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Rice-cold tolerance across reproductive stages

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Abstract. Cold temperature stress at the reproductive stage, particularly at booting and flowering stages can cause significant reductions in rice (*Oryza sativa* L.) yield particularly at high latitudes and elevation. Although genotypic variation for cold tolerance is known to exist, the tolerance mechanisms and genotypic consistency across the stages are yet to be understood for segregating populations. Three experiments were conducted under controlled temperature glasshouse conditions to determine floral characteristics that were associated with cold tolerance at the flowering stage and to determine genotypic consistency at the booting and flowering stages. Twenty F_5 Reiziq × Lijiangheigu lines from two extreme phenotypic bulks selected for cold tolerance at booting stage in the F_2 generation were utilised.

Spikelet sterility under cold stress at booting was significantly correlated with spikelet sterility under cold stress at flowering ($r = 0.62$**) with five lines identified as cold tolerant across reproductive stages. There was also a positive correlation ($r = 0.47$*) between spikelet sterility under cold stress at booting at the F_5 and at the F_2 generation. The quantitative trait loci (QTL; qLTSPKST10.1) previously identified on chromosome 10 contributing to spikelet sterility within the F_2 generation, was also identified in the F_5 generation. Additionally, genomic regions displaying significant segregation between the progenies contrasting for their cold tolerance response phenotype were identified on chromosomes 5 and 7 with Lijiangheigu as allelic donor and an estimated reduction in spikelet sterility of 25% and 27%, respectively. Although genotypic variation in spikelet sterility at the booting stage was not related to the development rate for heading or flowering, those cold-tolerant genotypes at the flowering stage were the quickest to complete flowering. Cold-tolerant genotypes at the flowering stage had larger numbers of dehisced anthers and subsequently pollen number on stigma, which contributed to reduced spikelet sterility. It is concluded that enhanced anther dehiscence plays a significant role in improved cold tolerance at the flowering stage.

Additional keywords: anther dehiscence, cold temperature stress, early generation selection, pollen number, spikelet sterility.

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Introduction

Cold temperature stress at the reproductive stage can cause large reductions in yield of rice (*Oryza sativa* L.). Early pollen microspore stage (10–12 days before heading) is the most sensitive to cold injury (Satake and Hayase 1970). The rice industry in Australia is based in the Riverina region in south-western New South Wales, and although rice is grown as a summer crop with temperatures reaching a mean daily maximum of 31°C in January (www.bom.gov.au, accessed 7 July 2016), it is not unusual for cold night temperatures to be a major cause of yield loss and variability. The optimum sowing time (October–November) for rice aims to ensure that the most cold-sensitive early microspore stage occurs when night temperatures are the warmest in late January to early February. However, the probability of the crop experiencing mean night temperatures less than 15°C for a period of 10 days is as high as 80% in November and 50% in late February with the latter coinciding with flowering (Farrell *et al*. 2006*b*). Yoshida (1981) considers flowering or anthesis to be the second most sensitive stage. Flowering stage cold is a problem particularly when crops are planted late in the season in Australia (Gunawardena and Fukai 2005). Although some consistency in cold tolerance among widely different varieties across different growth stages has been shown (Ye *et al*. 2009), it is not certain whether this applies to segregating populations and the mechanisms for such stability or growth stage specificity for cold tolerance are not known. While several major genes have been reported to contribute to tolerance at the reproductive stage there is growing support that multiple genes are involved (Andaya and Tai 2006; da Cruz *et al*. 2013). It is more than

likely that some tolerance genes are important across all growth stages whereas others provide tolerance to specific stages of development.

Spikelet sterility (SS) is widely accepted as an indicator for cold injury at the reproductive stages. Several varieties have been identified as cold tolerant, and some physiological characters (anther length, pollen number) were identified that confer cold tolerance at the booting stage (Ito 1978; Nishiyama 1982; Satake 1991; Farrell *et al*. 2004). Physiological characteristics associated with tolerance for flowering stage cold are less well understood. Using varieties contrasting in their cold tolerance response in northern Japan, Shinada *et al*. (2013) studied characteristics associated with cold tolerance at pollen maturation to fertilisation stages and identified pollen germination and pollen tube elongation to be associated with variation in SS. More recently, work on heat tolerance at flowering has identified the contribution of long basal dehiscence, which was found to enable the release of pollen grains from the thecae and was positively correlated with seed set (Matsui *et al*. 2005; Tian *et al*. 2010). This mechanism may equally apply to cold tolerance as the number of pollen shed on a stigma is likely to be a major constraint under cold conditions and may relate to the dehiscence length of anther theca, which determines pollination viability and SS under low temperature at flowering. Traditionally the work to identify physiological and morphological differences in floral traits has been conducted on varieties that can greatly differ in genetic background and consequently many other traits other than those of interest. Thus, these findings are not conclusive and require further investigation.

Introducing cold tolerance genes into Australian rice varieties is a major target for the New South Wales rice industry to overcome cold stress particularly at the booting stage (Farrell *et al*. 2001). Cold-tolerant lines exist and have been used to introgress cold tolerance genes into Australian-adapted varieties (Reinke *et al*. 2012). In earlier work, Ye *et al*. (2010) crossed rice varieties from China (Lijiangheigu) and Australia (Reiziq) to introduce cold tolerance genes into a well-adapted Australian line. Ye *et al*. (2010) identified a quantitative trait loci (QTL; qLTSPKST10.1) located within a 3.5-cM interval between simple sequence repeat (SSR) markers S10010.9 and S10014.4 on chromosome 10 in the F_2 cross of Lijiangheigu and Reiziq, which explained 20.5% of the variation of SS under cold stress at booting. 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.

In this paper we used the Lijiangheigu × Reiziq population developed by Ye *et al*. (2010) to determine whether selection at early generations can confer cold tolerance in later generations. In order to identify distinct and variable chromosomal regions between the parents we have performed a bulk segregant analysis with a genotyping-by-sequencing platform.

The objectives of the paper were to (1) identify plant and floral characteristics associated with cold tolerance at flowering, (2) determine consistency in cold tolerance as measured by SS at booting stage and flowering stage, (3) determine if F_5 lines maintained the cold tolerance phenotype of the F_2 lines from which they were derived, and (4) to validate the QTL on chromosome 10.

Materials and methods

Early booting and flowering stage cold stress experiments

A pair of experiments, an early booting stage cold stress and a flowering stage cold stress, were conducted in a controlled temperature glasshouse at the Gatton Campus, The University of Queensland (Latitude: –27.554404 , Longitude: 152.33864), Queensland, Australia from March to September 2014.

Genetic material

Two extreme phenotypic bulks consisting of the 10 most tolerant and 10 most susceptible F_2 progenies from Reiziq × Lijiangheigu cross identified by Ye *et al*. (2010) were selected based on SS. These were advanced by single seed decent to the F_5 generation and evaluated for cold tolerance alongside five cultivars (Reiziq, Lijiangheigu, Kyeema, Norin PL8 and Sherpa). However, one susceptible line was not tested at flowering stage. Lijiangheigu is a medium grain rice originating from China (Oliver *et al*. 2005) and has been identified previously as cold tolerant at all growth stages, whereas Reiziq is an Australian cultivar and is cold susceptible at both booting and flowering stages (Ye *et al*. 2009). NorinPL8 is a Japanese cold-tolerant cultivar (Saito *et al*. 1995) and Sherpa and Kyeema are Australian cultivars with Sherpa considered quite tolerant to cold temperature stress at the reproductive stage, whereas Kyeema is considered susceptible (Troldahl *et al*. 2014).

Treatments

Each experiment was conducted in a completely randomised within blocks design involving 25 rice genotypes with three replicates. In both experiments, plants were grown in a warm room (30°C/19°C day/night) except for 14 days of cold treatment (24°C/15°C day/night). The maximum temperature in the cold room varied between 27.6°C and 22.0°C whereas the night time temperature was constant throughout the experimental period at 15°C. In the flowering stage experiment, each pot was moved into the cold room when the individual plant reached the heading stage. The early booting experiment was planted 17 days after the flowering stage experiment, and when two out of three replications of a genotype in flowering stage experiment had reached the heading stage, all three replications of the same genotype in early booting experiment were moved into the cold room. As described by Ye *et al*. (2010), at the F_2 generation genotypes were moved into the cold room when the auricle of the flag leaf emerged (auricle distance greater than 0 mm).

Growing conditions

Plastic tube pots (50 mm × 120 mm) with an open mesh base were filled up with 250 mL of Lockyer prairie soil consisting of light, black clay [USDA Soil Taxonomy: Fluventic Hapludolls; (Isbell 2002)]. The soil physical and chemical characteristics was described in detail by Powell (1982). Five seeds were sown in each pot and seedlings were thinned gradually to leave one plant per pot at 16 days after sowing (DAS). Initially seedlings were grown aerobically and water level was increased gradually

(2 cm every 3 days) from 14 to 31 DAS after which water level was maintained at 4 cm above the soil surface. Plants within blocks were rotated weekly to remove any micro-environmental variation that may exist within the tanks.

Approximately 1 g of slow release Osmocote® Pro 3-4M fertiliser (17N-11P-10K+2MgO+TE, ICL Specialty Fertilizers) was applied to each pot before sowing. Iron sulfate was sprayed twice at 27 and 47 DAS as the plants started to show symptoms of iron deficiency. Fungicide (Banrot 400WP, ICL Specialty Fertilizers) and insecticide (Crown 225 SL and Procide 80SC; ICL Specialty Fertilizers) were sprayed to prevent fungus attack and to control red spider mites.

Measurements

The number of stems was counted three times during growth whereas the number of panicles was determined at maturity. A stem was counted when it had a minimum of two leaves per stem.

Heading was defined as the emergence of the first spikelet from the sheath. When the first spikelet protruded at least 2 cm from the sheath's base on the main-stem panicle, but before flowering, the main stem was tagged and plants were transferred into the cold room in flowering stage experiment. The dates of heading, first flowering and last flowering of the main-stem panicle were recorded. First flowering was defined as the appearance of the first extruded anthers from the spikelet. Last flowering was considered to be when the spikelets on the lower branches of the panicle had extruded anthers (i.e. the entire length of the panicle exhibited extruded anthers).

The number of spikelets on the main-stem panicle were counted and filled grain, unfilled grain i.e. spikelet that developed hull but were empty, and dead spikelets, were identified. Spikelet sterility was calculated as 100 (%) – filled grain (%). From a main-stem panicle, 100 grains were weighed to determine 100-grain weight.

At maturity, shoot (leaf, leaf sheath and stem) biomass was determined after oven drying at 65°C for 5 days. The main-stem panicles were separated from tiller panicles and dried at 37°C for 5 days and weight determined. Total dry matter was a sum of shoot and panicle dry weight.

Genotyping

Genomic DNA was extracted from newly emerged young leaves sourced from each genotype utilising the hexadecyl-trimethyl-ammonium-bromide technique, as described by Rogers and Bendich (1985).

The samples were genotyped following an integrated DArT and genotyping-by-sequencing methodology involving complexity reduction of the genomic DNA to remove repetitive sequences using methylation sensitive restrictive enzymes before sequencing on Next Generation sequencing platforms (DArT, www.diversityarrays.com, accessed 7 July 2016). The sequence data generated were then aligned to the rice reference genome assembly version 9 (ftp://ftp.jgi-psf.org/pub/compgen/phytozome/v9.0/Osativa/assembly/, accessed 7 July 2016) to identify single nucleotide polymorphism (SNP) markers.

The SNP containing ≥20% missing data were excluded from subsequent analyses. Lines were ordered according to phenotypic score for SS in the early booting experiment, and SNP filtered for distinct and significantly different (chi-square test $P \leq 0.01$) haplotypes (minimum of two markers within a 200-kb window) between the tolerant and susceptible lines and their respective parents.

Floral traits contributing to cold tolerance at flowering experiment

Genetic material, cultural and experimental design

A population of 60 F_6 Reiziq × Lijiangheigu lines was grown at the University of Queensland, St Lucia, Queensland, Australia, in August 2014. A subset of 20 of these lines, 11 of which were common to early booting and flowering experiments including the two parents, were sampled to identify floral characteristics that may confer cold tolerance when exposed to cold temperature at the flowering stage. Lines were sown in a glasshouse (mean temperature: 19.7/32.5°C; minimum temperature: 16.5/27.8°C; maximum temperature: 22.6/37.6°C night/day) and transferred to a cold room (mean temperature: 18.4°C; range: 14.3/21.5°C night/day) for 14 days after heading of each genotype (flowering stage cold). The plants were grown under flooded conditions imposed 21 DAS until maturity. Lines were grown in a completely randomised design with three replications and plants within blocks were rotated weekly.

Measurements

Six spikelets were collected from the middle of the main-stem panicle from each plant, shortly after floret opening but before floret closing (anther extrusion). Sampling was conducted during peak flowering time from 9.00am to 12.00pm. Samples were placed into a Petri dish and stored at room temperature until processed.

Three of the six spikelets collected were randomly sampled and dissected to separate the carpel (stigma, style and ovary) from the anthers. To measure anther dehiscence, two anthers, out of the six from each spikelet were randomly sampled. The anthers were lined up on the slide and an Olympus OSM digital micrometer and Olympus SZX10 microscope were used to measure the apical, basal and longitudinal dehiscence and anther length following Matsui and Kagata (2003). The stigma and style were then separated from the ovary and the stigma was stained with iodide solution and the number of pollen grains on the stigma was counted.

Statistical analyses

Analysis of variance based on a completely randomised design was carried out for all characters determined in the experiments using GENSTAT® version 14. Comparison of treatment means were carried out using the least significant difference test (l.s.d.) at 5% level of significance. Correlation analysis was determined on the means.

Principal component analysis (PCA) based on the correlation matrix of seven floral trait measurements was applied to produce a biplot using the R packages 'FactoMineR' (Husson *et al.* 2015) and 'factoextra' (Kassambara 2015). Vectors for floral traits measured were projected onto the genotype space in a PCA biplot.

Results

Booting stage cold stress

The commencement of the booting stage cold varied greatly among genotypes, and this was reflected in heading and subsequent flowering dates (Table 1). There was a significant ($P < 0.05$) positive correlation for days to heading ($r = 0.91$**) obtained under warm conditions (flowering stage experiment) and that of cold exposed lines (early booting experiment). There was a mean delay of 7 days in flowering due to the cold exposure during the booting stage. However, the variation was significant, ranging from no delay in one progeny line (RL-11) to a delay of 12 days in another progeny line (RL-25) and 13 days in Kyeema. The duration of heading to completion of flowering also varied greatly particularly among progeny lines (6–16 days).

Reiziq and Lijiangheigu were different in the pattern of stem and panicle number change as well as various panicle characteristics (Table 2). Reiziq increased the stem number from 68 DAS to maturity but not all stems produced panicles. However, Lijiangheigu did not produce new stems after 68 DAS, but all stems at maturity produced panicles. This cultivar also produced the largest main-stem and tiller panicle weight and total dry matter among five varieties, whereas Reiziq produced the smallest panicle weights. Spikelet number per panicle was higher in Lijiangheigu (107/main-stem panicle) than in Reiziq (58/main-stem panicle). The range in various characteristics among progeny lines was large, and some progeny lines (e.g. RL-10) produced panicle dry weight and total dry matter, which were similar to Lijiangheigu. They also produced the largest number of spikelets and 100-grain weight among all genotypes examined.

Mean SS for the progeny lines under booting stage cold was 60.3% at the F_5 generation (Table 3). The mean sterility of progeny lines that were selected as tolerant at F_2 stage was lower than that selected as susceptible (48.6% vs 72.2%). Seven progeny lines were classified as tolerant (not significantly different from Lijiangheigu, all <47.5% SS), three moderately tolerant (50.4–54.6% SS) and 10 susceptible (>60% SS). Among 10 progeny lines that were tolerant in the F_2 generation, eight had lower SS than the mean (60.3%) of progeny lines. From the nine progeny lines that were selected as susceptible in the F_2 only two (RL-156 and RL-230) had sterility less than the mean. Four varieties had sterility less than 30% whereas Reiziq had a high sterility of 70.1%. Sterility of lines RL-10 and RL-11 (16.6% and 12.2%) were as low as the strongly tolerant varieties Lijiangheigu and Norin PL8 (13.4% and 9.1%).

Spikelet sterility at the booting stage was not correlated with phenological development including delay in heading and duration of heading to flowering time or with tiller number and panicle number (Table 4). However, susceptible genotypes

Table 1. Phenological development (days after sowing) of Reiziq and Lijiangheigu, the lines derived from their cross, and three other varieties

Plants were exposed to cold at booting stage for 14 days. Commencement of cold treatment is also shown. *$P < 0.05$; **$P < 0.001$; n.a., not available

Genotype	Cold commenced	Heading	Heading delay (days)	First flower	Last flower	Heading – last flower (days)
Reiziq	80	101	6	106	112	11
Lijiangheigu	70	90	5	92	96	6
Line range	61 to 78	85 to 103	–1 to 12	90 to 107	92 to 110	6 to 16
Kyeema	84	111	13	115	121	10
Norin PL8	55	77	6	81	84	7
Sherpa	64	89	8	93	96	7
Mean	69	93**	7	97**	101**	8
l.s.d. (5%)	n.a.	5.3	n.a.	5.64	5.17	n.a.
CV (%)	n.a.	3.45	n.a.	3.49	3.08	n.a.

Table 2. Stem and panicle number and dry weights (g) and other characteristics of Reiziq and Lijiangheigu, the lines derived from their cross, and three varieties

The plants were exposed to cold at booting stage for 14 days

Genotype	Stem number			Panicle number	Dry weight at maturity					
	54 DAS	68 DAS	Maturity	Maturity	Shoot	Main-stem panicle	Tiller panicle	Total	Spikelet number/panicle	100-grain weight
Reiziq	5	5	9	6	10.6	0.69	2.86	14.2	58	2.89
Lijiangheigu	6	8	5	5	11.3	2.54	6.51	20.3	107	2.62
Line range	3–8	4–8	6–9	5–8	6.3–16.8	0.44–2.42	1.21–6.68	8.1–22.9	36–142	1.87–3.37
Kyeema	4	4	7	6	10.9	1.52	5.09	17.6	112	1.99
Norin PL8	3	4	9	8	5.1	1.51	4.86	11.5	69	2.33
Sherpa	5	5	6	6	7.7	1.41	5.22	14.4	80	2.30
Mean	5**	6**	7**	6*	10.5**	1.15**	3.93**	15.6**	79**	2.62**
l.s.d. (5%)	1.65	2.43	3.56	2.27	3.91	0.84	2.09	4.40	28.11	0.40
CV (%)	19.5	26.4	25.5	21.9	22.4	43.9	32.1	17.1	21.5	8.45

Table 3. Genotype, tolerance and susceptibility classification of 25 genotypes at F_2 and F_5 generation based on spikelet sterility exposed to cold temperature (24/15°C, day/night) for 14 days at booting stage (SSB in %) and at flowering stage (SSF in %)

Presence (✓) or absence (×) of cold-tolerant enhancing alleles donated from either Lijiangheigu (L) or Reiziq (R) is noted for three genomic regions identified by DArTseq analysis. The cold stress tolerance (T), moderate tolerance (MT) or susceptibility (S) of these progeny lines were obtained at F_2 generation at the booting stage cold exposure by Ye *et al.* (2010). Spikelet sterility of varieties and whether they were previously considered tolerant/susceptible is shown

Line code	SSB @ F_2	SSB @ F_5	Chr 5 (L)	Chr 7 (L)	Chr 10 (R)	SSF @ F_5
Reiziq	S	70.1 (S)	×	×	✓	26.3
Lijiangheigu	T	13.4 (T)	✓	✓	×	7.5
RL-11	13.3 (T)	12.2 (T)	✓	✓	✓	26.3
RL-10	7.9 (T)	16.6 (T)	✓	✓	✓	26.1
RL-226	10.3 (T)	43.4 (T)	✓	×	✓	58.5
RL-32	13.3 (T)	44.3 (T)	✓	×	✓	40.4
RL-5	16.9 (T)	47.5 (T)	✓	✓	×	31.1
RL-195	14.7 (T)	50.4 (MT)	✓	×	✓	18.7
RL-204	15.9 (T)	52.6 (MT)	✓	✓	✓	46.8
RL-170	16.0 (T)	54.6 (MT)	×	✓	✓	26.0
RL-37	14.6 (T)	74.6 (S)	×	✓	✓	89.1
RL-123	14.9 (T)	89.5 (S)	×	×	✓	55.1
Mean T	*13.8*	*48.6*				*41.8*
RL-156	91.7 (S)	23.4 (T)	×	✓	×	41.3
RL-230	73.2 (S)	43.3 (T)	✓	✓	✓	12.2
RL-206	73.3 (S)	65.5 (S)	✓	✓	×	27.7
RL-81	79.4 (S)	68.1 (S)	✓	×	×	25.4
RL-25	92.1 (S)	73.7 (S)	×	×	×	20.9
RL-144	80.0 (S)	80.3 (S)	×	✓	×	37.2
RL-227	73.7 (S)	84.6 (S)	×	×	×	41.3
RL-109	74.4 (S)	85.1 (S)	×	×	✓	n.a.
RL-243	85.7 (S)	96.8 (S)	✓	×	×	91.1
RL-232	82.2 (S)	99.6 (S)	×	×	×	96.8
Mean S	*80.6*	*72.2*	–	–	–	*43.8*
Sherpa	–	29.6	–	–	–	26.1
Norin PL8	–	9.1	–	–	–	7.0
Kyeema	–	27.0	–	–	–	42.0
Mean	–	54.2**	–	–	–	35.7**
l.s.d. (5%)	–	36.6	–	–	–	28.2
CV (%)	–	41.1	–	–	–	41.3

with high sterility had smaller panicle weights and 100-grain weight.

Spikelet sterility of 20 lines exposed to cold at early booting stage had a significant ($P < 0.05$) positive correlation ($r = 0.47$*) with SS determined at F_2 generation (Table 3) by Ye *et al.* (2010). Some progeny lines such as RL-10, RL-226, RL-32 and RL-11 were cold tolerant, whereas others such as RL-243 and RL-232 were susceptible in both generations.

Flowering stage cold stress

Norin PL8 headed more than 8 days before any other line (Table 5). Lijiangheigu headed 10 days before Reiziq, whereas some progeny lines headed earlier than Lijiangheigu. The interval between heading and last flowering varied from 6 to 9 days among the cultivars and 8–16 days among progeny lines. As each main-stem panicle was exposed to cold from the time of heading for 14 days all varieties completed flowering in the cold room, whereas five progeny lines did not complete flowering until they were transferred back to the warm room.

There was significant genotypic variation in most attributes determined in this experiment (Table 6). Stem number was similar among three measurement occasions and among five varieties but there was large variation among progeny lines. The stem number of some lines increased from 83 DAS to maturity, as late tillers were produced after the cold period. Panicle number at maturity followed the same genotypic pattern. Some progeny lines had much larger shoot dry matter and total dry matter than the varieties; however, the largest panicle weight of progeny lines was not significantly different from that of the parents and the other three varieties. Large variation was obtained among progeny lines for spikelet number per panicle and 100-grain weight.

Spikelet sterility (%) varied greatly among progeny lines, and RL-230 (12.2% SS) had similar low SS to that of tolerant varieties Lijiangheigu (7.5% SS) and Norin PL8 (7.0% SS) (Table 3). There was a group of lines that did not differ significantly from Lijiangheigu, including Sherpa, Reiziq and eight progeny lines (RL-10, RL-11, RL-5, RL-195, RL-170, RL-206, RL-81, RL-25). However, the mean SS of progeny lines previously identified as tolerant against booting stage cold at F_2

stage was similar to that of F_2 susceptible lines at flowering stage stress (41.8% vs 43.8%). SS of 24 genotypes under cold stress at flowering stage was positively correlated with several traits (Table 4). Although SS was not correlated with days to heading or first flowering, it was greater in genotypes that took longer to complete flowering ($r = 0.44$*). The more susceptible genotypes with higher sterility took a longer time from heading to first flowering ($r = 0.64$**) and to last flowering ($r = 0.77$**). There was a significant positive correlation between SS and the number of stems ($r = 0.82$**) and panicles ($r = 0.45$*) at maturity, although there was no correlation with stem number at earlier times. Susceptible lines also had smaller weight of individual panicles although 100-grain weight was not correlated with SS.

Table 4. Correlation coefficients between spikelet sterility and various traits in early booting stage cold and flowering stage cold experiments

Tiller number was determined at 54, 68 DAS and maturity in early booting experiment and 69, 83 DAS and maturity in flowering stage experiment. *$P < 0.05$; **$P < 0.001$; n.s., not significant

Traits	Booting stage cold	Flowering stage cold
Phenology: –		
Heading date	0.09n.s.	0.08n.s.
Delay in heading	0.21n.s.	–
First flowering date	0.21n.s.	0.28n.s.
Last flowering date	0.17n.s.	0.44*
Heading to first flowering	–	0.64**
heading to last flowering	0.32n.s.	0.82**
Tiller number: –		
54/69DAS	0.06n.s.	0.04n.s.
68/83DAS	0.21n.s.	0.22n.s.
Maturity	0.17n.s.	0.82**
Panicle number	–0.17n.s.	0.45*
Total spikelet number	–0.04n.s.	–0.26n.s.
100-grain weight	–0.36*	–0.25n.s.
Dry weight: –		
Shoot	0.23n.s.	0.29n.s.
Main stem panicle	–0.84**	–0.89**
Tiller panicle	–0.59**	–0.77**
Total	–0.22n.s.	–0.28n.s.

Consistency in SS obtained at flowering and booting stages

There was a highly significant ($P < 0.001$) positive correlation in SS between flowering stage and booting stage ($r = 0.62$**; Fig. 1). Almost all genotypes susceptible at the booting stage performed poorly at the flowering stage (e.g. RL-37, RL-232 and RL-243). Similarly, some genotypes that were tolerant at booting stage were also tolerant at flowering stage (e.g. Lijiangheigu, Norin PL8, Sherpa, RL-10 and RL-11).

Genotyping

A total of 14 563 SNP were generated and analysed utilising DArTseq across the 20 progeny and two parents. Initial data filtering revealed significant (P-value ≤ 0.0013) 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 (P-value ≤ 0.027), 7 (P-value ≤ 0.016) and 10 (P-value ≤ 0.024). The genomic region identified on chromosome 10 consisted of two haplotypes, the first consisting of 13 SNP (5.44–7.24 Mb) located partially within the QTL (qLTSPKST10.1; 4.58–5.82 Mb) identified by Ye *et al.* (2010) and a second haplotype

Table 5. Phenological development (days after sowing) of Reiziq and Lijiangheigu, the lines derived from their cross, and three other genotypes

Plants were exposed to cold for 14 days from heading (flowering stage cold experiment)

Genotypes	Heading	First flower	Last flower	Heading to last flower (days)
Reiziq	95	98	104	9
Lijiangheigu	85	86	92	7
Line range	79–95	84–99	90–106	8–16
Sherpa	81	87	90	9
Norin PL8	71	75	77	6
Kyeema	98	102	107	9
Mean	86	90	96	11
Probability	<0.001	<0.001	<0.001	–
l.s.d. (5%)	5.26	5.21	4.87	–
CV (%)	3.21	3.05	2.64	–

Table 6. Stem and panicle number and dry weight (g) and other characteristics of Reiziq and Lijiangheigu, the lines derived from their cross, and three other varieties

Plants were exposed to cold for 14 days from heading (flowering stage cold experiment)

	Stem number			Panicle number	Dry weight					
Genotypes	69 DAS	83 DAS	Maturity	Maturity	Shoot	Main-stem panicles	Tiller panicles	Total	Spikelet Number/panicle	100-grain weight
Reiziq	5	5	5	5	6.64	2.11	3.82	12.6	97	2.73
Lijiangheigu	7	6	5	5	7.71	2.61	5.51	15.8	106	2.51
Line range	3–9	3–8	3–12	3–9	6.45–17.35	0.39–2.37	0.78–5.25	10.3–24.4	61–109	2.18–3.39
Kyeema	5	5	5	4	6.92	1.78	4.36	13.1	121	2.22
Norin PL8	5	5	5	5	8.34	2.11	5.69	16.1	74	2.45
Sherpa	5	5	5	5	6.41	1.77	3.48	11.7	93	2.39
Mean	6*	5	6**	5*	9.01**	1.71**	3.67**	14.4**	89*	2.66**
l.s.d. (5%)	2.74	n.s.	2.12	1.98	4.32	0.96	2.34	4.34	29.3	0.37
CV (%)	24.9	24.4	19.3	20.8	25.1	29.42	33.37	15.78	17.5	6.91

of 14 SNP identified from 9.93 to 11.72 Mb. Eight lines classified as tolerant (T and MT) at the F_5 generation contained the Reiziq allele at the qLTSPKST10.1 QTL whereas eight 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%; Table 3). The genomic regions on chromosomes 5 and 7 consisted of 26 SNP (5.47–6.80 Mb) and 8 SNP (12.86–14.45 Mb), respectively. The genomic region on chromosome 5, donated by Lijiangheigu, had a significant main effect on SS, with an average of 49% SS in tolerant lines and 74% SS in susceptible lines harbouring the Reiziq allele. Likewise, the SNP markers identified on chromosome 7, had a predicted main effect on SS with an average of 47% SS in tolerant lines comprising of the Lijiangheigu allele and 74% in susceptible lines donated by Reiziq. When the additive effect of combinations was considered, four lines had the tolerant allele from the genomic regions on chromosome 5 and chromosome 7 (donated by Lijiangheigu) and the QTL on chromosome 10 (Reiziq), which had SS of only 31%. Lines missing all three alleles had a mean SS of 68%.

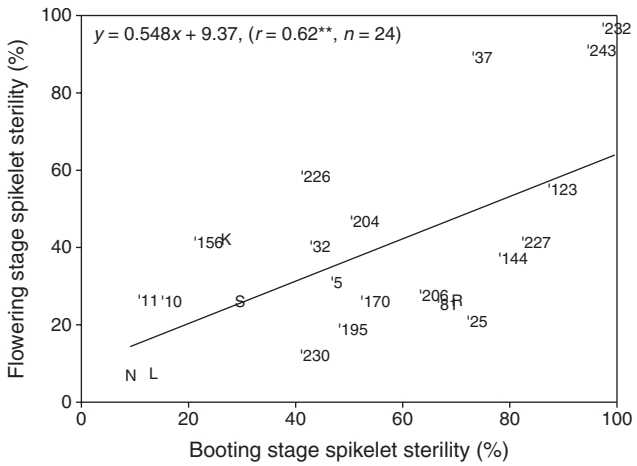

Fig. 1. Relationship between spikelet sterility at booting and flowering stages based on 24 lines exposed to cold temperature (24/15°C day/night) for 14 days. Line numbers indicated on the graph reflects line numbers as detailed in Table 3.Varieties are abbreviated with first initial: R: Reiziq, L: Lijiangheigu, N: NorinPL8, S: Sherpa, K: Kyeema.

Floral traits contributing to cold tolerance at flowering

There was significant ($P < 0.05$) or highly significant ($P < 0.01$) genotypic variation in all floral characteristics determined (Table 7). Lijiangheigu had larger anthers than Reiziq (1578 vs 1361 μm), although this was the only measurable difference between the parents. Thus, some progeny lines showed much higher/lower values compared with the parents. The number of dehisced anthers was particularly variable ranging from 0 to 5.8 (from a maximum of 6). The number of dehisced anthers was strongly correlated with the total dehiscence length of the theca ($r = 0.82$**); thus, genotypes which had a larger number of dehisced anthers showed greater dehiscence length for both the apical and basal positions and hence for the total dehiscence length (Table 8). These anther dehiscence lengths were in turn strongly correlated with the number of pollen grains on stigma ($r = 0.47$* to 0.80**) and in particular with apical dehiscence ($r = 0.80$**), and thus, there was also a significant correlation between the number of dehisced anthers and total pollen number on stigma ($r = 0.67$**). However, anther length was not significantly correlated with any characters determined. The significant relationships between pollen number on stigma and apical dehiscence length and the number of dehisced anthers are shown in Fig. 2*a*, *b*. PCA analysis explained a total of 81.8% of genotypic variation with 59.2% and 22.6% of the variation attributed to vector 1 and 2 respectively (Fig. 3). Anther length was developmentally determined before the cold exposure and was not correlated with the six floral traits influenced by cold treatment exposure, which were all similarly descriptive (loadings of 0.36–0.45) in explaining variation in PC1 vector although anther length and basal dehiscence described the greatest portion of the variation in PC2 and were weakly negatively correlated with each other. Basal dehiscence is thus identified as important in explaining variation in both vectors. Furthermore, these results indicate that counting the number of dehisced anthers per spikelet can equally capture the variation in genotype performance and thus provide a considerable saving in resource requirement compared with other characteristics measured herein.

There was a significant negative correlation between the SS from flowering stage cold stress experiment and some of the floral traits from floral trait experiment, in particular total pollen on stigma ($r = -0.65$*; Fig. 4*a*), the number of dehisced anthers ($r = -0.54$*; Fig. 4*b*), and the number of normal anthers ($r = -0.59$*), but other characteristics determined (anther length,

Table 7. Mean, range and parental values of various floral traits based on 20 genotypes including the parents Reiziq and Lijiangheigu exposed to cold temperatures at flowering in the floral trait identification experiment; number of dehisced anthers/spikelet (ND), number of normal anthers per spikelet (NNA), anther length (AL in μm), length of apical dehiscence (μm), length of basal dehiscence (μm), total dehiscence length (TD in μm), and total pollen number on stigma (TPNS)

Genotype	ND	NNA	AL	Apical	Basal	TD	TPNS
Lijiangheigu	4.1	5.4	1578	102.7	323.5	426.2	349
Reiziq	3.3	5.5	1361	85.9	554	640	255
Line range	0–5.8	3.7–6.0	1078–1733	0–334	2–834	2–951	1–753
Mean	3.44**	4.9*	1423**	127*	402**	529**	257**
l.s.d. (5%)	2.316	1.409	146.2	145.7	336.3	369	262.8
CV (%)	52.0	22.6	8.2	92.6	64.1	54.1	81.6

Table 8. Correlations among floral traits; number of dehisced anthers/spikelet (ND), number of normal anthers per spikelet (NNA), anther length (AL in μm), length of apical dehiscence (μm); length of basal dehiscence (μm); total dehiscence length (TD in μm), and total pollen number on stigma (TPNS) based on 20 genotypes exposed to cold temperature at flowering in the floral trait identification experiment

*$P < 0.05$; **$P < 0.001$

	ND	NNA	AL	Apical	Basal	TD	TPNS
ND	1	–	–	–	–	–	–
NNA	0.64**	1	–	–	–	–	–
AL	0.01	0.33	1	–	–	–	–
Apical	0.66**	0.39*	0.34	1	–	–	–
Basal	0.73**	0.45*	–0.31	0.33	1	–	–
TD	0.82**	0.51*	–0.17	0.58*	0.96**	1	–
TPNS	0.67**	0.66**	0.30	0.80**	0.47*	0.64*	1

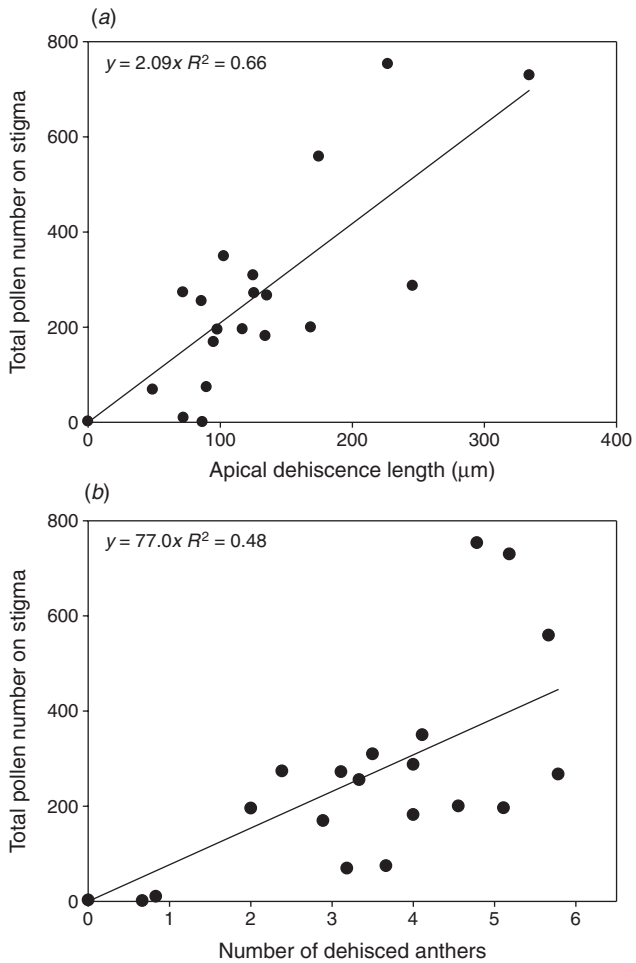

Fig. 2. Relationship between total pollen number on stigma and (*a*) apical dehiscence length and (*b*) the number of dehisced anthers based on 20 lines exposed to cold at flowering in the floral trait identification experiment.

apical dehiscence, basal dehiscence, and total dehiscence) were not significantly correlated with SS obtained in flowering stage cold stress experiment.

Discussion

Genotypic consistency in SS between flowering and booting stage cold stress

Almost all genotypes that were susceptible at the flowering stage performed poorly at the booting stage (e.g. RL-37, RL-232 and RL-243). Similarly, some genotypes that were tolerant at the flowering stage were also tolerant at the booting stage (e.g. Lijiangheigu, Norin PL8, Sherpa, RL-10 and RL-11). Ye *et al.* (2009) reported a positive correlation across development stages in 17 rice cultivars, and our results from a single cross also revealed a positive correlation ($r = 0.62$**) in SS between the flowering and booting stages. This suggests there are some common mechanisms for cold tolerance regardless of potential physiological mechanisms involved. However, others have suggested that cold tolerance genes will be developmental stage specific (e.g. da Cruz *et al.* 2013), and Ye *et al.* (2009) also found a few varieties were tolerant to cold only at specific stages.

Although the importance of cold temperature events at the booting stage is recognised by the Australian rice growing region (Reinke *et al.* 2012), cold temperatures at flowering could also be a major issue and general tolerance across development stages would be beneficial. As noted by Ye *et al.* (2009), Lijiangheigu has undergone long-term natural selection for cold tolerance as it originates from high elevation areas in Lijiang, China, and could confer cold tolerance across all reproductive growth stages. High elevation generally does not have large seasonal variation in temperature, and therefore general adaptation to cold would be required.

Genotypic consistency in performance between F_2 and F_5 generation in booting stage stress

There was consistency in performance across the F_2 and F_5 generations ($r = 0.47$*) with some progeny lines such as RL-10, RL-226, RL-32 and RL-11 consistently cold tolerant, whereas others such as RL-243 and RL-232 were susceptible at both generations. The results suggest that early generation selection was effective for improving cold tolerance in rice within this 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. Such relationships have been identified and exploited by the Australian rice breeding program for cold tolerance introgression in early generation from sources emanating from Yunnan province material (Reinke *et al.* 2012). Elsewhere, da Cruz *et al.* (2013) noted when non-additive effects are large, selection should be applied in advanced generations of breeding programs (F_4 or F_5 stage).

In the F_2 generation Ye *et al.* (2010) reported that the QTL, qLTSPKST10.1, explained 20.5% of the variation in SS caused by low temperature treatment at the booting stage, with the tolerant allele donated from Reiziq. In the present work based on DArTseq analysis, the lines with the Reiziq QTL allele at qLTSPKST10.1 were found to be more tolerant than those with the alternative allele. Additionally, the correlation between SS at F_2 and F_5 was high, even though of the nine tolerant lines at F_2 which contained the positive allele from Reiziq at the

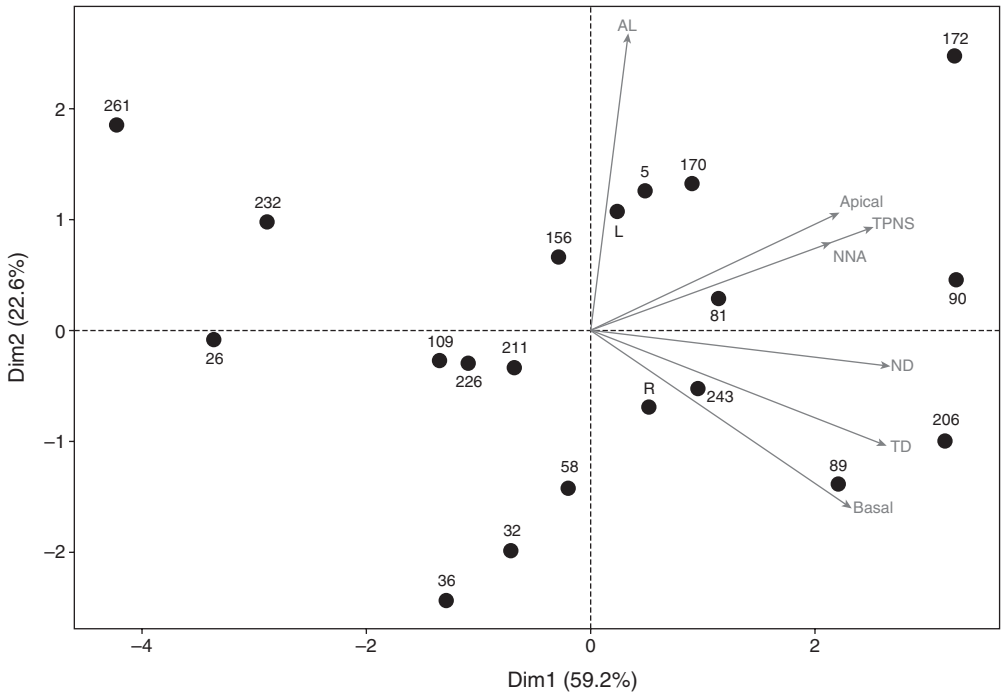

Fig. 3. PCA biplot of the first and second principal component vectors for the ordination of 20 genotypes (including the parents Reiziq (R) and Lijiangheigu (L) exposed to cold temperatures at flowering) and floral traits (number of dehisced anthers/spikelet (ND), number of normal anthers per spikelet (NNA), anther length (AL in µm), length of apical dehiscence (µm), length of basal dehiscence (µm), total dehiscence length (TD in µm), and total pollen number on stigma (TPNS). Eleven line numbers indicated on graph reflect line numbers as detailed in Table 3.

F_5 generation at qLTSPKST10.1, two of the lines were classified as susceptible at F_5.

In addition to the QTL identified on chromosome 10, two other regions significantly associated with improved cold tolerance were identified on chromosomes 5 and 7 with the cold tolerance alleles from Lijiangheigu. Although the present work does not have sufficient sample numbers to validate these genomic regions as QTL, we plan to develop and screen a larger number of QTL near isogenic lines derived from the Reiziq × Lijiangheigu cross and pyramid these genomic regions into genetic backgrounds such as the susceptible parent Reiziq. Such work would build on previous research efforts such as that of Shinada *et al*. (2014), whose work suggests that pyramiding QTL into genotypes contributes to enhancing their cold tolerance.

Floral traits associated with cold tolerance at flowering stage

Genotypic variation in SS at the flowering stage was related to the rate of flowering in the cold room. The duration from first flower to last flower on the main-stem panicle was shortest in tolerant lines, whereas susceptible lines took longer to flower across the entire length of the panicle, and for five lines flowering was completed after the 14-day cold period. Spikelet damage caused susceptible lines to produce late tillers and new panicles, resulting in a positive correlation between SS and the number of stems and panicles at maturity. Nishiyama (1984) suggested that under cool daytime temperatures that spikelets would remain closed ('dormancy strategy') and flowering could be delayed until temperatures increased. In contrast, when some flowers opened under cool conditions high sterility resulted. In the present experiment with a 14 days' cold spell (with a minimum temperature of 15°C) a 'dormancy' strategy appeared not to be operating with most lines continuing to flower albeit at a slower rate and those lines that had long flowering duration across the panicle resulted in high SS, a result supported by Nishiyama (1984). It should be pointed out that 14 days of 15°C would be considered a frequent cold period in the Australian rice growing area and elsewhere. Farrell *et al*. (2006*a*) estimated that there is a 30–50% probability of mean minimum temperature below 15°C for 10 consecutive days in late January– late February. This dormancy strategy together with photo-period sensitivity (Farrell *et al.* 2006*b*), has historically been a feature of Australian varieties like Amaroo, a stalwart of the Australian production system for over 20 years. However, such avoidance mechanisms, do not address the emerging need for short-season varieties, which have minimal delays in crop duration and a degree of yield certainty. The dormancy strategy may work for a shorter cold period and hence this needs be tested further.

Cold-tolerant genotypes at the flowering stage had larger numbers of dehisced anthers and subsequently pollen number on stigma, which contributed to reducing SS. Our result on the effect of dehiscence reflects the work of Matsui and Omasa (2002), who showed that high temperature stress was associated with inhibited dehiscence and lower fertilisation due to inability of pollen shedding onto the stigma. A more recent paper on heat stress showed that basal dehiscence and the percentage of

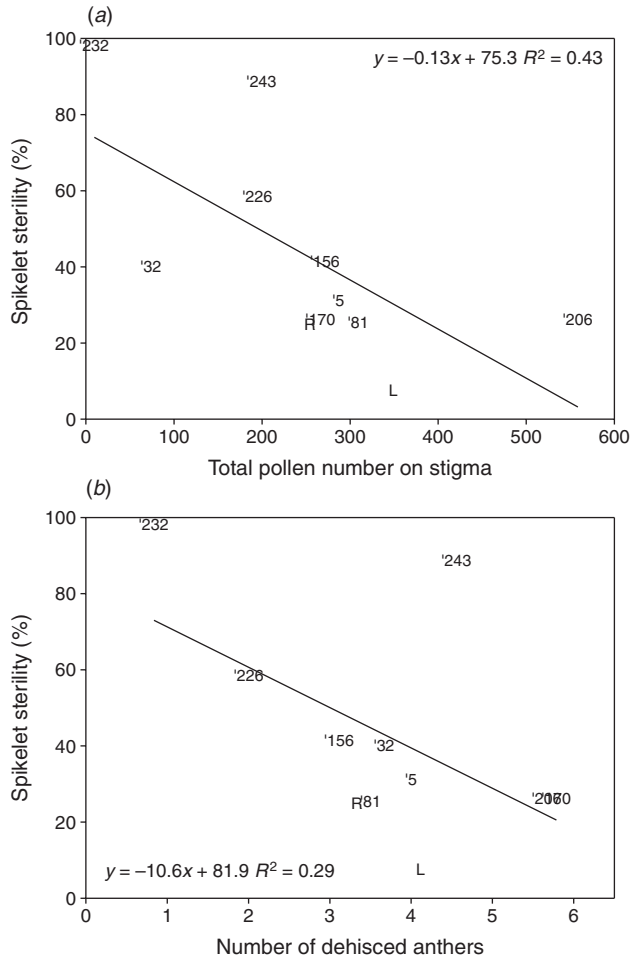

Fig. 4. The relationship of spikelet sterility of nine Reiziq × Lijiangheigu lines and their parents exposed to cold temperatures at flowering stage to (*a*) pollen number on stigma and (*b*) number of dehisced anthers obtained in the floral trait identification experiment. Line numbers indicated on graph reflects line numbers as detailed in Table 3.

dehisced anthers was strongly correlated with the number of pollinated florets (Kobayashi *et al*. 2011).

Khan *et al*. (1986) also showed a negative association between SS and the number of pollen grains intercepted by the stigma at low temperatures (20−21°C) for 5 days at flowering in tropical rice cultivars. The warm exposed lines had an average of 44.7 pollen grains per stigma, compared with an average of 21.3 grains for the low temperature treatment.

Comparison of the floral trait data with SS data for the F_5 Reiziq × Lijihangheigu population in the present work showed that there were significant negative correlations between SS and total pollen number on stigma (r = –0.65), number of dehisced anthers (r = –0.54), and number of normal anthers (r = –0.59). Thus, the main traits contributing to cold tolerance at flowering as measured by SS within an F_5 Lijihangheigu × Reiziq population appears to be total pollen number on stigma. This was largely influenced by the number of dehisced anthers and the apical dehiscence length. It appears that by simply counting the number of dehisced anthers we can predict the likelihood of lines performing well when exposed to cold temperature stress at flowering.

There may be other floral characteristics that contribute to SS. For example, Shinada *et al*. (2013) demonstrated that susceptible varieties had lower pollen germination and pollen tube elongation. It is possible that all these characteristics contribute to SS in flowering stage cold. Pollen number on stigma was greater than 50 in 10 of the 11 lines tested, and these values would be considered to be more than sufficient for fertilisation provided conditions were suitable for germination. According to Satake and Yoshida (1978) it is essential to have 10 germinated pollen grains on the stigma to ensure fertilisation, whereas Gunawardena *et al*. (2003) showed that 20 germinated pollen are required to ensure less than 10% sterility. Assuming that 50% of pollen grains on stigma germinate, Matsui and Kagata (2003) considered 40 and 80 pollen grains on stigma were sufficient and ample to ensure fertilisation. Thus, it is likely that several pollen on stigma will not have germinated in the flowering stage cold experiment, and hence the ability to germinate under the cold requires further investigation.

Conclusion

Key floral traits in which lines differed in cold tolerance at the flowering stage were the total pollen number on stigma, the number of dehisced anthers per spikelet and the mean apical dehiscence. Significant relationships between the number of dehisced anthers and total pollen number on stigma; between mean apical dehiscence and total pollen number on stigma; and between number of dehisced anthers and the percentage pollen intercepted by the stigma existed. Enhanced anther dehiscence plays a significant role in cold tolerance at the flowering stage.

There was consistency in performance of the F_5 Reiziq × Lijiangheigu lines between exposure to cold temperature at booting and flowering stages. Two lines were identified as tolerant and three susceptible to cold across the reproductive stages. There was also consistency in performance across the F_2 and F_5 generations, the result suggesting that early generation selection was effective for improving cold tolerance in rice within this population where the parental line Lijiangheigu is 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. In addition to the QTL located on chromosome 10 there were two other regions identified as potential QTL for cold tolerance on chromosomes 5 and 7. 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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