Closing the gap: linking phenotype to genotype in rice lines contrasting for cold adaptation

Jaquie Mitchell[1], Siti Zulkafli[1], Brad Campbell[1], Peter Snell[2] Ian Godwin[1], Shu Fukai[1]
[1]The University of Queensland, School of Agriculture and Food Science, St Lucia, Queensland, 4072, jaquie.mitchell@uq.edu.au
[2]Department of Primary Industries, Yanco Agricultural Institute, Yanco, 2703, NSW, Australia.

Abstract
Rice (*Oryza sativa* L.) production can be severely affected by low temperatures (15-19ºC) during the reproductive stage. Previously a major quantitative trait loci (QTL) was mapped on chromosome 10 which explained 20.5% of the variation in low temperature induced spikelet sterility (SS) at the booting stage of an F2 Reiziq x Lijiangheigu (RL) population. Extreme bulks (10 tolerant and 10 susceptible) selected from the F2 stage were advanced to the F5 generation and again exposed to low temperature (24/15°C) for 14 days at booting and flowering stage. The objectives of this study were to determine i) whether lines identified as tolerant or susceptible to cold at booting at F2 generation maintained their classification at F5 ii) whether F5 RL lines identified as cold tolerant at the booting stage were also tolerant at the flowering stage and iii) whether lines identified as tolerant contained segregating markers in the vicinity of the previously identified QTL region.
SS at the booting stage (54.2%) was more severe than at the flowering stage (35.8%) indicating higher susceptibility to low temperature at booting. There was a positive correlation (r=0.62**) between SS at booting and flowering with 5 lines performing consistently well. There was also good consistency in performance across the generations with 8 of the F5 generation progeny lines maintaining high to medium tolerance (MT) to cold temperature at booting while 8 susceptible F2 lines were also susceptible at F5. SNP markers of potential importance were identified on chromosomes 5, 7 and 10. Lines containing the QTL region located on the short arm of chromosome 5 and 7, with Lijiangheigu as the allelic donor, had a reduction in SS of ≈ 25 and 27% respectively. Early generation selection in this population was effective for improving cold tolerance and the utilisation of a high density genome by sequence (GBS) marker system allowed the detection of additional QTLs which may prove useful in marker assisted breeding strategies.

Keywords spikelet sterility, early generation selection, cold temperature stress, early microspore stage,

Introduction
The rice industry is based in the Riverina region in south-western NSW and while grown as a summer crop with temperatures reaching a mean daily maximum of 31ºC in January (*www.**bom**.gov.au*), typically cold night air temperatures are a major cause of yield loss and variability. The optimum sowing time (October-November) for rice aims to ensure that the most cold sensitive young microspore stage occurs when night temperatures are the warmest in late January to early February. However, the probability of the crop experiencing night temperatures less than 15°C is as high as 80% in November and 50% in late February the latter coinciding with flowering (Farrell et al. (2006). Thus, there is value in identifying lines that have adaptation to cold tolerance across the whole reproductive period.
Ye et al (2010) reported that the QTL, qLTSPKST10.1, located within a 3.5 cM interval between SSR markers S10010.9 and S10014.4 on chromosome 10, could explain 20.5% of the variation in SS caused by low temperature treatment at the booting stage. In addition this QTL had a strong additive effect and could increase the SS by 14% in genotypes carrying the allele from Lijiangheigu. i.e. the allele from Lijiangheigu increased the SS, and allele from Reiziq decreased the SS. While a number of major genes have been reported to contribute to tolerance at the reproductive stage there is growing support that multiple genes are involved (Andaya & Tai 2006; da Cruz et al. 2013). It is more than likely that some tolerance genes are important across all growth stages while others provide tolerance to specific stages of development.
In this paper we examined a population which was known to possess a QTL with large additive effect to determine whether selection can be successful in early generation (F2 stage). In order to gain a much finer resolution of the chromosomal regions displaying segregation between the parents we have performed a bulk segregant analysis (BSA) with a genotyping by sequencing system (DArTseq), with the advantage of markers directly anchored to the sequenced genome of rice.

Materials and methods
Phenotyping
Two experiments, flowering stage cold stress, and booting stage cold stress, were conducted in a controlled temperature glasshouse at the Gatton Campus of The University of Queensland (Latitude: -27.554404 |

Longitude: 152.33864) from March to September 2014. In both experiments, 25 genotypes consisting of five varieties (Reiziq, Lijiangheigu, Kyeema, Norin PL8 and Sherpa) and 20 (10 tolerant and 10 susceptible) F5 lines derived from single seed descent were advanced from the F2 generation of RL cross (Ye et al. 2010), except for 1 susceptible line which was lost during the flowering stage experiment. The tolerance or susceptibility of each line was determined by SS in F2 generation and was on average 13.8% (all <17%) and 80.6% (all >73%) for the two extreme bulks respectively. Lijiangheigu, is a medium grain rice originated from Lijiang (alt. 2390m a.s.l.), China and is considered cold tolerant at all growth stages (Ye et al. 2009) and NorinPL8 is a Japanese cold tolerant parental line (Saito et al. 1995). Reiziq, Sherpa and Kyeema are Australian varieties with Sherpa considered quite tolerant to cold temperature stress at the reproductive stage (Troldahl et al. 2014).

Each experiment was conducted in a completely randomized design with three replicates. All plants were grown in a warm room (30ºC/19ºC ±2ºC day/night) except for 14 days of cold treatment (24°C/15°C day/night). The maximum temperature in the cold room varied between 22.0 and 27.6°C while the night time temperature was constant throughout the experimental period. In the flowering stage experiment, each pot was moved into the cold room when the individual plant reached the heading stage. The booting stage experiment was planted 17 days after the flowering stage experiment, and when 2 out of 3 replications of a line in the flowering stage experiment had reached the heading stage, all three replications of the same line in the booting stage experiment were moved into the cold room.

Plastic tube pots (50x120mm) were filled with 250 ml of alluvial Lockyer prairie soil consisting of light, black clay (Isbell 2002). Five seeds were sown in each pot and seedlings thinned to leave one plant per pot 16 DAS (days after sowing). Initially seedlings were grown aerobically and 14 DAS, the water level was increased gradually (2 cm every 3 days) until 31 DAS when the water level was maintained at 4 cm above the soil surface. One gram of slow release Osmocote® Pro 3-4M fertiliser (17N-11P-10K-2MgO-TE) was applied to each pot before sowing. Iron sulphate was sprayed twice at 27 and 47 DAS as the plants started to show symptoms of iron deficiency.

Head emergence was defined as the emergence of the first spikelet from the sheath. When the first spikelet protruded at least 2cm from the sheath's base on the main stem panicle, but before flowering, the main stem was tagged and plants transferred into the cold room for the flowering stage. The number of filled spiklelets; unfilled grain i.e. spikelets that developed hull but were empty; and dead spikelets i.e. spikelets that failed to develop hull of spikelets on the main stem panicle were counted for each line. SS was calculated as 100 (%) – filled grain (%). All data were statistically analysed using Genstat V14.

Genotyping

Genomic DNA was extracted from young leaf tissue sourced from each line utilising the hexadecyltrimethylammoniumbromide (CTAB) technique, as described by Rogers and Bendich (1985). DNA was prepared for genotyping according to the recommendations of diversity arrays technology (www.diversityarrays.com) and genotyped utilising the DArTseq system and Rice array (Oryza *sativa*). The model genome used in sequence alignments was sourced from Phytozome assembly v9 downloaded from ftp://ftp.jgi-psf.org/pub/compgen/phytozome/v9.0/Osativa/assembly/. Marker data was compiled and analysed utilising Microsoft Office Excel 2007, with markers containing ≥ 20% missing data removed. After sorting lines according to phenotypic score for SS, markers were filtered for clear segregation patterns between the tolerant and susceptible lines and their respective parents. This consisted of a minimum of 65% of lines in both the tolerant and susceptible group sharing one or the other parental genotypes. For DArT markers, only those that had at least 2 or more markers consecutively located within 50,000 physical map units of each other were retained and for SNPs, at least 1 DArT marker within 50,000 physical map units of a SNP were retained.

Results and Discussion

Genotypic consistency in performance (SS) between flowering and booting stage stress

There was a highly significant ($p<0.001$) positive correlation in SS between the flowering and booting stages ($r=0.62^{**}$; Figure 1). Significant positive correlations across growth stages have been reported previously by Ye et al (2009) among others when examining response of varieties. Herein we show similar positive correlations are observed when working with a population of lines from a single cross and offers the promise of an underlying tolerance regardless of potential physiological mechanisms involved. While the importance of cold temperature events at the booting stage is recognised by the Australian rice growing region, cold temperatures at flowering could also be a major issue and general tolerance across development stages would be beneficial. Almost all genotypes that were susceptible at the flowering stage performed similarly at the booting stage e.g. RL-37, RL-232 and RL-243. Similarly, some genotypes that were tolerant at flowering stage were also tolerant at the booting stage e.g. Lijiangheigu, Norin PL8, Sherpa, RL-10 and RL-11. As

noted by Ye et al 2009, Lijiangheigu has undergone long-term natural selection for cold tolerance as it originates from high elevation areas and could explain cold tolerance for all growth stages.

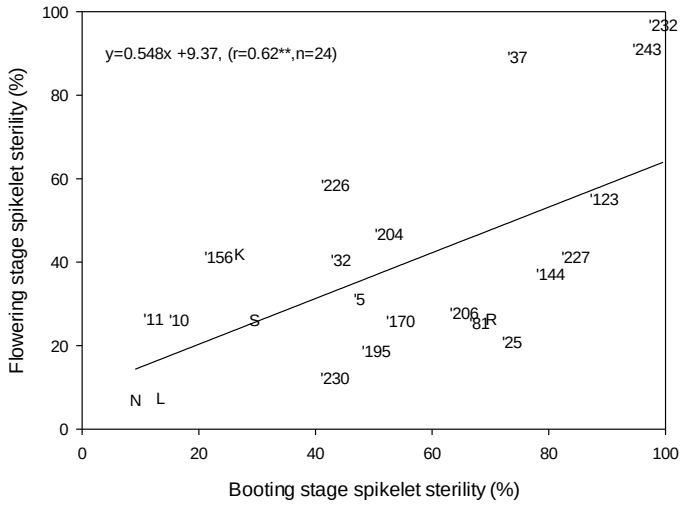

Figure 1. Relationship between spikelet sterility when 24 lines were exposed to cold temperature (24/15°C day/night), for 14 days at booting and flowering stages. Numbers are consistent with Line code numbers listed in Table 1 while varieties are abbreviated with first initial: R= Reiziq; L= Lijiangheigu, N= NorinPL8; S= Sherpa, K= Kyeema.

Table 1. Variety, tolerance and susceptibility classification of 22 lines at F2 and F5 generation based on percentage spikelet sterility (SSB) exposed to cold temperature (24/15°C, day/night) for 14 days at booting stage. Presence (√) or absence (X) of cold tolerant enhancing alleles donated from either Lijiangheigu (L) or Reiziq (R) is noted for 3 QTL regions identified by DArTseq analysis.

Line code	@ F2	SSB(%)	sig	@ F5	Chr 5 (L)	Chr 7 (L)	Chr 10 (R)
RL-11	T	12.2	a	T	√	√	√
Lijiangheigu	T	13.4	a	T	√	√	X
RL-10	T	16.6	ab	T	√	√	√
RL-156	S	23.4	abc	T	X	√	X
RL-230	S	43.3	abcd	T	√	√	√
RL-226	T	43.4	abcd	T	√	X	√
RL-32	T	44.3	abcde	T	√	X	√
RL-5	T	47.5	abcde	T	√	√	X
RL-195	T	50.4	bcdef	T	√	X	√
RL-204	T	52.6	bcdef	MT	√	√	√
RL-170	T	54.6	cdefg	MT	X	√	√
RL-206	S	65.5	defgh	S	√	√	X
RL-81	S	68.1	defgh	S	√	X	X
Reiziq	S	70.1	defgh	S	X	X	√
RL-25	S	73.7	defgh	S	X	X	X
RL-37	T	74.6	defgh	S	X	√	√
RL-144	S	80.3	efgh	S	X	√	X
RL-227	S	84.6	fgh	S	X	X	X
RL-109	S	85.1	fgh	S	X	X	√
RL-123	T	89.5	gh	S	X	X	√
RL-243	S	96.8	h	S	√	X	X
RL-232	S	99.6	h	S	X	X	X
Mean		54.2**					
LSD (5%)		36.6					
CV (%)		41.1					

Genotypic consistency in performance between F2 and F5 generation in booting stage stress and QTLs

Mean SS under the booting stage cold was 54.2% at the F5 generation (Table 1). The mean sterility of progeny lines that were selected as tolerant at F2 stage was lower than that selected as susceptible (48.6 vs 72.0%). Among the 10 progeny lines that were tolerant in F2 generation, 7 had lower SS than the mean of 25 genotypes. Comparatively, among the 9 progeny lines that were selected at F2 as susceptible, only 2 (RL-156 and RL230) had sterility less than the mean. Four varieties had sterility less than 30% while Reiziq had a high sterility of 70.1%. Sterility of lines RL-10 and RL-11 (16.6 and 12.2%) were similarly as low to the strongly tolerant varieties Lijiangheigu and Norin PL8 (13.4 and 9.1%). Some progeny lines such as RL-10, RL-226, RL-32

and RL-11 were cold tolerant, while others such as RL-243 and RL-232 were susceptible at both generations. Thus, SS of 20 lines exposed to cold at booting stage at F5 generation had a significant ($p<0.05$) positive correlation ($r=0.47^*$) with SS determined at the F2 generation, suggesting that additive effects were large.
As noted by Cruz et al. 2013 when non-additive effects are large, selection should be applied in advanced generations of breeding programs (F4 or F5 stage).
A total of 14,563 SNPs were generated and analysed utilising DArTseq. Initial data filtering revealed significant distinctive haplotypes on chromosomes 3, 5, 7, 8, 10 and 11. Of these, only three of the genomic regions had a predicted significant effect on SS% and these were located on chromosomes 5, 7 and 10. Eight lines classified as tolerant (T & MT) at the F_5 generation contained the Reiziq allele at the qLTSPKST10.1 QTL while 8 of the susceptible lines had the allele from Lijiangheigu. A significant phenotypic difference in SS existed between those lines that contained the allele from Reiziq or Lijiangheigu at this QTL on chromosome 10 (48 vs 73%). The genomic regions on chromosome 5 and 7 donated by Lijiangheigu, had an average of 49 and 47% SS in tolerant lines and 74% SS in susceptible lines harbouring the Reiziq allele. When the additive effect of genomic combinations was considered, four lines had the tolerant allele from the genomic regions on chromosome 5, 7 and the QTL on chromosome 10 which resulted in SS of only 31%.

Conclusion

There was consistency in performance across the F2 and F5 generations. Four genotypes had sterility less than 30% with lines RL-10 and RL-11 strongly tolerant. This result demonstrates early generation selection was effective for improving cold tolerance in rice within the RL population where the parental line Lijiangheigu, is a line considered to have a general cold tolerance across growth stages and where additive effects appear large. Whether cold tolerance donated by this line holds true in populations of a different genetic background requires further investigation. There was also consistency in performance of the F5 RL lines between exposure to cold temperature at booting and flowering stages with 5 genotypes performing consistently well.
In addition to the QTL identified on Chromosome 10 there were two other regions identified as potentially offering improvements in cold tolerance on Chromosome 5 and 7. When the additive effect of combinations of genomic regions and QTL was considered, lines that had the tolerant allele on chromosomes 5 (L), 7 (L) and 10 (R) conferred significant tolerance to cold exposure at booting. The results of this work suggest that the incorporation of Lijiangheigu material into the breeding program will provide general improvement in cold tolerance.

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