1685 | 1787      | 2072 | 0.41 |

Results
Phenotypic evaluation of parental lines and mapping population. Mean days to flowering (DTF), plant height (PH), grain yield (GY), heritability (H), range and least significant difference at 5% (LSD_{0.05}) in four parents of two targeted mapping populations under non-stress (NS), severe stress (SS) and moderate stress (MS) conditions during 2016WS and 2017DS is presented in Table 1. The results indicated reduction in mean grain yield of parents and lines under reproductive stage drought (RS) compared with NS conditions in all the 12 experiments clearly indicating that drought stress imposed during 2016 wet season (WS) and 2017 dry season (DS) was effective. Severity of imposed drought during 2016WS was much higher than 2017DS. Drought stress delayed mean DTF in Swarna*2/Dular population by 9 days under MS while the population was accelerated by 6 days in SS compared to their mean NS trials. Mean PH was drastically reduced under both SS and MS conditions, respectively; while that of the recipient parent (Swarna) was reduced from 4600 kg ha^{-1} under SS, to 0.0 kg ha^{-1} under MS and NS, to 4064 kg ha^{-1} (NS) to 485 kg ha^{-1} and 1382 kg ha^{-1} under SS and MS conditions, respectively; while that of the recipient parent (Swarna) was reduced from 4600 kg ha^{-1} under SS, to 0.0 kg ha^{-1} and 561 kg ha^{-1} under SS and MS, respectively. The mean GY of Swarna*2/Dular population was 2894 kg ha^{-1}, 1211 kg ha^{-1} and 1382 kg ha^{-1} in NS, SS and MS conditions, respectively with percent mean grain yield reduction varying from 83% in SS to 52% under MS compared to NS condition. The heritability (H) estimate ranged from 0.28 under SS to 0.30 under SS and 0.42 under MS for Swarna*2/Dular population.

Similarly, mean DTF, PH and GY, H, and LSD_{0.05} of parents and lines for IR11N121*2/Aus 196 population is also presented in Table 1. Mapping population showed mean GY 589 kg ha^{-1} under SS (2016WS) and 1685 kg ha^{-1} under MS (2017DS). Aus 196 (drought donor) showed 26% higher GY under MS while 116% higher GY under SS compared to IR11N121 recipient. The mean GY in IR11N121*2/Aus 196 population showed 34% and 21% higher yield than mean GY of Swarna*2/Dular population under SS (2016WS) and MS (2017DS) conditions respectively. Under NS, mean GY for IR11N121*2/Aus 196 population was also higher than GY mean of Swarna*2/Dular population in both the years during 2016 and 2017. Heritability/repeatability (H) for GY under SS was 0.49 (SS) and 0.41 (MS) while under NS ranged from 0.36 (2016WS) to 0.39 (2017DS). Mean for other yield related traits (DTF and PH) for IR11N121*2/Aus 196 population is presented in Table 1. Statistical analysis of the validation panel along with donor (Dular and Aus 196) and recipient parents (TDK1, Swarna and IR11N121) is summarized in Table 2. The mean GY of validation panel was 618 kg ha^{-1}, 1211 kg ha^{-1} and 2840 kg ha^{-1} under SS, MS and NS conditions and most of the best performing lines under drought was derived from TDK 1*2/Aus196 with an average mean of 945 kg ha^{-1}. 

GBS offers a powerful approach in producing large number of SNPs for genotyping and genetic analysis for implementing in genome wide association studies (GWAS), diversity analysis, genomic selection (GS), marker and gene discovery, genome profiling and high-resolution QTL mapping. QTL mapping through GBS using high density linkage maps has been studied for various traits like fusarium wilt resistance and sterility mosaic resistance in pigeon pea, drought tolerance in chickpea and rust resistance and flag leaf traits in wheat, plant architecture and yield traits in maize and grain weight, grain length in rice.

In the present study, we genotyped two mapping populations segregating for grain yield using a high throughput genotyping strategy called genotyping-by-sequencing approach. The identified high-quality SNP markers were used to construct high density linkage maps in order to find consistent grain yield QTLs under drought stress situation. Further, the identified stable QTL was validated in the subset of 250 lines derived from five mapping populations.
**Table 2.** Trial means for DTF, PHT and GY parameters analyzed from 250 lines derived from five mapping populations (TDK1*2/Dular, TDK1*2/Aus196, Swarna*2/Dular, IR11N121*2/Aus 196, IR11N121*2/Dular) under NS and RS conditions. Note: DTF = days to flowering, PHT = plant height in cm, GY (kg ha⁻¹) = grain yield in kg per hectare, NS = non-stress, SS = severe stress, MS = moderate stress, DS = dry season, WS = wet season, M = population mean, LSD₀.₀₅ = least significant difference at 5% confidence level, H = heritability.

| Designation | DTF (days) | PHT (cm) | GY (kg ha⁻¹) |
|-------------|------------|----------|---------------|
|             | 2017/DS    | 2017/WS  | 2017/DS       | 2017/WS       | 2017/DS       | 2017/WS       |
| Dular       | 80         | 81       | 80            | 82            | 135           | 99            | 162           | 137           | 6679         | 2673         | 4069         | 1087         |
| Aus196      | 87         | 89       | 89            | 93            | 117           | 87            | 146           | 105           | 7617         | 1365         | 4314         | 871          |
| Swarna      | 100        | 112      | 110           | 117           | 84            | 65            | 104           | 86            | 7121         | 371          | 4500         | 258          |
| TDK1        | 91         | 103      | 106           | 116           | 100           | 70            | 120           | 92            | 6569         | 500          | 4891         | 343          |
| IR11N121    | 88         | 85       | 93            | 100           | 101           | 75            | 111           | 80            | 5706         | 1149         | 4743         | 679          |
| Anjali       | 83         | 75       | 85            | 69            | 100           | 82            | 125           | 81            | 4151         | 748          | 3900         | 232          |
| M           | 86         | 88       | 91            | 95            | 118           | 86            | 144           | 105           | 5814         | 1492         | 4228         | 514          |
| LSD₀.₀₅     | 9          | 10       | 8             | 15            | 18            | 15            | 32            | 49            | 2840         | 1211         | 2446         | 618          |
| H           | 0.87       | 0.89     | 0.85          | 0.84          | 0.84          | 0.79          | 0.39          | 0.42          | 0.54         | 0.56         | 0.37         | 0.57         |

High throughput sequencing and SNP discovery. A total of 41.2 GB (307.64 million reads) and 34.8 GB (276.98 million reads) sequencing data were generated for Swarna*2/Dular and IR11N121*2/Aus 196 mapping populations, respectively (Supplementary Table S1). The sequence reads from individual lines mapped to reference genome varied from 0.23 to 11.05 million reads in Swarna and 0.01 to 1.31 million reads for IR11N121*2/Aus 196. The sequence reads were mapped to reference genome Nipponbare IRGSP1.0 (http://rapdb.dna.affrc.go.jp/) and aligned, cleaned GBS reads were used in pipeline for SNP calling. The alignment of reads to reference genome for both the populations used in this study are provided in Supplementary Tables S2 and S3. As a result, a total of 81,152 and 52,169 SNPs was identified for Swarna*2/Dular and IR11N121*2/Aus 196 mapping populations, respectively. Further, SNPs were filtered out for missing data (≤ 90%) and minor allele frequency (MAF) at threshold of 0.05 and in total, 6243 and 4247 high quality SNPs were generated for both the populations to validate the allelic variations between parents and lines (Supplementary Table S4 and S5). There is a significant reduction in the numbers of SNPs from several thousands to few thousands, due to stringent selection criteria used in the present analysis. SNP calls from a nucleotide-based format were converted to parent-based format using ABH-plugin in TASSEL pipeline. After converting the generated SNPs into parent-based format, in total 3929 SNPs (Swarna*2/Dular) and 1191 SNPs (IR11N121*2/Aus 196) with contrasting alleles in parental genotype were retained to be used for construction of linkage maps.

SNP based high density linkage maps. The genome wide polymorphic SNPs were evaluated for expected segregation ratio using Chi-square analyses in both Swarna*2/Dular and IR11N121*2/Aus 196 populations. For the Swarna*2/Dular population, a total 3929 SNPs with contrasting alleles between the parents were mapped on all 12 chromosomes while 1191 polymorphic SNPs were mapped for IR11N121*2/Aus 196 population (Supplementary Tables S6 and S7). In total, 20 SNPs for Swarna*2/Dular and 8 SNPs for IR11N121*2/Aus 196 population had shown segregation distortion and unable to locate on their respective linkage maps. The total length of genetic map computed for Swarna*2/Dular population was 1454.68 cm (varied from 89.60 cm in chromosome 9 to 169.52 cm in chromosome 1). Average genetic distance between two SNPs was 0.37 cm across the chromosomes, reflecting its utility in fine mapping of QTLs/gene. The number of mapped SNPs to each chromosome for this population. A calculated average genetic distance between two SNPs across the chromosomes was 1.18 cm (ranged from 0.70 cm on chromosome 4 to 2.12 cm on chromosome 6). The developed high-density genetic maps using filtered polymorphic SNPs were integrated with data for grain yield under drought and its associated traits for QTL analysis (Fig. 1).

QTL analysis. Swarna*2/Dular population. A total of six qDTY QTLs were identified for GY under severe (SS) and moderate (MS) drought conditions through composite interval mapping (CIM) during the years of 2016 and 2017. Over the two years of testing, three drought QTLs (qDTY₁, qDTY₃, and qDTY₆) were detected in both SS and MS conditions during 2016WS and 2017DS while the remaining three QTLs (qDTY₂, qDTY₄, and qDTY₅) were found only under SS condition during 2016WS (Table 4 and Fig. 2). These identified QTLs explained phenotypic variance (PVE) from 4.34 to 13.50% with LOD scores ranging from 2.87 to 32.05. The majority of the GY QTLs under SS and MS had positive additive effect indicating that alleles contributed from parent Dular increased the phenotypic values. Three QTLs namely qDTY₁, qDTY₃, and qDTY₆ were identified as a consistent effect grain yield QTL expressed across the seasons under both SS and MS conditions of drought. The consistent effect QTL qDTY₁ within the marker interval of S₁_40013502--S₁_41216734 was positioned at 159.9–161.4 cm on chromosome 1 and explained PVE ranging from 9.45% to 10.90% with LOD scores of 3.13 to 3.89 during 2016 and 2017 respectively. The QTLs qDTY₃ detected on chromosome 3 with PVE 13.50%
and qDTY₆ on chromosome 6 with PVE of 8.62% were novel and had consistent effect on grain yield under drought. Two QTLs qDTY₁,₂ and qDTY₁,₁ explained the highest % PVE for GY under drought from Swarna*2/Dular population.

Five QTLs for days to flowering (DTF) including two stable ones were expressed under both SS and MS conditions while three QTLs for PH were identified in MS conditions only (Table 4 and Fig. 2). Two significant QTLs (qDTF₁,₁ and qDTF₁,₃) for DTF mapped under both SS and MS conditions were consistent and one of them (qDTF₁,₃) was located at similar genetic locations with the grain yield QTL qDTY₁,₁ in Swarna*2/Dular population. One of the DTF QTL (qDTF₁,₃) detected under SS in marker interval of S1_42655097S1_42885648 was detected nearly 1 Mb away from the GY QTL under drought, qDTY₁,₁ identified in Swarna*2/Dular population. Phenotypic variations explained by DTF QTLs were significantly low (4 to 6%) as compared to GY QTLs under SS conditions while three QTLs for PH were identified in MS conditions only (Table 4 and Fig. 2). Two significant QTLs (qPH₁,₁ and qPH₁,₃) were detected with PVE ranged from 3 to 27% under MS on chromosomes 1 and 5. No stable QTL for PH under drought had been identified in Swarna*2/Dular population.

IR11N121*2/Aus 196 population. A total of eight qDTY QTLs for GY under severe (SS) and moderate (MS) drought conditions were detected from IR11N121*2/Aus 196 population during the years of 2016WS and 2017DS (Table 5 and Fig. 3). These QTLs explained from 4.56 to 14.92% of the PVE and were distributed over the six chromosomes (1, 2, 3, 4, 8 and 11). Four QTLs (qDTY₁,₁, qDTY₁,₄, qDTY₁,₃ and qDTY₁,₆) were detected under SS in 2016 and two QTLs (qDTY₁,₂ and qDTY₁,₃) identified under MS in 2017. Two QTLs (qDTY₂,₁ and qDTY₂,₁₂) was found as a stable QTLs identified in both the environments (MS and SS) during 2016 and 2017. The stable QTL qDTY₂,₁ was identified nearly 1 Mb away from the GY QTL under drought, qDTY₁,₁ identified in Swarna*2/Dular population. Co-localization of qDTY QTLs. Many of the previously reported qDTY QTLs including qDTY₁,₁ were linked with undesirable alleles of tallness/earliness and fine mapping approach was to be followed before introgression of any such QTLs. In the present study, we have detected four stable QTLs (qDTY₁,₂, qDTY₁,₃, qDTY₆,₄ and qDTY₁,₁₁), One QTL (qDTY₁,₂) was co-located at the same position as previously reported. We found that qDTY₂,₁ was linked with the QTL for DTF (qDTF₁,₁) and qDTY₁,₁₁ with the QTLs for DTF (qDTF₁,₁) and PH (qPH₁,₁). Validation of qDTY₁,₁ allele across the five mapping populations. We identified a common grain yield QTL (qDTY₁,₁) between 40.01 to 42.11 Mb (1.2 Mb) expressed in both the mapping populations (Swarna*2/Dular and IR11N121*2/Aus 196). qDTY₁,₁ was also detected in Swarna*2/Dular population.

| Chromosome Number | Filtered SNPs | SNPs mapped | Distance (cM) | Average marker distance | Filtered SNPs | SNPs mapped | Distance (cM) | Average marker distance |
|-------------------|--------------|-------------|--------------|------------------------|--------------|-------------|--------------|------------------------|
| 1                 | 786          | 484         | 169.52       | 0.35                   | 547          | 171         | 168.97       | 0.99                   |
| 2                 | 590          | 360         | 140.63       | 0.39                   | 410          | 95          | 130.23       | 1.37                   |
| 3                 | 477          | 307         | 142.53       | 0.46                   | 314          | 79          | 139.23       | 1.76                   |
| 4                 | 610          | 417         | 139.16       | 0.33                   | 482          | 198         | 139.16       | 0.70                   |
| 5                 | 492          | 348         | 116.67       | 0.34                   | 326          | 116         | 110.15       | 0.95                   |
| 6                 | 579          | 367         | 121.80       | 0.33                   | 257          | 47          | 99.53        | 2.12                   |
| 7                 | 511          | 311         | 113.32       | 0.36                   | 329          | 108         | 109.84       | 1.02                   |
| 8                 | 345          | 230         | 110.37       | 0.48                   | 216          | 60          | 107.87       | 1.80                   |
| 9                 | 412          | 300         | 89.60        | 0.30                   | 309          | 99          | 88.87        | 0.90                   |
| 10                | 459          | 245         | 90.15        | 0.37                   | 385          | 78          | 89.60        | 1.15                   |
| 11                | 548          | 310         | 113.27       | 0.37                   | 369          | 58          | 110.40       | 1.90                   |
| 12                | 434          | 250         | 107.66       | 0.43                   | 303          | 82          | 105.78       | 1.29                   |
| Total             | 6243         | 3929        | 1454.68      | 0.37                   | 4247         | 1191        | 1399.68      | 1.18                   |

Table 3. Features of the genetic maps in Swarna*2 × Dular and IR11N121*2 × Aus 196 drought mapping populations in rice.
Dular and IR11N121 (Aus 196) under RS drought conditions and it was overlapped with the chromosomal regions carrying \( q_{DTY} \) QTLs detected within 36.75–40.70 Mb earlier by Vikram et al.\(^1\). The SSR markers present within the vicinity of the newly mapped QTL region was utilized on 250 lines derived from five mapping populations to validate the \( q_{DTY1} \). Out of tested six SSRs, two markers (RM12091 and RM12146 at 40.21 Mb & 40.7 Mb respectively) were found polymorphic between the parents and were utilized for the validation. SSR markers reported in the study, presented in the vicinity of the fine mapped QTLs (1.2 Mb), free from undesirable linkages shall be utilized in the breeding programs. Lines with and without \( q_{DTY1} \) QTL and their GY data under drought is provided in Table S8. A significant difference was found in comparative mean for grain yield under drought among the lines with having \( q_{DTY1} \) and lines without having \( q_{DTY1} \) QTL (Table S9). The estimation of QTL effect had shown an advantage of 473.43 kg ha\(^{-1}\) in the lines with having positive allele for \( q_{DTY1} \) compared to the lines without \( q_{DTY1} \) QTL (Table S9).

**Candidate genomic regions for breeding for drought tolerance.** In this study, three novel \( q_{DTY} \)s (\( q_{DTY2}, q_{DTY3}, \) and \( q_{DTY6} \)) constitutively expressed under variable situations of drought from moderate to severe conditions were found consistent on grain yield under drought with the maximum variation of 14.92% explained by \( q_{DTY2} \). The QTL size for above mentioned QTLs was varied from 0.1–1.0 Mb and free from undesirable linkages to plant height. One of the stable QTL (\( q_{DTY1} \)) detected between 40.01 to 41.21 Mb (1.2 Mb) in this study was located near to the previously mapped \( q_{DTY1} \) within 36.75–40.70 Mb on the long arm of chromosome 1. The undesirable linkages such as tallness and earliness in maturity started from 36.70–38.89 Mb has been linked with drought QTL (\( q_{DTY1} \)) mapped upto 40.70 Mb in the previous study by Vikram et al.\(^1\). The \( q_{DTY1} \) with detected in this study was free from undesirable linkages due to minimal QTL size of 1.2 Mb compared to 3.95 Mb QTL size of previous mapped by Vikram et al.\(^1\). A comparative map showing the narrowed down genomic region of previously mapped \( q_{DTY1} \) using high density linkage map in the present study has been depicted in Supplementary Fig. S1(a,b). The genomic regions underlying these fine mapped QTLs (\( q_{DTY2}, q_{DTY3}, \) and \( q_{DTY6} \)) including \( q_{DTY1} \) will be an important candidate region for its utilization in marker assisted selection, sequencing and allele mining for drought tolerance in rice.

**Discussion**

Marker assisted breeding had enormous potential to achieve desirable phenotypic variation in less time through deployment of markers linked to QTLs for desirable trait\(^{37}\). However, discovery and development of SSR markers, their scoring across populations is time consuming, labor intensive and costly process\(^{38,39}\). Even large QTL regions identified through low density SSR markers may introduce undesirable linkages through MAS and can make
the introgression line unacceptable for release and cultivation\(^\text{40}\). Such limitations of SSR marker makes it unrealistic in fine mapping of complex traits and for full use in accelerated breeding programme compare to recent available sequencing technologies at much cheaper cost. In some of the recent studies, undesirable linkages were successfully eliminated using next generation markers such as SNPs by identifying the recombinants to break

**Table 4.** Results of QTL analysis in Swarna\(^*\)/Dular backcross mapping population in rice. Note: \(^{*}\)QTLs detected in both the years (2016 and 2017) under SS and MS conditions of drought. \(^{\dagger}\)Chromosome number on which QTL was identified. \(^{\ddagger}\)The scanning position in cM on the chromosome. \(^{§}\)LOD score calculated from composite interval mapping. \(^{¶}\)Phenotypic variation explained by QTL. \(^{††}\)Estimated additive effect of QTL. \(^{‡‡}\)Dom: Estimated dominance effect of QTL. \(^{#}\)Confidence interval calculated by one-LOD drop from the estimated QTL position, DTF = days to flowering in days, PH = plant height in cm, GY (kg ha\(^{-1}\)) = grain yield in kg per hectare.

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**Figure 2.** Genotyping-by-sequencing (GBS) derived high density genetic map and distribution of QTLs associated with drought tolerance in Swarna\(^*\)/Dular population. The twelve chromosomes were shown as vertical bars and each horizontal line on the bar represent single SNP marker. Aggregation on horizontal lines reflects higher marker density on that chromosome. The scale on left side represents genetic position in cM.

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the introgression line unacceptable for release and cultivation\(^\text{40}\). Such limitations of SSR marker makes it unrealistic in fine mapping of complex traits and for full use in accelerated breeding programme compare to recent available sequencing technologies at much cheaper cost. In some of the recent studies, undesirable linkages were successfully eliminated using next generation markers such as SNPs by identifying the recombinants to break
Table 5. Results of QTL analysis in IR11N121*2/AUS 196 backcross mapping population in rice. Note: *QTLs detected in both the years (2016 and 2017) under SS and MS conditions of drought. *Chromosome number on which QTL was identified. $The scanning position in cM on the chromosome, §LOD score calculated from composite interval mapping, ¶Phenotypic variation explained by QTL, ††Estimated additive effect of QTL, ‡‡Dom: Estimated dominance effect of QTL, #Confidence interval calculated by one-LOD drop from the estimated QTL position, DTF = days to flowering in days, PH = plant height in cm, GY (kg ha⁻¹) = grain yield in kg per hectare.
linkage a favorable allele conferring drought tolerance and an unfavorable allele for tall plant height. Currently, next generation sequencing (NGS) technologies have become powerful tools for discovery of millions of SNPs in cost effective manner to develop high density linkage maps, dissect the complex traits and identify key genomic regions underlying the associated traits.

A rapid, robust and cost-effective genotypic platform called GBS is nowadays becoming more feasible in genomics assisted breeding by providing many markers for QTL/gene discovery at much cheaper rate. In this study, we have developed two high density genetic maps using GBS approach to identify QTLs for grain yield under various drought conditions (SS and MS) using multi-season phenotypic data from two mapping populations. The average inter-marker distance between two SNPs varied from 0.37 cM to 1.18 cM in both populations used in this study which may be one of the most saturated genetic maps developed for QTL identification in rice for drought tolerance. Drought donors (Dular and Aus 196) used in this study for population development belong to a distinct genetic group so called *aus*-type and known for valuable genetic resource for abiotic stress tolerance including drought. Recently, drought-responsive metabolite associated with tolerance had been identified in two *Aus* rice varieties (Dular and N22) underlying genes and pathways for drought tolerance in rice. Both donors (Dular and Aus 196) used in this study gave significantly higher yields than the recipient parents (Swarna, IR11N121 and TDK1) under various drought conditions tested over two years, suggesting their usefulness and reliability in utilization as donors.

Drought screening in rain-out shelter during 2016WS was more effective than field screening in 2017 DS and could be attributed to precise control and monitoring of the amount and timing of irrigation. Broad-sense heritability (H) for GY was moderate under SS, MS and NS conditions while it was moderate to high for DTF and PH in both the populations and in both years. Previous studies also reported moderate heritability of grain yield under drought and high heritability for DTF and PH under non-stress and stress conditions.

A total of fourteen QTLs for GY under SS and MS conditions were detected from the two populations used in this study. Most of the GY QTLs identified in this study were expressed either in SS or MS conditions of drought and only few of them were detected in both SS and MS drought across the years 2016 and 2017. Three such QTLs (*qDTY1*, *qDTY3* and *qDTY6*) for Swarna*2/Dular and two QTLs (*qDTY4* and *qDTY12*) in IR11N121*2/Aus 196 population were found stable across the environments/seasons. The lack of stability of QTL effects across the environments/genetic backgrounds has been one of the most limiting factors in successful deployment of QTLs through MAS breeding for various complex traits including drought.

One of the stable grain yield QTL *qDTY1* was identified in our study was located far away from previously mapped *qDTY1* at 30–31 Mb physical position in rice genome reported by Venuprasad et al. One of the GY QTLs *qDTY1* was identified at 0.7–0.8 Mb in Swarna*2/Dular population under SS was co-located with *qDTY1* reported earlier by Swamy et al. However, this QTL was not detected under moderate level of drought stress in the present study. It is interesting to note that we have detected QTL *qDTY1* with significant effects under varying severity of drought (SS and MS) in both the populations used in this study. Also, the physical position (S1_40013502–S1_41216734) of this QTL was nearly same of previously identified QTL *qDTY1* in a most relevant grain yield QTL under drought. It indicates authenticity, reliability of this study and usefulness of the stable QTLs under drought.

Furthermore, we have validated the effect of this QTL *qDTY1* in five alternate mapping populations derived from three genetic backgrounds (TDK1, Swarna and IR11N121) developed in this study. Consistency of *qDTY1* in multiple genetic backgrounds were also found in many previous studies in both (lowland and upland) the ecosystems of rice. The positive alleles of *qDTY1* was found in 64% and >50% of the drought tolerant lines from a panel of random drought-tolerant lines used. These findings clearly suggest that the *qDTY1* on chromosome 1 could be a hot spot for alleles with positive effect on GY under drought and useful candidate region for explore in genomics assisted breeding.

Two of the consistent QTL *qDTY2* and *qDTY3* in IR11N121*2/Aus 196 population located at 17 Mb and 27 Mb were far from *qDTY1* and *qDTY1* detected in this study, which was low, despite using the highly saturated genetic map and multiple season of precise phenotyping. However, use of high-density maps could be helpful in precise detection of QTLs for complex traits by reducing the chances of getting false positive. Similar finding was discussed in earlier reports, where up to 15% PVE was achieved using GBS based QTL mapping for fusarium wilt resistance in pigeon pea and flag leaf traits in bread wheat. Most of previously identified major effect drought QTLs except *qDTY1* had explained 10 to 30% PVE with yield advantage of 300–500 kg ha−1 under RS drought stress and QTL pyramiding looks a feasible strategy here to achieve the desired level of phenotypic variation (yield advantage of 1.0 ha−1) under severe drought stress.

In this study, a stable GY QTL *qDTY6* was co-located with QTLs for DTF *qDTF1* in Swarna*2/Dular population while *qDTY1* was co-located with *qDTF1* and *qPH1* in IR11N121*2/Aus 196 population. Many of the previous studies also reported the co-existence of drought grain yield QTLs with QTLs for DTF and PH under stress. For instance, *qDTY1* was coinciding with QTLs for DTF and PH, *qDTY1* co-located with DTF and *qDTY2* with QTLs for DTF, PH and other morphological traits. Three QTLs (*qDTY1, qDTY3, qDTY6*) found in this study were critical and desirable loci without any linkage drag and can be introgressed in multiple genetic backgrounds to find their individual and combined effects. Transfer of major grain yield QTLs under drought co-located with PH is not preferable in MAS breeding as positive alleles of tallness/earliness linked with QTLs for PH and DTF and *qDTY6* had linked with QTLs for DTF. Ingressed of QY QTLs unlinked from PH QTLs will led to development of rice varieties tolerant to drought with optimal plant stature and higher yield. The association of QTL for DTF and PH with grain yield QTLs under drought prevailed for two *qDTYs* found in this study and a suitable breeding strategy should be followed before introgression.
of such QTLs in elite varieties of rice. Recently, marker assisted linkage elimination strategy was followed to remove the undesirable linkages of PH QTLs from grain yield QTLs such as qDTY$_{1,1}$, qDTY$_{5,3}$ and qDTY$_{12,1}$.

Conclusion
The developed high-density genetic maps in this study could be a strong foundation for fine mapping of grain yield QTLs under drought stress and identified genomics regions could be utilized in breeding programs. We have identified some novel candidate genomic regions from two populations that contained four stable QTLs for grain yield under drought in multiple environments. Two novel qDTY QTLs (qDTY$_{5,3}$ and qDTY$_{12,1}$) along with qDTY$_{5,3}$ detected at same position of previously known drought QTL qDTY$_{5,3}$ in rice genome, free from any undesirable linkages have suggested the importance and utility of these QTL cluster regions for rice breeders to be utilized in MAS work and candidate gene identification for higher grain yield under drought stress situation.

Methods

Plant materials. Two drought tolerant donors (Dular and Aus196) and three recipient parents (Swarna, IR11N121, and TDK1), were utilized for the development of five BC$_{1}$F$_{3}$ bi-parental mapping populations (Swarna*2/Dular, IR11N121*2/Dular, TDK1*2/Dular, IR11N121*2/Aus196 and TDK1*2/Aus196). Dular, a drought-tolerant donor identified at IRRI is an early maturing, low yielding, blast resistant, traditional cultivar originated from India. Aus 196, an improved drought tolerant cultivar belongs to aus subspecies and originated from Eastern India. Recipient parent, Swarna is a long duration, high yielding, mega variety for rainfed and irrigated rice ecosystems of India, Nepal and Bangladesh but highly susceptible to reproductive stage (RS) drought stress. IR11N121 is a high yielding rice variety released for lowland rice ecology of South East Asia, susceptible to drought. TDK1 is a high yielding, long duration, gluttonous Lao variety, highly susceptible to drought and submergence.

Phenotypic evaluation and statistical analysis. A total of 12 experiments were conducted using five mapping populations during 2016 and 2017 under non-stress (NS) and reproductive stage drought (RS) conditions at IRRI Los Baños, Laguna, Philippines (14°30′N, 121°15′E). Two BC$_{1}$F$_{3}$ mapping populations (Swarna*2/Dular and IR11N121*2/Aus196) consisted of 350 lines each were used for phenotyping and genotyping in the process of QTL identification. A validation panel consisted of 250 lines randomly selected from five mapping populations [TDK1*2/Dular (50 lines), TDK1*2/Aus196 (50 lines), Swarna*2/Dular (50 lines), IR11N121*2/Aus196 (50 lines), IR11N121*2/Dular (50 lines)] was utilized for the confirmation of positive alleles of any stable QTLs found in the present study.

The two mapping populations (Swarna*2/Dular and IR11N121*2/Aus196) were screened under RS and NS conditions during 2016 and 2017. The 2016 wet season (WS) reproductive stage drought stress phenotyping screening was performed at IRRI rain out shelter facility while 2017 dry season (DS) screening was carried out directly in field. Experiments were laid out in augmented-RCBD design using repeated drought tolerant checks (Sahbhagi dhan) and susceptible checks (Swarna, IR64 and MTU1010), along with parents in 5-meter row plot for computation of means, LSD and heritability (H). Experiments with grain yield reduction of more than 65% (GYKGPHA) from NS and RS trials were collected and analyzed using PBTools (http://bbi.irri.org/products) for computation of means, LSD and heritability (H). Experiments with grain yield reduction of more than 65% were classified as severe stress (SS), while 31–64% yield reduction was classified as moderate stress (MS). Linear mixed model was used for analysis of variance considering the lines/genotypes as fixed and the effect of replications and blocks within replications as random.

The model used for augmented-RCBD design was:

\[ Y_{ijk} = M + G_i + B_l + E_{ilk} \]

where, \( Y_{ijk} \) is measurement recorded in plot, \( M \) is the overall mean of plot, \( G_i \) is the effect of the \( i^{th} \) genotype, \( B_l \) is the effect of the \( l^{th} \) block and \( E_{ilk} \) is the experimental error.

The model used for alpha-lattice design was:

\[ Y_{ijk} = M + G_i + R_j + B_{lj} + E_{ilk} \]

where, \( Y_{ijk} \) is measurement recorded in plot, \( M \) is the overall mean of plot, \( G_i \) is the effect of the \( i^{th} \) genotype, \( R_j \) is the effect of the \( j^{th} \) replicate, \( B_{lj} \) is the effect of the \( l^{th} \) block within \( j^{th} \) replicate, and \( E_{ilk} \) is the experimental error.

To estimate broad sense heritability (H), variance components were estimated for a model using PBTools software packages by considering all the variables and genotypes as random. Broad sense heritability (H) or repeatability was calculated as
$$H = \frac{\sigma_G^2}{\sigma_G^2 + \sigma_E^2/r}$$

where, $H$ is broad sense heritability, $\sigma_G^2$ represents the genetic variance, $\sigma_E^2$ the error variance and $r$ the number of replications.

The QTL effect on grain yield under drought stress were estimated using mixed model analysis (REML) in CROPSTAT version 7.2.3 using the lines with and without QTL. The effects of the QTL and genotypes within the QTL are considered as fixed while the replicate and blocks within replicate effects are considered random. We first computed the genotype means using PB tools by adjusting blocks in augmented RCBD design and in second stage these genotype means were used to analyze the QTL mean comparisons involving the two classes “with” and “without” QTLs using CROPSTAT.

**Leaf tissue sampling, DNA extraction and preparation of GBS libraries.** The fresh leaf tissue samples from six plants per line were collected at 42 DAS. High throughput automated leaf sampling and genomic DNA extraction from leaf tissue was performed using Brooks PlantTrak Hx rice leaf tissue sampler and LGC Genomics oKtopure systems at IRRI genotyping service laboratory. The assessment of DNA quality and quality was done by running on 1% agarose gel. Using this platform, a high-quality DNA yield ranges from 40–60 ng/µl was achieved for SNP genotyping. GBS libraries were prepared using the protocol adapted from Elshire et al. For GBS a type II restriction endonuclease (ApeKI) was used for DNA digestion, and the digested DNAs were ligated to the adapter, and then 96-plex library was constructed as per GBS protocol. GBS was carried out using HiSeq2500 sequencing platform with Macrogen Inc. (Korea).

**SNP identification and genotyping.** The sequence reads generated in FASTQ file were processed and analyzed for SNP identification using TASSELGBS analysis pipeline. Pipeline allows searching of all raw sequencing reads with perfectly matched barcode and expected remnant bp of restriction cut site and reads were further sorted, de-multiplexed and trimmed to create a unique, 64-bp long sequences called tags. These good quality tag sequences were aligned with the reference genome using Burrows-Wheeler Alignment (BWA) software, while reads carrying “N” within first 64 bases had removed from further analysis. The perfectly matched and aligned sequences was processed further for SNP calling and genotyping through GBS analysis pipeline. SNPs were further filtered for minor allele frequency (MAF) below 0.05 and for the percentage of missing data (<99%) per SNP using TASSEL GBS analysis pipeline using default parameters. Filtered data file having final set of SNPs in nucleotide-based hap map format was converted to an ABH-based format using ABH-plugin in TASSEL pipeline where “A” represents donor allele, “B” represents recipient allele and “H” represents heterozygous allele. Finally, imputed SNPs of lines were filtered against parental alleles and only polymorphic SNPs were retained to be used in construction of linkage map.

**Linkage map construction and QTL mapping.** The genotypic data for 350 lines from each mapping population with filtered SNP markers was used for linkage map construction using the linkage mapping function implemented in the QTL IciMapping software v4. The grouping and ordering of 3929 and 1191 polymorphic SNP markers for both the populations were carried out using regression mapping algorithm RECORD (REcombination Counting and ORDering) based on recombination events between adjacent markers. Further, Rippling was done for fine-tuning of the ordered markers on their respective chromosomes by sum of adjacent recombination fractions (SARF) algorithm with a default window size. QTL mapping for GY QTLs and other traits under drought was performed using composite interval mapping (CIM) functions implemented in the QTL IciMapping software v4. The threshold LOD value to declare a significant QTL was computed by a permutation test involving 1000 runs at a significance level of $p = 0.05$. After completion of permutation test, window size of 10 cM and walk speed of 1 cM was set to start analysis of composite interval mapping.

**Data Availability**

The data sets supporting the results of this article are included within the article.

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Author Contributions
S.Y. was involved in conducting the experiments, taking observations, experimental data analysis, Q.T.L. mapping and drafting the manuscript; N.S. was involved interpretation of data and revising the manuscript; V.K.S. was involved in drafting and correction of the manuscript, M.C. helped in sample preparation, S.S.R. genotyping for validation work. A.K. was involved in the design of the experiment and in the critical revision of the manuscript. All authors approved the final version of the manuscript.

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