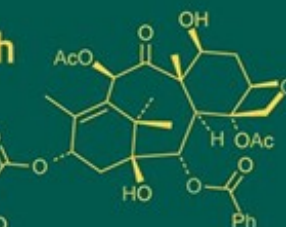


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Marker-assisted identification of *Rf3* and *Rf4* fertility restorer lines in rice (*Oryza Sativa* L.) and their evaluation for osmotic stress tolerance at the seedling stage and grain yield under rainfed drought

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ABSTRACT

Hybrid rice seed production under the wild abortive cytoplasmic male sterility (WA-CMS) system depends on restorer lines, yet most restorers in use were bred for irrigated conditions and lose yield when water is short. Sixty rice lines were screened with markers linked to the fertility restorer loci *Rf3* and *Rf4* to identify restorer candidates that also tolerate drought. The assay classified 14 lines as *Rf3* only, 18 as *Rf4* only, 10 as carrying both loci and 18 as carrying neither. The 28 *Rf4*-positive lines were grown hydroponically in a completely randomised design with three replications under control, 15% and 25% PEG-6000, and 20 of them were then evaluated for nine agronomic traits under irrigated and rainfed field conditions with three replications. Osmotic stress at 25% PEG reduced root length, shoot length and seedling biomass by 32.6 to 35.7% relative to the control, and the genotype $\times$ stress interaction was significant ($P < 0.001$) for all six seedling traits. Under rainfed conditions, mean grain yield fell from 25.86 to 22.91 g plant⁻¹ (11.4%) and unfilled grains per panicle rose by 31.5%, while days to 50% flowering did not differ between environments ($P = 0.071$). Yield reduction among lines ranged from -1.24% in ML-59 to 32.04% in ML-12. Rainfed grain yield was closely associated with spikelet fertility ($r = 0.98$) and with shoot dry weight at 25% PEG ($r = 0.97$). Lines ML-02, ML-13, ML-57, ML-32 and ML-05, three of them carrying both restorer loci, ranked highest in both seedling and field selection indices and are recommended for test crossing with WA-CMS lines and multilocation evaluation.

Keywords: Cytoplasmic male sterility, Three-line hybrid, Water deficit, Polyethylene glycol, Spikelet fertility, Selection index

Introduction

Rice (*Oryza sativa* L.) hybrids yield 15 to 20% more than the best inbred varieties grown under comparable management, and this advantage has made hybrid rice a central element of yield intensification programmes in South and East Asia (Virmani, 1994) [1]. In India, adoption has lagged behind expectation. Seed cost, inconsistent heterosis across seasons and the weak performance of many hybrids outside irrigated ecologies have all limited farmer uptake (Spielman *et al.*, 2013) [2]. Almost all commercial hybrids in the country are three-line hybrids built on the wild abortive cytoplasmic male sterility (WA-CMS) system, in which a male sterile A line is crossed with a restorer or R line carrying nuclear genes that suppress the sterility. The R line thus contributes half of the hybrid genome and much of its adaptation, and a hybrid rarely tolerates stress better than the restorer from which it was made.

Fertility restoration in the WA-CMS system is governed mainly by two dominant loci, *Rf3* on chromosome 1 and *Rf4* on chromosome 10, with *Rf4* usually exerting the larger effect (Yao *et al.*, 1997) [3]. Both loci were first placed on linkage maps with RFLP and RAPD markers (Zhang *et al.*, 1997) [4]. *Rf4* was later cloned and shown to encode a mitochondria-targeted pentatricopeptide repeat protein that lowers the abundance of transcripts of *WA352*, the mitochondrial chimeric gene responsible for sterility (Tang *et al.*, 2014) [5]. The *WA352* protein interacts with a nuclear-encoded mitochondrial protein and triggers premature degeneration of the tapetum. Restoration is in effect the outcome of nuclear control over a specific mitochondrial transcript, and the dosage and background of the restorer alleles determine how completely pollen fertility is recovered in the F₁ (Chen *et al.*, 2014) [6].

Conventional identification of restorers requires test crosses with a CMS line and scoring of pollen and spikelet fertility in the resulting hybrids over at least one additional season. Marker-assisted screening shortens this cycle considerably. Sattari *et al.* (2007) [7] developed a two-gene marker system that detected *Rf3* and *Rf4* alleles with high accuracy across diverse germplasm, and Sheeba *et al.* (2009) [8] validated several SSR markers linked to these loci in Indian breeding material. Fine mapping later narrowed both intervals

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and produced tightly linked markers that predicted restorer status with considerably fewer false positives (Suresh *et al.*, 2012) [9]. Screening large sets of elite lines with such markers before test crossing now allows breeders to concentrate field resources on candidates with a high probability of carrying functional restorer alleles.

Drought is the most damaging abiotic constraint on rice in rainfed lowland and upland ecologies, which together account for a large share of rice area in eastern and central India. Water deficit around flowering is especially harmful because it impairs pollen viability, anther dehiscence and panicle exertion, and the resulting spikelet sterility cannot be compensated later in the season (Pantuwan *et al.*, 2002) [10]. Grain yield under stress has proved to be a more dependable selection criterion than most secondary traits, provided stress is applied uniformly and heritability under stress remains adequate (Venuprasad *et al.*, 2007) [11]. Over the past decade, direct selection for yield under managed drought, supported by large-effect quantitative trait loci, has produced several drought-tolerant inbred varieties (Kumar *et al.*, 2014) [12]. Comparable effort has rarely been directed at restorer lines, and most WA-CMS restorers in current use were developed and tested only under irrigation.

Seedling-stage screening with polyethylene glycol (PEG) offers a rapid way to reduce a large set of lines to a manageable number before field testing. PEG of high molecular weight does not enter root cells and lowers the water potential of the nutrient solution in a predictable way (Michel *et al.*, 1973) [13]. Seedling root and shoot growth under PEG-induced osmotic stress has discriminated tolerant from susceptible rice varieties in several studies (Swapna *et al.*, 2017) [14]. Whether seedling tolerance predicts reproductive-stage performance is less certain, because drought resistance at flowering depends on processes such as spikelet fertility that are not expressed in young seedlings (Fukai *et al.*, 1995) [15]. A combined approach that uses markers to confirm restorer genotype, seedling assays to discard clearly susceptible lines and field trials to confirm yield under stress has seldom been reported for restorer germplasm.

The present study addressed this gap in a set of 60 rice lines. Its objectives were (i) to classify the lines for *Rf3* and *Rf4* using linked molecular markers, (ii) to evaluate the *Rf4*-positive lines for seedling growth under PEG-induced osmotic stress, (iii) to assess the best of these lines for yield and yield components under irrigated and rainfed conditions, and (iv) to identify restorer candidates combining marker-confirmed restorer alleles with stable yield under water deficit.

Materials and Methods

Plant material and experimental site

Sixty rice lines, coded ML-01 to ML-60, formed the base population. Seed of all lines was multiplied in a single season before the experiments to minimise differences in seed age and vigour. The marker assay and the seedling experiment were carried out in the laboratory and controlled-environment facilities of B.N.P.G. College, Rath, Hamirpur, Uttar Pradesh. The field experiment was conducted at the research farm of B.N.P.G. College, Rath, Hamirpur, Uttar Pradesh, during 2024-2025. Weather conditions during the crop season are presented in Figure-1.

Genotyping for *Rf3* and *Rf4*

Genomic DNA was extracted from young leaves of each line by the CTAB method (Murray *et al.*, 1980) [16], and its quality and concentration were checked on agarose gel and by spectrophotometry. Each

line was genotyped with markers linked to *Rf3* on chromosome 1 and *Rf4* on chromosome 10, namely RMS-SF21-5 for *Rf3* and RMS-PPR9-1 for *Rf4*, which have been validated for restorer identification in Indian germplasm (Sheeba *et al.*, 2009) [8] and refined by fine mapping (Suresh *et al.*, 2012) [9]. A known restorer and a maintainer line were included in every run as positive and negative controls. A line was scored P (present) when it amplified the allele of the restorer control and A (absent) when it amplified the allele of the maintainer control. Lines were then grouped into four marker classes, namely *Rf3* only, *Rf4* only, *Rf3* + *Rf4* and neither.

The mapped effect of *Rf4* on fertility restoration is larger than that of *Rf3* in the WA-CMS system (Yao *et al.*, 1997) [3], and lines carrying the *Rf4* allele were given priority for phenotyping. All 28 lines positive for *Rf4*, whether alone or together with *Rf3*, were advanced to the seedling experiment.

Seedling screening under PEG-induced osmotic stress

Surface-sterilised seeds were germinated on moist filter paper, and uniform seedlings were transferred to Yoshida nutrient solution (Yoshida *et al.*, 1976) [17]. After acclimatisation, seedlings were exposed to three osmotic treatments, namely control (nutrient solution alone), 15% (w/v) PEG-6000 and 25% (w/v) PEG-6000. The osmotic potential of each PEG solution was derived from concentration and solution temperature following Michel *et al.* (1973) [13]. Solutions were renewed at regular intervals to maintain concentration and pH. The experiment followed a completely randomised design with three replications for each of the 84 genotype × treatment combinations. Visual injury under 25% PEG was scored on the 1 to 9 scale of the Standard Evaluation System for rice, where 1 indicates no visible symptoms and 9 indicates nearly all leaves dried (IRRI, 2013) [18]. Root length (RL, cm) and shoot length (SL, cm) were measured on the sampled seedlings. Root fresh weight (RFW) and shoot fresh weight (SFW) were recorded immediately after separation and blotting, and root dry weight (RDW) and shoot dry weight (SDW) after oven drying to constant weight. All weights are expressed in g per seedling.

Field evaluation under irrigated and rainfed conditions

Twenty of the 28 lines, spanning the full range of seedling response from highly tolerant to susceptible, were evaluated in two adjacent blocks. The irrigated block was kept flooded from transplanting to physiological maturity. In the rainfed block, irrigation was withheld before the reproductive stage and the crop depended on rainfall until maturity, following the approach to managed reproductive-stage stress used in drought breeding of rice (Venuprasad *et al.*, 2007) [11]. Each environment had three replications. The crop was raised following the recommended package of practices for the region, with uniform fertiliser application and plant protection in both blocks.

Observations were recorded on randomly selected competitive plants in each plot. Days to 50% flowering (DFF) was noted on a plot basis. Plant height (PH, cm), number of effective tillers per plant (ET), panicle length (PL, cm), filled grains per panicle (FG), unfilled grains per panicle (UFG), spikelet fertility (SF, %), grain length (GL, mm) and grain yield per plant (GY, g plant⁻¹) were recorded following the Standard Evaluation System (IRRI, 2013). Spikelet fertility was calculated as the ratio of filled grains to total spikelets per panicle, expressed as a percentage.

Yield reduction (YR) under rainfed conditions was calculated for each line as

$$YR(\%) = \left[(GY_{\text{irrigated}} - GY_{\text{rainfed}}) / GY_{\text{irrigated}} \right] \times 100$$

where GY is the mean grain yield of a line in the respective environment. The same expression, applied to seedling trait means, gave the percentage reduction under each PEG level relative to the control.

Statistical analysis

Replicate observations were analysed as two-factor experiments, genotype × stress level for the seedling traits and genotype × environment for the field traits, following Gomez *et al.* (1984) [19], with both factors treated as fixed. Standard error of the mean,

$SE(m)_{\pm} = \sqrt{\frac{MSE}{r}}$, and critical difference at $P = 0.05$, $CD = t_{0.05} \times \sqrt{(2MSE/r)}$, were derived from the pooled error mean square, with $r = 3$. The coefficient of variation was calculated as the ratio of the error

standard deviation to the grand mean of the trait.

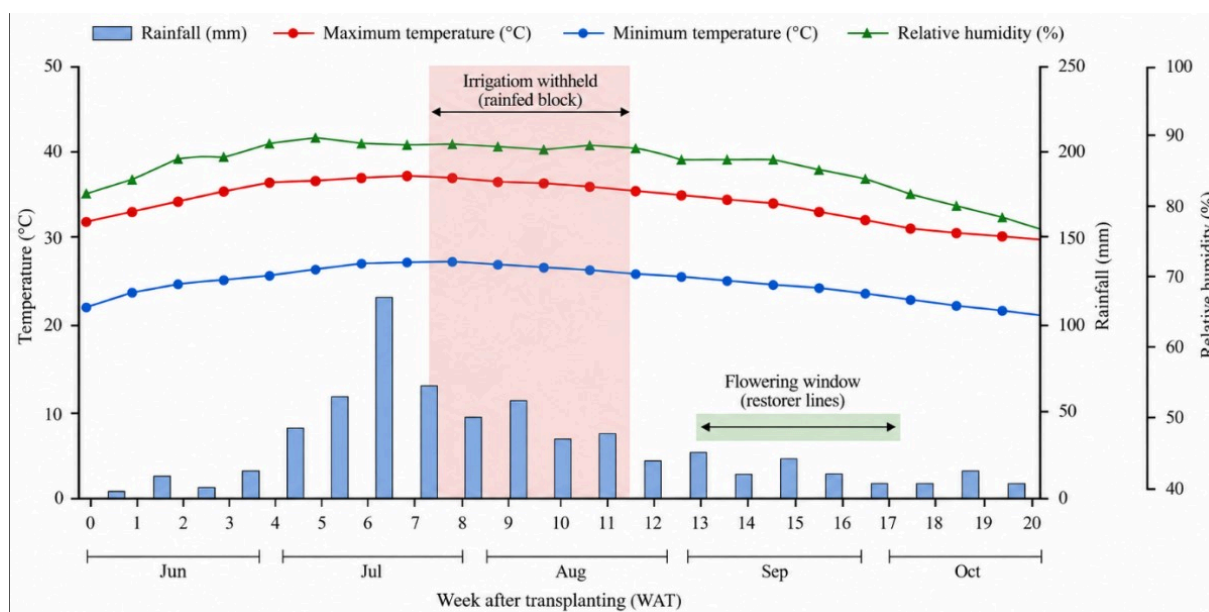


Figure 1. Weekly mean maximum and minimum air temperature (°C), relative humidity (%) and total rainfall (mm) at the experimental site during the crop season, with the period of withheld irrigation in the rainfed block and the flowering window of the test lines marked on the time axis

Pearson correlation coefficients were computed among seedling traits at 25% PEG and among field traits under rainfed conditions using genotype means. Principal component analysis on standardised genotype means was used to examine how variation was distributed across traits (Jolliffe *et al.*, 2016) [20]. Lines were grouped by k-means clustering on standardised means with $k = 3$ (Hartigan *et al.*, 1979) [21]. A standardised multi-trait selection index, scaled from 0 to 1 with higher scores denoting closer proximity to the ideotype, was computed for each line from seedling traits at 25% PEG and from field traits under rainfed conditions. Agreement between seedling and field indices was tested by Spearman rank correlation. Analyses were performed in Python 3.11 with the statsmodels (version 0.15) and SciPy (version 1.17) libraries.

Results

Marker classification of the 60 lines

The two markers separated the lines into four classes of unequal size. Fourteen lines (23.3%) carried the restorer allele at *Rf3* alone and 18 (30.0%) at *Rf4* alone. Ten lines (16.7%) were positive at both loci, and the remaining 18 (30.0%) amplified the maintainer allele at both. Taken together, 28 lines (46.7%) carried the *Rf4* restorer allele and were advanced to phenotyping, while the 14 lines positive only for *Rf3* and the 18 double-negative lines were set aside.

Seedling growth under PEG-induced osmotic stress

Osmotic stress depressed every seedling trait, and the depression deepened with PEG concentration. Averaged over the 28 lines, 15% PEG reduced the six traits by 12.9% (*RL*) to 15.9% (*SDW*) relative to the control. At 25% PEG the reductions rose to between 32.6% (*RL*) and 35.7% (*SFW*), roughly two and a half times the loss at the lower level. Root length responded least in relative terms at both concentrations, whereas the fresh and dry weights of the shoot responded most. Analysis of variance showed highly significant effects of genotype, stress level and their interaction for all six traits ($P < 0.001$). Stress level contributed by far the largest variance ratio ($F = 2504.85$ to 3145.82), followed by genotype ($F = 56.83$ to 415.03). The genotype

$\times$ stress interaction, though smaller ($F = 3.24$ to 14.96), was significant throughout and was strongest for *RDW*. Lines thus changed rank as stress intensified, and a ranking made under control conditions would not have identified the most tolerant lines. Error coefficients of variation stayed between 3.38 and 3.96%, which indicates good control of experimental error in the hydroponic system.

Table 1 presents the means of the 28 lines at 25% PEG, the level used for selection. ML-02 recorded the highest *RL*, *SL*, *SFW*, *RDW* and *SDW*, and ML-13 the highest *RFW*. ML-32, ML-05, ML-57 and ML-59 followed closely, and the differences among these lines were often within the critical difference for individual traits. At the opposite end, ML-52 gave the lowest values for five of the six traits, and ML-45, ML-22, ML-53 and ML-47 were similarly poor. The spread between best and poorest lines was wide. *SDW* at 25% PEG ranged from 0.067 to 0.163 g, a 2.4-fold difference, and *RDW* from 0.0156 to 0.0422 g, a 2.7-fold difference.

Values are means of three replications $\pm$ standard error of the replicate observations. *RL*, root length; *SL*, shoot length; *RFW*, root fresh weight; *SFW*, shoot fresh weight; *RDW*, root dry weight; *SDW*, shoot dry weight; weights are per seedling. $SE(m)_{\pm}$, CD ($p = 0.05$) and CV (%) are derived from the pooled error mean square of the genotype $\times$ stress analysis (error $df = 168$) and apply to comparisons of genotype means within a stress level. Effects of genotype, stress and genotype $\times$ stress were significant at $P < 0.001$ for all traits.

Relative reduction at 25% PEG also varied considerably among lines (Figure-2). Loss of root length ranged from 22.8% in ML-15 to 41.7% in ML-49, and loss of shoot dry weight from 24.1% in ML-05 to 47.6% in ML-45. A line with vigorous growth under control conditions did not necessarily retain it under stress. ML-49, for example, had among the longest roots in the control but lost more root length than any other line at 25% PEG.

Visual injury scores at 25% PEG agreed well with the growth measurements. Two lines, ML-02 and ML-32, scored 1 (highly tolerant) and seven scored 3 (tolerant), namely ML-05, ML-08, ML-09, ML-13, ML-51, ML-57 and ML-59. Eleven lines were moderately tolerant (score 5), six susceptible (score 7) and two, ML-22 and ML-52, highly susceptible (score 9).

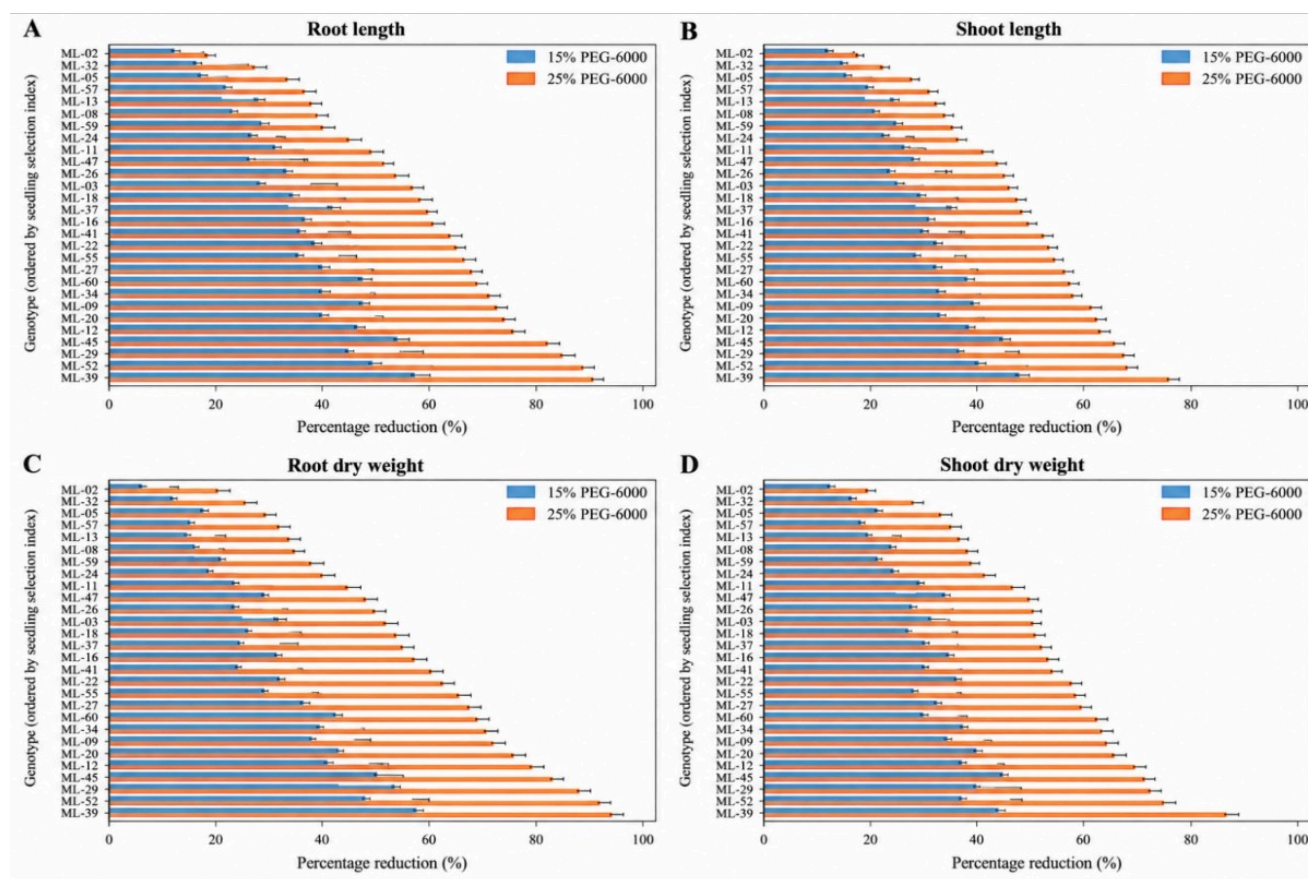


Figure 2. Percentage reduction in root length, shoot length, root dry weight and shoot dry weight of 28 *Rf4*-positive rice lines at 15% and 25% PEG-6000 relative to the unstressed control. Lines are ordered by the seedling selection index. Vertical bars indicate standard error of the mean (n = 3)

Table 1. Mean seedling traits ($\pm$ SE) of 28 *Rf4*-positive rice lines grown in nutrient solution with 25% PEG-6000, listed in descending order of the seedling selection index

Genotype	RL (cm)	SL (cm)	RFW (g)	SFW (g)	RDW (g)	SDW (g)
ML-02	6.34 $\pm$ 0.079	29.95 $\pm$ 0.282	0.229 $\pm$ 0.0071	0.618 $\pm$ 0.0031	0.0422 $\pm$ 0.0011	0.163 $\pm$ 0.0028
ML-32	6.26 $\pm$ 0.219	27.99 $\pm$ 0.342	0.221 $\pm$ 0.0034	0.577 $\pm$ 0.0181	0.0393 $\pm$ 0.0006	0.155 $\pm$ 0.0009
ML-05	5.67 $\pm$ 0.059	29.02 $\pm$ 0.381	0.240 $\pm$ 0.0030	0.609 $\pm$ 0.0117	0.0336 $\pm$ 0.0002	0.163 $\pm$ 0.0022
ML-57	5.75 $\pm$ 0.025	27.94 $\pm$ 0.107	0.231 $\pm$ 0.0054	0.567 $\pm$ 0.0055	0.0375 $\pm$ 0.0013	0.158 $\pm$ 0.0023
ML-13	5.79 $\pm$ 0.038	27.46 $\pm$ 0.269	0.243 $\pm$ 0.0041	0.567 $\pm$ 0.0125	0.0367 $\pm$ 0.0007	0.149 $\pm$ 0.0016
ML-08	5.69 $\pm$ 0.082	27.81 $\pm$ 0.285	0.221 $\pm$ 0.0022	0.515 $\pm$ 0.0095	0.0359 $\pm$ 0.0008	0.158 $\pm$ 0.0046
ML-59	5.92 $\pm$ 0.005	28.12 $\pm$ 0.354	0.222 $\pm$ 0.0076	0.559 $\pm$ 0.0042	0.0337 $\pm$ 0.0009	0.147 $\pm$ 0.0037
ML-51	5.87 $\pm$ 0.076	26.99 $\pm$ 0.220	0.227 $\pm$ 0.0011	0.528 $\pm$ 0.0126	0.0363 $\pm$ 0.0001	0.146 $\pm$ 0.0019
ML-09	5.82 $\pm$ 0.084	27.50 $\pm$ 0.311	0.211 $\pm$ 0.0017	0.515 $\pm$ 0.0104	0.0355 $\pm$ 0.0008	0.150 $\pm$ 0.0029
ML-43	5.10 $\pm$ 0.153	26.67 $\pm$ 0.234	0.222 $\pm$ 0.0064	0.514 $\pm$ 0.0097	0.0369 $\pm$ 0.0008	0.143 $\pm$ 0.0006
ML-10	5.51 $\pm$ 0.074	26.39 $\pm$ 0.782	0.207 $\pm$ 0.0054	0.503 $\pm$ 0.0194	0.0371 $\pm$ 0.0007	0.141 $\pm$ 0.0011
ML-49	5.10 $\pm$ 0.052	26.67 $\pm$ 0.277	0.200 $\pm$ 0.0015	0.471 $\pm$ 0.0056	0.0314 $\pm$ 0.0003	0.135 $\pm$ 0.0030
ML-29	4.84 $\pm$ 0.081	24.83 $\pm$ 0.566	0.190 $\pm$ 0.0031	0.456 $\pm$ 0.0072	0.0288 $\pm$ 0.0006	0.132 $\pm$ 0.0042
ML-15	5.45 $\pm$ 0.056	24.88 $\pm$ 0.861	0.184 $\pm$ 0.0012	0.442 $\pm$ 0.0130	0.0299 $\pm$ 0.0007	0.128 $\pm$ 0.0004
ML-21	5.02 $\pm$ 0.120	24.35 $\pm$ 0.071	0.175 $\pm$ 0.0021	0.426 $\pm$ 0.0095	0.0285 $\pm$ 0.0003	0.127 $\pm$ 0.0029
ML-28	4.23 $\pm$ 0.077	24.15 $\pm$ 0.382	0.171 $\pm$ 0.0078	0.425 $\pm$ 0.0091	0.0216 $\pm$ 0.0003	0.116 $\pm$ 0.0041
ML-60	4.35 $\pm$ 0.155	23.34 $\pm$ 0.558	0.163 $\pm$ 0.0040	0.393 $\pm$ 0.0065	0.0219 $\pm$ 0.0002	0.108 $\pm$ 0.0016
ML-20	4.41 $\pm$ 0.065	24.35 $\pm$ 0.294	0.157 $\pm$ 0.0010	0.405 $\pm$ 0.0051	0.0223 $\pm$ 0.0004	0.108 $\pm$ 0.0010
ML-33	4.48 $\pm$ 0.088	23.62 $\pm$ 0.357	0.174 $\pm$ 0.0013	0.355 $\pm$ 0.0038	0.0236 $\pm$ 0.0012	0.105 $\pm$ 0.0008
ML-50	4.57 $\pm$ 0.091	22.16 $\pm$ 0.071	0.147 $\pm$ 0.0021	0.363 $\pm$ 0.0126	0.0223 $\pm$ 0.0004	0.095 $\pm$ 0.0019
ML-12	4.28 $\pm$ 0.092	23.01 $\pm$ 0.181	0.145 $\pm$ 0.0045	0.338 $\pm$ 0.0015	0.0195 $\pm$ 0.0004	0.102 $\pm$ 0.0021
ML-55	4.17 $\pm$ 0.056	22.33 $\pm$ 0.850	0.136 $\pm$ 0.0039	0.303 $\pm$ 0.0142	0.0196 $\pm$ 0.0002	0.094 $\pm$ 0.0020
ML-39	4.16 $\pm$ 0.037	20.78 $\pm$ 0.178	0.145 $\pm$ 0.0017	0.305 $\pm$ 0.0096	0.0189 $\pm$ 0.0005	0.090 $\pm$ 0.0023
ML-53	3.81 $\pm$ 0.030	21.53 $\pm$ 0.244	0.129 $\pm$ 0.0011	0.345 $\pm$ 0.0044	0.0186 $\pm$ 0.0002	0.083 $\pm$ 0.0010
ML-22	3.65 $\pm$ 0.097	21.12 $\pm$ 0.908	0.131 $\pm$ 0.0054	0.344 $\pm$ 0.0079	0.0191 $\pm$ 0.0006	0.091 $\pm$ 0.0044

(continued on next page)

Genotype	RL (cm)	SL (cm)	RFW (g)	SFW (g)	RDW (g)	SDW (g)
ML-47	4.07 ± 0.045	21.48 ± 0.735	0.128 ± 0.0030	0.299 ± 0.0067	0.0167 ± 0.0001	0.091 ± 0.0030
ML-45	4.00 ± 0.039	22.06 ± 0.476	0.128 ± 0.0042	0.303 ± 0.0071	0.0177 ± 0.0002	0.067 ± 0.0003
ML-52	3.41 ± 0.070	19.73 ± 0.104	0.125 ± 0.0040	0.288 ± 0.0038	0.0156 ± 0.0001	0.067 ± 0.0008
Mean	4.92	24.87	0.182	0.441	0.0279	0.122
SE _m ±	0.125	0.620	0.0046	0.0130	0.00078	0.0033
CD (<i>p</i> = 0.05)	0.348	1.730	0.0129	0.0362	0.00217	0.0093
CV (%)	3.49	3.38	3.44	3.96	3.82	3.71

All six seedling traits at 25% PEG were strongly and positively inter-correlated, with coefficients between 0.93 and 0.97. The first principal component alone accounted for 96.3% of the variation among line means, and no later component explained more than 1.5%. Seedling performance under osmotic stress thus behaved as a single composite of general vigour retention rather than as independent root and shoot responses. K-means clustering placed 11 lines in a vigorous group (cluster 1), nine in a stress-sensitive group (cluster 2) and eight in an intermediate group (cluster 3). The seedling selection index ranked ML-02 (0.983), ML-32 (0.912), ML-05 (0.891), ML-57 (0.869), ML-13 (0.855), ML-08 (0.830) and ML-59 (0.821) highest, and all seven belonged to cluster 1. ML-52 had the lowest index (0.008).

Field performance under irrigated and rainfed conditions

Water deficit in the rainfed block lowered the mean of every field trait except days to flowering and raised the number of unfilled grains. Relative to the irrigated block, mean grain yield of the 20 lines fell by 11.4%, from 25.86 to 22.91 g plant⁻¹. The largest relative changes were a 31.5% increase in unfilled grains and a 10.2% loss of filled grains per panicle. Spikelet fertility fell by 6.1 percentage points and effective tillers by 7.0%, while plant height, panicle length and grain length declined by only 1.2 to 3.6%. Mean days to 50% flowering was almost identical in the two environments.

The environment effect was significant for eight of the nine traits (*P* < 0.001), the exception being *DFF* (*F* = 3.35, *P* = 0.071). Genotype and genotype × environment effects were significant for every trait, including *DFF* (*P* < 0.001). The interaction was strongest for spikelet fertility (*F* = 38.93), filled grains (*F* = 30.63) and grain yield (*F* = 25.85), which are precisely the traits that decide yield under stress. Lines thus differed not only in yield potential but also in the degree to which that potential was lost under water deficit. Field coefficients of variation were low, from 0.60% for *DFF* to 4.71% for *UFG*.

Under rainfed conditions (Table 2), ML-57 (28.38 g plant⁻¹), ML-13 (27.96 g plant⁻¹), ML-32 and ML-05 (both 27.72 g plant⁻¹) and ML-02 (27.08 g plant⁻¹) gave the highest grain yields, and these five lines did not differ from one another by more than the critical difference in most yield components. They combined the largest number of filled grains per panicle (132 to 146), spikelet fertility above 84% and ten or more effective tillers per plant. ML-12, ML-20, ML-60, ML-28 and ML-33 formed the poorest group, with grain yield between 14.75 and 17.21 g plant⁻¹, spikelet fertility below 68% and more than 23 unfilled grains per panicle. These low yielders also flowered later under stress. ML-28 and ML-60 reached 50% flowering in about 97 days, compared with 91 to 95 days for most of the high yielders.

Table 2. Mean agronomic traits (± SE) of 20 rice restorer candidates under rainfed conditions, listed in descending order of the rainfed selection index

Genotype	DFF (d)	PH (cm)	ET (no.)	PL (cm)	FG (no.)	UFG (no.)	SF (%)	GL (mm)	GY (g plant ⁻¹)
ML-02	92.61 ± 0.17	114.44 ± 0.61	11.16 ± 0.06	23.83 ± 0.17	144.80 ± 0.23	10.93 ± 0.46	88.77 ± 0.76	6.50 ± 0.01	27.08 ± 0.27
ML-13	91.17 ± 0.31	111.06 ± 1.03	11.08 ± 0.14	23.62 ± 0.16	146.00 ± 0.69	13.91 ± 0.59	88.66 ± 0.57	6.47 ± 0.00	27.96 ± 0.51
ML-57	94.66 ± 0.12	112.08 ± 0.36	11.43 ± 0.11	23.62 ± 0.18	139.78 ± 1.02	12.68 ± 0.27	86.02 ± 0.41	6.55 ± 0.03	28.38 ± 0.10
ML-32	93.70 ± 0.16	112.51 ± 0.54	10.58 ± 0.21	24.38 ± 0.15	137.79 ± 0.68	13.64 ± 0.38	88.16 ± 1.12	6.62 ± 0.02	27.72 ± 0.36
ML-05	93.45 ± 0.09	113.48 ± 0.40	11.12 ± 0.16	23.69 ± 0.08	132.37 ± 1.05	16.79 ± 0.56	84.52 ± 0.66	6.40 ± 0.02	27.72 ± 0.35
ML-51	94.78 ± 0.22	111.23 ± 0.73	10.64 ± 0.21	23.14 ± 0.06	141.93 ± 1.27	15.08 ± 0.24	86.92 ± 0.22	6.48 ± 0.02	25.41 ± 0.52
ML-59	95.57 ± 0.28	108.41 ± 0.50	10.21 ± 0.18	23.34 ± 0.10	133.20 ± 0.79	16.58 ± 0.45	87.50 ± 0.09	6.47 ± 0.02	26.23 ± 0.34
ML-08	95.14 ± 0.20	110.72 ± 0.39	10.22 ± 0.15	23.71 ± 0.06	131.70 ± 0.38	14.01 ± 0.52	84.02 ± 0.29	6.41 ± 0.03	25.86 ± 0.43
ML-09	94.36 ± 0.33	107.80 ± 0.53	9.85 ± 0.14	23.56 ± 0.02	126.87 ± 0.68	16.26 ± 0.31	84.21 ± 0.37	6.46 ± 0.03	25.62 ± 0.06
ML-10	94.08 ± 0.27	110.65 ± 0.08	9.99 ± 0.10	22.44 ± 0.04	130.25 ± 1.15	16.70 ± 0.02	80.49 ± 0.67	6.44 ± 0.01	25.01 ± 0.34
ML-43	94.56 ± 0.09	107.76 ± 0.47	9.98 ± 0.23	22.30 ± 0.32	123.44 ± 1.69	16.68 ± 0.48	82.54 ± 0.65	6.31 ± 0.03	23.78 ± 0.18
ML-49	96.56 ± 0.20	107.57 ± 0.62	9.71 ± 0.09	22.00 ± 0.14	117.96 ± 1.75	18.19 ± 0.31	77.84 ± 0.42	6.27 ± 0.04	23.63 ± 0.29

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Genotype	DFF (d)	PH (cm)	ET (no.)	PL (cm)	FG (no.)	UFG (no.)	SF (%)	GL (mm)	GY (g plant ⁻¹)
ML-15	92.54 ± 0.21	105.60 ± 0.15	9.71 ± 0.11	22.17 ± 0.13	113.59 ± 1.55	18.24 ± 0.40	74.61 ± 0.52	6.24 ± 0.02	22.04 ± 0.35
ML-29	95.28 ± 0.13	107.80 ± 0.50	9.50 ± 0.04	21.73 ± 0.02	117.27 ± 0.97	18.16 ± 0.12	74.98 ± 0.46	6.26 ± 0.03	21.19 ± 0.32
ML-21	92.53 ± 0.17	105.78 ± 0.89	8.43 ± 0.05	22.02 ± 0.06	104.55 ± 1.10	20.81 ± 0.46	74.46 ± 0.76	6.27 ± 0.01	20.39 ± 0.06
ML-33	93.12 ± 0.17	102.59 ± 0.26	8.51 ± 0.13	21.18 ± 0.04	102.00 ± 0.36	23.81 ± 0.40	67.64 ± 0.57	6.04 ± 0.00	17.21 ± 0.31
ML-28	97.26 ± 0.17	101.77 ± 0.83	8.20 ± 0.03	21.48 ± 0.20	102.13 ± 1.54	23.80 ± 0.36	66.43 ± 0.54	6.11 ± 0.03	16.31 ± 0.24
ML-60	96.92 ± 0.42	99.90 ± 0.48	8.15 ± 0.04	20.89 ± 0.12	91.88 ± 1.08	23.49 ± 0.44	65.53 ± 0.65	6.09 ± 0.02	16.12 ± 0.29
ML-20	95.72 ± 0.45	99.66 ± 0.21	7.89 ± 0.04	20.16 ± 0.03	92.76 ± 0.96	23.84 ± 0.45	64.68 ± 0.19	5.97 ± 0.02	15.71 ± 0.37
ML-12	95.40 ± 0.56	103.13 ± 0.76	8.17 ± 0.15	20.38 ± 0.15	89.96 ± 0.73	25.34 ± 0.51	64.37 ± 0.25	6.03 ± 0.02	14.75 ± 0.36
Mean	94.47	107.70	9.73	22.48	121.01	17.95	78.62	6.32	22.91
SE _m ±	0.330	0.565	0.127	0.143	1.203	0.429	0.581	0.025	0.353
CD (<i>p</i> = 0.05)	0.928	1.589	0.358	0.402	3.387	1.208	1.636	0.070	0.993
CV (%)	0.60	0.89	2.18	1.08	1.63	4.71	1.23	0.67	2.51

Values are means of three replications ± standard error of the replicate observations. *DFF*, days to 50% flowering; *PH*, plant height; *ET*, effective tillers per plant; *PL*, panicle length; *FG*, filled grains per panicle; *UFG*, unfilled grains per panicle; *SF*, spikelet fertility; *GL*, grain length; *GY*, grain yield per plant. SE (m)±, CD (*p* = 0.05) and CV (%) are derived from the pooled error mean square of the genotype × environment analysis (error df = 80). Genotype and genotype × environment effects were significant at *P* < 0.001 for all traits; the environment effect was significant at *P* < 0.001 for all traits except *DFF* (*P* = 0.071).

Yield reduction under rainfed conditions differed widely among lines (Table 3). ML-59 yielded slightly more under rainfed than under irrigated conditions (−1.24%), and ML-10, ML-49 and ML-57 lost less than 2%. Most of the high-yielding lines lost between 3.5 and 8.2%.

At the other extreme, ML-12, ML-20, ML-28, ML-60 and ML-33 lost 26.0 to 32.0% of their irrigated yield. The loss of spikelet fertility followed the same pattern. ML-51, ML-32, ML-59, ML-09 and ML-02 lost no more than 0.34 percentage points of spikelet fertility, and the first four recorded marginally higher fertility under rainfed conditions, whereas ML-60, ML-20 and ML-28 lost 13 to 14 points.

Low yield reduction did not by itself identify the best restorer candidates. ML-10 and ML-49 were stable but had modest irrigated yields (25.33 and 24.02 g plant⁻¹), so their rainfed yield remained below that of lines such as ML-13 and ML-05, which lost around 6% from a higher starting point. Rainfed and irrigated grain yield were closely correlated across the 20 lines (*r* = 0.92, *P* < 0.001), which shows that yield potential contributed strongly to rainfed performance in this material.

Table 3. Marker class, seedling response to 25% PEG-6000 and grain yield under irrigated and rainfed conditions of 20 rice restorer candidates, listed in descending order of the rainfed selection index

Genotype	Marker class	VIS at 25% PEG	Seedling index	GY irrigated (g plant ⁻¹)	GY rainfed (g plant ⁻¹)	Yield reduction (%)	Field index
ML-02	<i>Rf3 + Rf4</i>	1	0.983	28.13 ± 0.11	27.08 ± 0.27	3.73	0.976
ML-13	<i>Rf3 + Rf4</i>	3	0.855	29.75 ± 0.52	27.96 ± 0.51	6.05	0.947
ML-57	<i>Rf4</i>	3	0.869	28.85 ± 0.35	28.38 ± 0.10	1.63	0.942
ML-32	<i>Rf3 + Rf4</i>	1	0.912	28.80 ± 0.28	27.72 ± 0.36	3.74	0.905
ML-05	<i>Rf4</i>	3	0.891	29.50 ± 0.16	27.72 ± 0.35	6.06	0.864
ML-51	<i>Rf3 + Rf4</i>	3	0.802	26.88 ± 0.82	25.41 ± 0.52	5.49	0.849
ML-59	<i>Rf3 + Rf4</i>	3	0.821	25.90 ± 0.36	26.23 ± 0.34	−1.24	0.814
ML-08	<i>Rf3 + Rf4</i>	3	0.830	28.15 ± 0.07	25.86 ± 0.43	8.15	0.784
ML-09	<i>Rf3 + Rf4</i>	3	0.802	26.57 ± 0.32	25.62 ± 0.06	3.55	0.715
ML-10	<i>Rf4</i>	5	0.716	25.33 ± 0.17	25.01 ± 0.34	1.28	0.691
ML-43	<i>Rf3 + Rf4</i>	5	0.738	26.85 ± 0.02	23.78 ± 0.18	11.43	0.652
ML-49	<i>Rf4</i>	5	0.607	24.02 ± 0.16	23.63 ± 0.29	1.62	0.546
ML-15	<i>Rf4</i>	5	0.561	25.40 ± 0.71	22.04 ± 0.35	13.22	0.498
ML-29	<i>Rf3 + Rf4</i>	5	0.573	24.57 ± 0.55	21.19 ± 0.32	13.74	0.472
ML-21	<i>Rf4</i>	5	0.503	24.64 ± 0.06	20.39 ± 0.06	17.22	0.338
ML-33	<i>Rf4</i>	5	0.319	23.25 ± 0.13	17.21 ± 0.31	26.00	0.198
ML-28	<i>Rf4</i>	5	0.370	23.58 ± 0.21	16.31 ± 0.24	30.85	0.140
ML-60	<i>Rf4</i>	5	0.342	22.47 ± 0.49	16.12 ± 0.29	28.27	0.068
ML-20	<i>Rf4</i>	5	0.323	22.87 ± 0.29	15.71 ± 0.37	31.30	0.038

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Genotype	Marker class	VIS at 25% PEG	Seedling index	GY irrigated (g plant ⁻¹)	GY rainfed (g plant ⁻¹)	Yield reduction (%)	Field index
ML-12	<i>Rf4</i>	7	0.226	21.71 ± 0.43	14.75 ± 0.36	32.04	0.036
Mean				25.86	22.91	11.43	
$SE_m \pm (G \times E)$				0.353	0.353		
CD ($p = 0.05$) ($G \times E$)				0.993	0.993		
CV (%)				2.51	2.51		

Marker class from genotyping with markers linked to *Rf3* and *Rf4*. VIS, visual injury score on the 1 to 9 scale of IRRI (2013), where 1 is highly tolerant and 9 highly susceptible. Seedling and field index, standardised multi-trait selection index computed from seedling traits at 25% PEG and from field traits under rainfed conditions respectively, with higher values indicating closer proximity to the ideotype. GY, grain yield per plant, mean ± SE ($n = 3$). Yield reduction = (GY irrigated – GY rainfed)/GY irrigated × 100; a negative value indicates higher yield under rainfed conditions. SE (m)±, CD and CV apply to grain yield (genotype × environment, error df = 80).

Associations among traits and agreement between seedling and field screening

Under rainfed conditions, grain yield correlated most closely with spikelet fertility ($r = 0.98$), followed by filled grains per panicle, effective tillers and grain length ($r = 0.96$ each), panicle length ($r = 0.95$) and plant height ($r = 0.94$). Unfilled grains correlated negatively with yield ($r = -0.95$). DFF showed a moderate negative association with all yield-related traits ($r = -0.39$ to -0.48), so later-flowering lines tended to yield less under stress. The first principal component explained 87.0% of the variation among rainfed line means and the second 8.6%, so that two components captured 95.6% of the total (Figure-3).

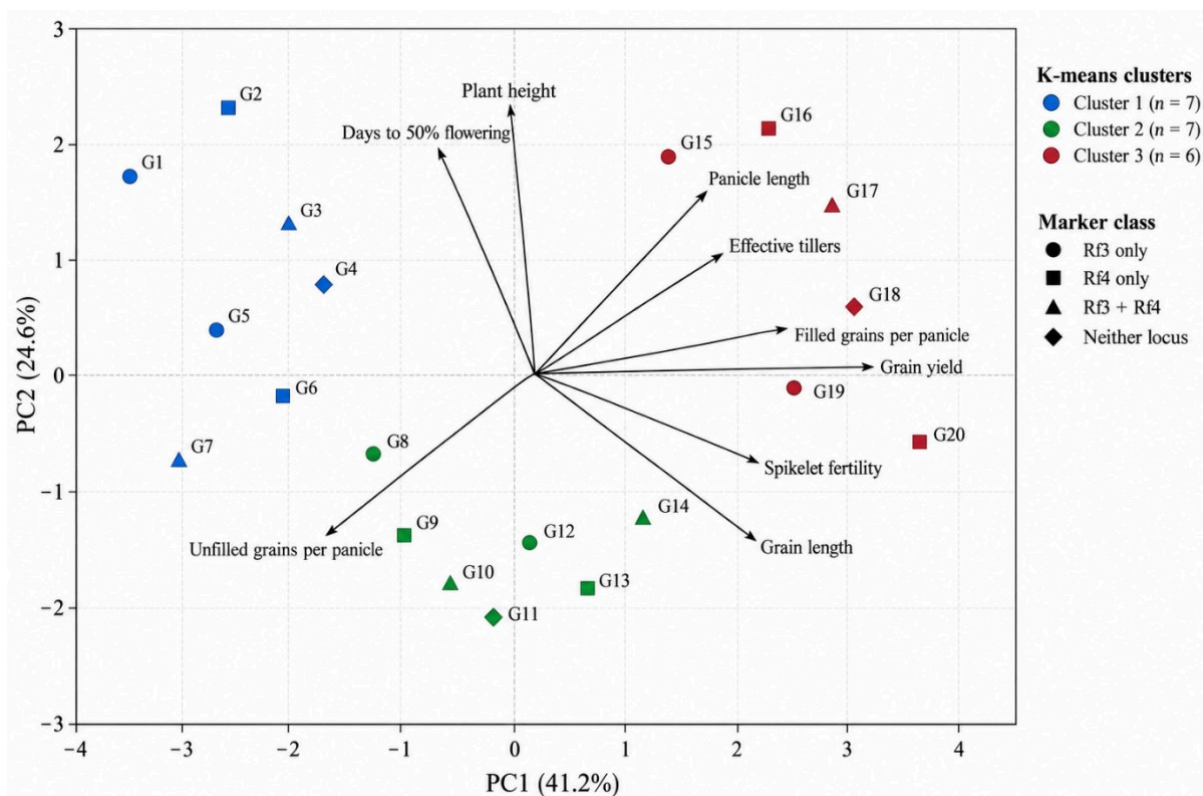


Figure 3. Biplot of the first two principal components of nine agronomic traits of 20 rice restorer candidates under rainfed conditions, with lines coloured by k-means cluster membership and marker class indicated by symbol shape

K-means clustering on rainfed traits reproduced the seedling grouping to a large extent. The ten lines of field cluster 1 (ML-02, ML-05, ML-08, ML-09, ML-10, ML-13, ML-32, ML-51, ML-57 and ML-59) had all belonged to seedling cluster 1. ML-43 was the one exception, moving from the vigorous seedling cluster to the intermediate field cluster together with ML-15, ML-21, ML-29 and ML-49. ML-12, ML-20, ML-28, ML-33 and ML-60 formed the poorest field cluster. The seedling and field selection indices were strongly concordant across the 20 lines (Spearman $\rho = 0.97$, $P < 0.001$). Seedling traits at 25% PEG predicted rainfed grain yield well, with correlations of 0.97 for *SDW*, 0.95 for *RDW* and 0.94 for *RL* (all $P < 0.001$, $n = 20$). Root

length at 25% PEG was also negatively associated with yield reduction ($r = -0.89$, $P < 0.001$). Five of the seven lines with the highest seedling index, namely ML-02, ML-32, ML-05, ML-57 and ML-13, were also among the six best lines in the field index, the sixth being ML-51.

The marker classes were not evenly distributed along the tolerance gradient. Of the nine lines that scored 1 or 3 for visual injury, seven carried both *Rf3* and *Rf4*, and only two (ML-05 and ML-57) carried *Rf4* alone. Of the 18 lines positive for *Rf4* alone, 16 scored 5 or higher. The association was not absolute, since the double-positive

line ML-47 scored 7 and had one of the lowest seedling indices (0.111).

Discussion

Frequency and meaning of restorer marker classes

Nearly half the lines (46.7%) carried the *Rf4* restorer allele and one in six carried both loci. Such frequencies are typical of breeding material with *indica* ancestry, in which restorer alleles are common because many elite *indica* lines trace back to restorer parents used in early hybrid programmes (Virmani, 1994) [1]. The prominence of *Rf4* in the present set agrees with the view that this locus is the principal restorer of WA-CMS and is under positive selection wherever restorers have been bred deliberately (Yao *et al.*, 1997) [3]. A marker score, it must be stressed, is a prediction of restorer status and not proof of it. Markers linked to a locus rather than lying within the causal gene can be separated from the functional allele by recombination, and the predictive accuracy of earlier SSR markers varied across genetic backgrounds for exactly this reason (Sheeba *et al.*, 2009) [8]. Gene-based markers developed after the cloning of *Rf4* reduce this risk considerably (Tang *et al.*, 2014) [5], but even they cannot account for modifier loci and background effects on pollen fertility.

The decision to advance only *Rf4*-positive lines was a practical one and carries a cost. Lines positive for *Rf3* alone may still restore fertility partially, and in some backgrounds *Rf3* contributes substantially to seed set in the hybrid. Sattari *et al.* (2007) [7] found that lines carrying restorer alleles at both loci gave the most complete and consistent restoration, which supports giving double-positive lines priority but does not exclude single-locus restorers altogether. Test crossing the selected lines with one or more WA-CMS lines and scoring pollen and spikelet fertility in the F_1 is the necessary next step before any of them is described as a restorer in the strict sense.

Why seedling growth declined under osmotic stress

PEG-6000 lowers the water potential of the root medium without being taken up, so the seedling must lower its own tissue water potential to continue absorbing water. The first process to suffer is cell expansion, which depends on turgor and responds to water deficits smaller than those needed to reduce photosynthesis or stomatal conductance (Hsiao, 1973) [22]. This explains why length and fresh weight fell sharply even at 15% PEG. At 25% PEG the reduction in dry weight was of similar magnitude to that in length, which suggests that carbon assimilation was restricted as well as expansion, a pattern commonly reported once osmotic stress becomes moderate to severe (Farooq *et al.*, 2009) [23].

Root length was the least affected trait at both PEG levels. Roots of many species sustain elongation at water potentials that halt shoot growth altogether, through osmotic adjustment in the growing zone and through changes in cell-wall extensibility that allow expansion at reduced turgor (Sharp *et al.*, 2004) [24]. The smaller relative loss in root length than in shoot biomass seen here is consistent with this preferential maintenance of root growth. Lines that kept their roots growing under 25% PEG, such as ML-02, ML-32 and ML-59, would in principle be better placed to exploit deeper soil moisture in the field, a relationship that gained genetic support when the *DRO1* allele for steeper root angle was shown to raise yield under drought (Uga *et al.*, 2013) [25].

The significant genotype $\times$ stress interaction for all seedling traits has a direct bearing on selection. ML-49 had one of the longest roots under control conditions but suffered the largest loss of root length at 25% PEG, whereas ML-15 had shorter roots initially but lost the least. Seedling vigour under favourable conditions is thus a poor guide to tolerance, and screening must be done at a stress level strong enough to reveal the interaction. The discrimination achieved with 25% PEG in this study is in line with the report of Swapna *et al.* (2017) [14], who found that PEG-induced osmotic stress separated rice varieties into tolerance groups that corresponded with their known field reaction. The very high intercorrelation of seedling traits,

with 96.3% of variation on the first principal component, needs cautious interpretation. Length and biomass of the same organ are allometrically linked, and part of the agreement among traits reflects this dependence rather than independent physiological evidence of tolerance.

Yield formation under rainfed conditions

The mean yield loss of 11.4% under rainfed conditions was modest compared with the losses usually reported when water is withheld over flowering in managed drought nurseries, where yield frequently falls by half or more (Venuprasad *et al.*, 2007) [11]. The stress in the present trial was thus moderate in its effect on yield. Stress severity changes which lines appear tolerant, and the ranking of rice lines under drought can shift considerably between mild and severe stress (Kumar *et al.*, 2014) [12], so the present classification applies to stress of similar intensity.

The traits most affected were those formed at and after flowering. Unfilled grains rose by almost a third while grain length and panicle length hardly changed. Panicle length and grain dimensions are largely fixed before heading, whereas the proportion of spikelets that set grain is decided in a narrow window around anthesis. Water deficit during meiosis and anthesis reduces pollen viability, impairs anther dehiscence and lowers the number of pollen grains landing on the stigma (Saini *et al.*, 1999) [26]. Genotypic differences in the sensitivity of anther dehiscence to water deficit have been demonstrated directly in rice (Liu *et al.*, 2006) [27], and such differences could explain why lines such as ML-32, ML-51 and ML-59 kept their spikelet fertility intact while ML-20, ML-28 and ML-60 lost 13 to 14 percentage points. The correlation of 0.98 between spikelet fertility and grain yield under rainfed conditions confirms that spikelet fertility, rather than tiller number or grain size, was the main limit on yield under stress in this material.

Days to 50% flowering did not differ between environments on average, yet the genotype $\times$ environment interaction for *DFF* was significant. Some lines flowered earlier and others later under rainfed conditions, so the mean concealed opposite responses. Delay in flowering under drought is a well-established sign of susceptibility in rice, because stressed plants slow panicle development and the delay is associated with lower grain yield under stress (Pantawan *et al.*, 2002) [10]. The negative correlation between *DFF* and yield under rainfed conditions, and the late flowering of ML-28 and ML-60, agree with this interpretation. Earlier-flowering lines may also have partly escaped the most severe period of stress, and this escape component cannot be separated from true tolerance in a single-season trial.

Yield potential, stability and the choice of selection criteria

Rainfed yield correlated closely with irrigated yield ($r = 0.92$), so under the stress level of this trial most of the ranking under rainfed conditions reflected differences in yield potential. This relationship typically weakens as stress becomes severe, and in upland and rainfed lowland trials with intense drought the correlation between yield under stress and yield without stress can fall close to zero (Bernier *et al.*, 2008) [28]. Under mild to moderate stress, as here, selecting lines with high potential remains an efficient strategy, provided the lines do not lose that potential disproportionately.

Yield reduction alone would have favoured lines such as ML-10 and ML-49, which were stable largely because their irrigated yield was already lower. Indices based solely on relative yield loss are known to reward low-yielding but stable genotypes, a limitation recognised since the drought susceptibility index was first proposed for wheat (Fischer *et al.*, 1978) [29]. The combined consideration of rainfed yield, yield reduction and a multi-trait index avoided this bias and identified lines that were both productive and stable. ML-57 illustrates the ideal case, combining the highest rainfed yield with a reduction of only 1.63%. ML-59 yielded slightly more under rainfed conditions than under irrigation. With three replications and a difference smaller than the critical difference, this is best read as full stability rather than a genuine positive response to stress.

Agreement between seedling and field screening

The close agreement between seedling and field indices ($p = 0.97$) and the high correlation of seedling dry weight at 25% PEG with rainfed grain yield were stronger than expected. Reviews of drought breeding in rice have repeatedly cautioned that seedling traits measured in artificial media often fail to predict yield under reproductive-stage stress, because the processes limiting yield in the field operate at flowering and depend on soil water extraction by the whole root system (Fukai *et al.*, 1995) [15]. Kamoshita *et al.* (2008) [30] reached a similar conclusion after reviewing a large body of phenotypic and genetic studies. Several features of the present study may explain the difference. The stress in the field was moderate and yield potential dominated rainfed yield, so any trait reflecting general vigour would be expected to correlate with it. The 20 field lines were themselves selected on seedling performance, and although this restriction of range would normally weaken a correlation, the lines that were advanced still spanned the full distance from highly tolerant to susceptible. Finally, the indices at both stages were built from sets of strongly intercorrelated traits, which tends to inflate agreement between them. The concordance is encouraging, but it should be confirmed in unselected material and under more severe stress before seedling PEG screening is used to replace field evaluation rather than to precede it.

Restorer loci and drought tolerance

Lines carrying both *Rf3* and *Rf4* were over-represented among the tolerant group. Seven of the nine lines with injury scores of 1 or 3 were double positive, while 16 of the 18 lines with *Rf4* alone scored 5 or higher. No mechanism links fertility restorer genes to drought tolerance. *Rf4* encodes a pentatricopeptide repeat protein acting on a single mitochondrial transcript in the anther (Chen *et al.*, 2014) [6], and the *Rf3* region carries no gene known to influence water relations. The association is more plausibly explained by shared ancestry. Lines carrying both loci are likely to descend from a small number of elite restorer parents that were also selected for vigour and broad adaptation, and pedigree analysis of the test lines would allow this explanation to be tested directly. ML-47, a double-positive line with poor seedling tolerance, shows that restorer markers cannot be used as surrogates for drought tolerance. Their value lies in confirming restorer status, while tolerance must still be phenotyped.

The fact that three of the five best lines in both screens carried both restorer loci is nevertheless of practical value. Lines with restorer alleles at both loci restore WA-CMS more completely and more consistently across environments than single-locus restorers (Sattari *et al.*, 2007) [7]. If ML-02, ML-13 and ML-32 are confirmed as effective restorers in test crosses, they would combine robust fertility restoration with stable yield under water deficit, which is the combination required of male parents for hybrids intended for rainfed ecologies. The *Rf4*-only lines ML-57 and ML-05 performed as well in the field and deserve equal attention, since a single dominant *Rf4* allele is often sufficient for restoration in *indica* backgrounds.

Limitations of the study

The field evaluation was conducted in a single season at one location. Genotype $\times$ season interaction is large for yield under drought, and the ranking of lines may change in a year with an earlier or more severe dry spell (Kumar *et al.*, 2007) [31]. Restorer status rests on linked markers, and test cross data confirming fertility restoration are not yet available. Grain yield was recorded on a per-plant basis, which does not capture competition effects and plot-level yield. Physiological traits that would explain the mechanism of tolerance, such as leaf water potential, canopy temperature, relative water content and root distribution in the soil profile, were not measured. PEG-induced stress in nutrient solution also differs from soil drying in the absence of mechanical impedance and in the speed at which stress develops (Price *et al.*, 2002) [32]. These gaps define the programme of work needed before the candidate lines are released into hybrid breeding.

Conclusion

Marker screening for *Rf3* and *Rf4* reduced 60 rice lines to 28 *Rf4*-positive restorer candidates, and a two-stage evaluation separated these candidates clearly for drought response. Osmotic stress at 25% PEG cut seedling growth by about one third on average, with a significant genotype $\times$ stress interaction that allowed tolerant and susceptible lines to be distinguished. Under rainfed conditions grain yield fell by 11.4% on average, and the loss was governed mainly by spikelet fertility. Seedling performance under 25% PEG ranked lines in close agreement with their rainfed yield. ML-02, ML-13 and ML-32, which carry restorer alleles at both loci, and the *Rf4*-positive lines ML-57 and ML-05 combined high rainfed yield with small yield reduction and strong seedling tolerance. These five lines are recommended as male parents for hybrids targeted at rainfed ecologies, subject to confirmation of fertility restoration through test crosses with WA-CMS lines. Future work should test the hybrids and their parents over several seasons and sites under quantified reproductive-stage stress, measure the physiological basis of spikelet fertility maintenance, and use gene-based markers to confirm the restorer alleles.

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