Improving low-temperature tolerance in rice

Christopher Proud[1], Jaquie Mitchell[1], Bradley Campbell[1], Zuziana Suzanti[1,2], Ian Godwin[1], Ben Ovenden[3], Peter Snell[3] and Shu Fukai[1]

[1] The University of Queensland, School of Agriculture and Food Sciences, St Lucia, QLD, 4072, Email: c.proud@uq.edu.au
[2] Indonesian Centre for Rice Research, Agency for Agricultural Research and Development,
Jalan Raya 9 Sukamandi, Subang, West-Java.
[3] Department of Primary Industries, Yanco Agricultural Institute, Yanco, 2703, NSW, Australia.

Abstract
The occurrence of low temperature (15-19^{o}C) events particularly during the young microspore stage (YMS) is a major constraint facing the temperate rice industry leading to reduced fertility and yield. A series of experiments have been conducted to improve our understanding of low-temperature tolerance in terms of underlying physiological mechanisms and the molecular basis of traits involved in low-temperature tolerance at the YMS in populations pertinent to the Australian breeding program. Our research has identified that anther dehiscence is a floral trait critical to ensuring low-temperature tolerance. Single nucleotide polymorphisms (SNPs) were identified utilising genome wide association analysis with 6 putative QTL identified for spikelet sterility that co-located with number of dehisced anthers. The use of putative markers for spikelet fertility and underlying floral traits will lead to increased efficiency in breeding for low-temperature tolerance in rice.

Key Words
Spikelet sterility, yield, cold tolerance, temperate production, molecular markers, anther dehiscence.

Introduction
In Australia's NSW rice industry, reduced seed set as a result of low temperatures (<20°C) at the YMS is the major constraint to yield stability and is believed to be the result of damage to developing pollen (Farrell 2006). Spikelet sterility is widely accepted as an indicator for low-temperature injury at the reproductive stages. Marker assisted breeding is often proposed as an efficient method for improving low-temperature tolerance in a breeding population as the result of the low repeatability of field screening and the intensiveness of controlled temperature screening. There have been numerous studies identifying quantitative trait loci (QTL) associated with low-temperature tolerance (Saito 2001, Andaya and Mackill 2003), but few utilise a robust highly repeatable phenotyping method which is necessary for the identification of QTL. Mitchell (2016) developed a repeatable two-set screening method that clearly identifies YMS and identified 3 QTL associated with cold tolerance using bulk-segregant analysis.

In late 1990s, the NSW DPI identified a number of varieties as potential donors for low-temperature tolerance at YMS, and some floral characters (anther length, pollen number) were identified that confer low-temperature tolerance (Farrell 2006). However, because of large variation in genetic background among these varieties, these findings were not conclusive and the use of segregating populations are required for further testing of the importance of these physiological characters. The physiological basis for low-temperature tolerance at YMS of a Kyeema//Kyeema/Norin PL8 (KKN) population was recently published and highlighted the importance of traits "pollen in anther" and "greater numbers and length of dehisced anthers", which enabled the release of pollen grains from the thecae and was positively correlated with seed set (Susanti 2019). Using the same low-temperature tolerant donor, Norin PL8, Saito (2001) demonstrated that two QTL that improved grain set on chromosome 4 was associated with increased anther length. However, there have been limited examination on the genetic associations with floral traits such as the number of dehisced anthers and pollen on stigma which are directly influenced by low temperatures at YMS. In this paper, we utilised the phenotypic screening method described by Mitchell (2016) to identify genomic regions associated with spikelet sterility after low-temperature exposure at YMS, and examined the genetic associations between spikelet sterility and key floral traits in the aforementioned KKN population.

Methods
KKN population
This study phenotyped 117 F6, KKN lines, a population developed through single seed descent from BC2F2 head selection, taken by the breeders. A backcrossed population was employed as it was more likely to generate progeny of merit to the breeding program, whose breeding objective, in this case, was a low-temperature

tolerant fragrant long grain replacement for Kyeema. To surmise, two F2 tolerant lines identified in the F2 cold nursery of Kyeema/Norin PL8 were backcrossed to Kyeema from which two F1 populations (YC08258 and YC08259) were selfed, and the F2 populations were screened in a cold nursery. Two tolerant individuals were identified in YC08258 from 55 panicles and 7 individuals from 69 panicles in YC08259. Approximately, 15 F3 seed from each selected F2 individual were expedited through SSD to the F6.

Phenotyping
YMS low temperature experiments were conducted in controlled temperature glasshouse rooms at The University of Queensland. These glasshouse rooms were located next to each other, and other than temperature, growing conditions were identical. A two-stage phenotyping system described by Mitchell (2016) was utilised to ensure the identification of YMS (booting) for each genotype. Briefly this system consists of two sets of each genotype sown 18 days apart. Set 1 grown entirely under warm conditions (28°C/22°C day/night) identified the heading stage at which time the 3 replications of Set 2 were moved to the low temperature (21°C/15°C day/night) room for 14 days, after which they were returned to the warm room.

Measurements and Statistical Analysis
At maturity, the total number of spikelets along main-stem panicle were counted. Spikelet stertility (SS) was calculated as a ratio of unfilled spikelets to total spikelets (× 100%) to assess low-temperature tolerance in rice for a particular genotype. Floral trait analysis was conducted as detailed in Susanti (2019). Briefly, total pollen number in anther (PIA), anther length (AL), anther dehiscence length (ADL), number of dehisced anthers (NDA) and pollen number on stigma (POS) were measured after 14 days low-temperature exposure. Best Linear Unbiased Estimates (BLUEs) were modelled from the phenotypic data using Genstat.

Genotyping and GWAS
DNA was extracted from newly emerged young leaves utilising the hexadecyl-trimethylammonium-bromide technique. The samples were genotyped following an integrated DArT and genotyping-by-sequencing methodology, DArTseq™. Each marker was scored as 0 or 1 representing homozygosity for the two alleles. The generated sequence data was aligned on the rice reference genome assembly version 9 to identify single nucleotide polymorphism (SNP) markers. A total of 8,765 SNPs were utilised for subsequent analysis after removal of those markers that were monomorphic, duplicated, with more than 20% missing values, or had a minor allele frequency below 0.05.

A genome wide association analysis was performed on the BLUEs for spikelet sterility, pollen in anther, number of dehisced anthers and pollen on stigma (Susanti 2019). A compressed linear mixed model approach was implemented in the package 'rrblup'(Endelman 2011) in the R environment (R Core Team 2019). The function was modified to estimate marker effects. A leave-one-approach was adopted which removes the chromosome being tested in the formation of the kinship matrix (**K**) to prevent the double fitting of the candidate marker as a fixed effect and a random effect in **K**. In the selection of markers for the calculation of **K**, a clustering analysis was performed to identify highly correlated markers (r^2>0.25) on each chromosome. Only one marker was randomly selected from each cluster in the generation of **K** to avoid weighting positions with a higher density of SNPs. A significant threshold of LOG 2.9 was determined using the Bonferroni correction (0.05/n) where n was the number of linkage groups, and based on the direction of the allelic score, we assigned tolerant alleles (negative effect) to each marker. Genetic estimated breeding values (GEBVs) were modelled for each trait with AsReml-R by fitting **K** to the BLUEs. Additive genetic correlations were estimated by the variance parameters from separate bivariate models between spikelet sterility and floral traits.

Results and Discussion
Genomic regions associated with spikelet sterility after low-temperature exposure at the young microspore stage.
In total, 14 significant markers were identified to be associated with SS and were grouped into 7 QTLs containing 1-3 markers (Figure 1a). The peak marker on the QTL on chromosome 10 had the strongest signal (5.5) which Norin PL8 donated the tolerant allele. A further 4 QTL (chromosome 2, 3, 5 and 7) were present where the Norin PL8 allele decreased the level of spikelet sterility. Kyeema was the donor for remaining two QTL on chromosome 1 and 11. The two previously identified QTL identified from the low-temperature tolerant donor Norin PL8, *Ctb-1* and *Ctb-2*, on chromosome 4 (Saito 2001), were not detected in the GWAS

of the KKN. The sequence of the parents in the region (Saito 2001) was fixed in both parents. This supports the phenotypic results which suggest that Kyeema does have a degree of low-temperature tolerance. The susceptible rating of Kyeema given by industry is largely because it is a tall variety which results in limited protection from permanent deep water.

All of the lines contained at least one tolerant allele from the 7 QTL identified, however, 64% of the population contained between 4-6 and highlights the shift in allele frequencies as a result of selection in earlier generations. There was a highly significant ($p<0.01$) negative linear trend between the number of tolerant associated alleles at QTLs and SS with the model accounting for 42% of the additive genetic variance (Figure 1b). The linear trend indicated an average sterility decrease of 8% per tolerant allele, and demonstrates the potential of stacking multiple loci to improve the level of cold tolerance. However, the identified markers first need to be shown to be effective across a range of genetic diversity before being integrated into a breeding program (Cobb 2018).

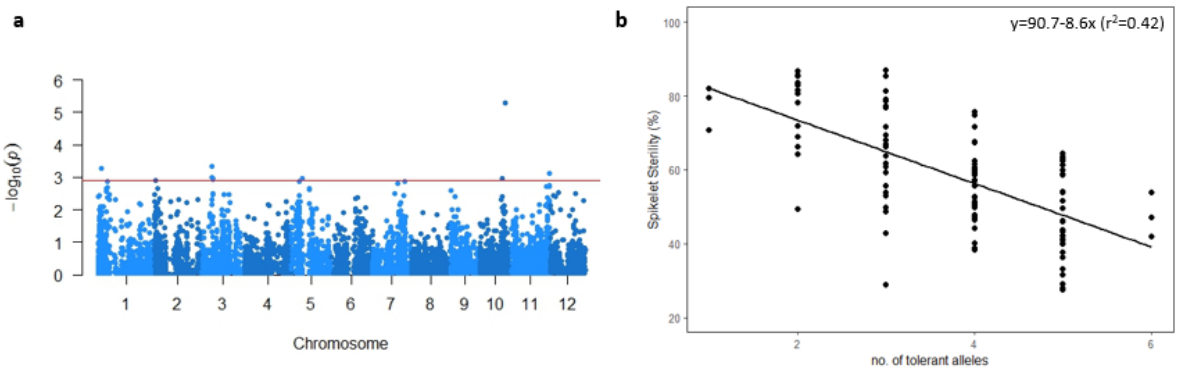

Figure 1. Marker associations with spikelet sterility (a). The relationship between spikelet sterility in the KKN population exposed to low temperature at YMS and the number of tolerant alleles (b).

QTL in common between spikelet sterility and floral traits

All QTL identified for SS were co-located with at least one of three floral traits: PIA, -NDA and POS. The tolerant alleles associated at QTLs for SS demonstrated a positive effect for all floral traits that had significant associations, providing evidence that SS after low-temperature exposure at YMS was the direct result of the failure of the male reproductive organs. The congruency between loci for SS and floral traits is consistent with the phenotypic correlations demonstrated by Susanti (2019) and strong additive genetic correlations demonstrated herein (r_g= -0.76** to -0.98**). NDA was associated with all loci with the exception of one located on chromosome 11, while PIA had significant association with the loci on chromosomes 2, 5 and 10. Supporting the hypothesis that overall pollen abundance is an important factor in driving the swelling of the anther for dehiscence and the successful release of POS. POS had significant associations with the loci identified for SS on chromosome 5, 7 and 11. The QTL on chromosome 5 was associated with all three floral traits. In contrast to the finding of Saito (2001), no co-locating loci between SS and AL was observed which suggest it may be background specific (data not shown). The biplot of the first two principal components of the GEBVs for SS and the three floral traits demonstrated that the stacking of QTL associated with reduced SS will also lead to increases in POS, NDA and PIA (Figure 2). A regression analysis estimated increases in 10 POS, 0.64 NDA (total possible of 6) and 82 PIA per QTL.

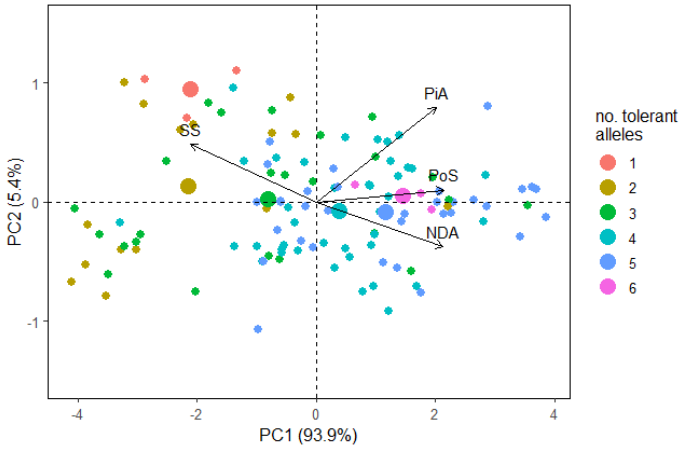

Figure 2. Principal components between the genetic estimated breeding values of spikelet sterility (SS), pollen in anther (PIA), the number of dehisced anthers (NDA) and pollen on stigma (POS). Individuals are grouped according to the number of tolerant associated alleles present for QTLs for SS.

Conclusions

Seven putative QTL associated with SS after low-temperature exposure at YMS in the KKN population were identified. The stacking of the tolerant alleles led to significant improvement in low-temperature tolerance and appears to be an attractive breeding strategy to improve the level of low-temperature tolerance within a population. However, further exploration in alternate backgrounds is required. The genome wide association analysis highlighted that each QTL associated with SS was also associated with at least one floral trait, which demonstrates for the first time, to the best of our knowledge, the direct genetic linkage between SS and injury to the male reproductive traits. The importance of anther dehiscence to grain set was reaffirmed with 6 of 7 QTL associated with SS also associated with the NDA. Current experimentation is underway to validate the putative QTL in alternate backgrounds and further examine the effectiveness of QTL stacking.

Acknowledgements

The authors wish to thank Agrifutures (PRJ-007580) for financial support.

References

Andaya VC and Mackill DJ (2003). QTLs conferring cold tolerance at the booting stage of rice using recombinant inbred lines from a *japonica* x *indica* cross. Theoretical and Applied Genetics 106**,** 1084-1090.

Cobb JN, Biswas PS and Platten JD (2018). Back to the future: revisiting MAS as a tool for modern plant breeding. Theoretical and Applied Genetics 132**,** 647-667.

Endelman JB (2011). Ridge Regression and Other Kernels for Genomic Selection with R Package rrBLUP. Plant Genome 4**,** 250-255.

Farrell TC, Fox KM, Williams RL, Fukai S and Lewin LG (2006). Minimising cold damage during reproductive development among temperate rice genotypes. II. Genotypic variation and flowering traits related to cold tolerance screening. Australian Journal of Agricultural Research 57**,** 89-100.

Mitchell JH, Zulkafli SL, Bosse J, Campbell B, Snell P, Mace ES, Godwin ID and Fukai S (2016). Rice-cold tolerance across reproductive stages. Crop & Pasture Science 67**,** 823-833.

R Core Team. (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. Available: https://www.R-project.org/.

Saito K, Miura K, Nagano K, Hayano-Saito Y, Araki H and Kato A (2001). Identifaction of two closely linked quantitative trait loci for cold tolerance on chromosome 4 of rice and their association with anther length. Theoretical and Applied Genetics 103**,** 862-868.

Susanti Z, Snell P, Fukai S and Mitchell JH (2019). Importance of anther dehiscence for low-temperature tolerance in rice at the young microspore and flowering stages. Crop and Pasture Science 70**,** 113-120.