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QTL mapping of physiological traits at the booting stage in rice under low temperature combined with nitrogen fertilization

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Abstract: Further dissection of physiological molecular mechanisms is indispensable to alleviate rice yield losses resulting from cold injury. By using 105 near-isogenic lines (NILs) derived from a backcross between cv. Lijiangxintuanheigu (LTH) and cv. Towada, we detected quantitative trait loci (QTLs) for physiological traits of the rice flag leaf, based on polymorphic simple sequence repeat (SSR) markers, inclusive composite interval mapping (ICIM), mixed composite interval mapping (MCIM) approaches and phenotypic value subjected to combine with cold-water stress and three nitrogen application rates. By using ICIM, a total of 34 QTLs with additive effects (A-QTLs) were identified on chromosomes 1, 3, 4, 5, 6, 7 and 10, and the phenotypic variation (R^2) explained by each QTL ranged from 8.46 to 29.14%. By using MCIM, 20 A-QTLs and 14 pairs of QTLs with epistatic × environment interaction effects (Epistatic QTLs) were detected, the contribution of environment interaction (H^2AE) was 0.87 to 7.36%, while the contribution rates of E-QTL were from 0.97 to 3.58%. Fourteen A-QTLs were detected by ICIM and MCIM, which may serve as a basis for fine-mapping and candidate gene studies, and providing strategies for the development of cold-tolerant rice cultivars and nitrogen application to alleviate chilling stress.

Keywords: antioxidant enzymes; cold stress; japonica rice (*Oryza sativa* L.); nitrogen; QTLs

Abbreviations: CAT – catalase; FAA – free amino acid; H^2AE – contribution of additive × environment interaction effect; H^2AAE – contribution of epistatic × environment interaction effect; ICIM – inclusive composite interval mapping; MCIM – mixed composite interval mapping; MDA – malondialdehyde; N – nitrogen; NR – nitrate reductase; NILs – near-isogenic lines; PRO – proline; POD – peroxidase; QTL – quantitative trait loci; SP – soluble protein; SS – soluble sugar

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Rice (*Oryza sativa* L.) is one of the staple food crops, with increasing production to play a crucial role in global food security (FAO 2015). Nevertheless, humanity confronts a challenge for chilling damage that significantly reduces potential productivity during the booting stage in rice, with up to 10% losses per year (Shimono *et al*. 2016), which has been documented across Korea (Endo *et al*. 2016), Japan (Shimono *et al*. 2016), high-latitude or high-altitude regions of China (Yang *et al*. 2018). In parallel, the cold events of subtropical and tropical rice-producing areas are likely to occur more frequently in the future climate change scenario (IPCC 2013). Across previous studies, physiological mechanisms of plant resistance against cold stress were extensively described, i.e. increased electrolyte leakage (Song *et al*. 2011), and a higher accumulation of proline (Faruk *et al*. 2015), soluble protein (SP) (Theocharis *et al*. 2012), free amino acids (FAA) (Yang *et al*. 2012), soluble sugars (SS) (Zhang *et al*. 2010). Besides, enhanced activities of antioxidant enzymes superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) (Gill & Tuteja 2010), and a drop in photosynthesis (Faruk *et al*. 2015). Despite induced genes and signal transduction with roles in increasing plant cold tolerance have been explained (Rihan *et al*. 2017), deciphering of molecular changes in rice remains limited, and genetic dissection is indispensable for successful efforts in developing cold-resistant cultivars.

Nitrogen (N) belongs to main yield-determining and yield-stabilising nutrient factors, appropriate N supply could alleviate the negative impact of cold stress in plants (Waraich *et al*. 2012), whereas low temperature at the reproductive stage significantly induced spikelet sterility and reduced spikelet numbers per plant in rice under the application of high N rates, leading to a reduction in the engorged pollen number per anther (Gunawardena & Fukai 2005). The previous studies demonstrated that N uptake, transport, assimilation, and remobilization are regulated by interacting genetic and environmental factors (Xu *et al*. 2012). Further, the abundance of glutamine synthetase and RuBisCO is also linked closely to severity of cold stress, whereas under a root-zone ecosystem with low soil and water temperature, rice N uptake and utilization efficiency are reduced as a result of the inhibited activity of enzymes and transporters (Gutiérrez 2012), especially in lower N mineralization rate or inorganic N conditions (Nasholm *et al*. 2009). Despite these consequences, the regulation of physiological quantitative trait loci (QTLs) has not yet been clearly described on a coupling chilling and nitrogen level. Here, we exploited largest-effect QTL of cv. Lijiangxintuanheigu at the booting stage with three levels of applied nitrogen under low temperature, in an attempt to promote guidelines for future understanding the cold-tolerant physiological mechanisms in rice.

MATERIAL AND METHODS

Plant material. We developed a set of 105 BC_4F_{10} and BC_4F_{11} near-isogenic lines (NIL) derived from a backcrossing of cv. Lijiangxintuanheigu (LTH; as a donor) to cv. Towada (as a recipient). LTH is a *japonica* landrace cold-tolerant at the booting stage of Yunnan province in China (Shirasawa *et al*. 2012), and Towada is a cold-sensitive elite *japonica* cultivar in Japan (Yang *et al*. 2018).

Experimental design and cold-stress treatment. The field experiments were conducted under cold-water stress and different N rates in two consecutive years (2016 and 2017) in SanDan (25.04°N, 102.49°E, and altitude 2171 m) of Yunnan Province, China. The soil type was clay loam, with the characteristics of pH 6.5 (1 : 2.5 soil/water ratio), organic matter (2.81%), total N (2.36%), alkaline N (98.71 mg/kg), available P (22.34 mg/kg) and available K (143.52 mg/kg). Fifteen plants per each line were transplanted in a single row at a spacing of 15/25 cm between plants and rows with one seedling per hill according to a randomized complete block design with three replicates. Three N levels in the form of urea with an N content of 46% were applied, 0 kg N/ha (N1), 120 kg N/ha (N2) and 240 kg N/ha (N3). Basal nitrogen was applied at 50% of the total amount before transplanting, and remaining N was split-applied at tillering (20%) and booting stage (30%), respectively. Entire phosphorus and potassium fertilizers were applied into the soil pre-transplanting as superphosphate and potassium sulphate at rates of 80 kg/ha (P_2O_5) and 80 kg/ha (K_2O). According to the method described by Endo *et al*. (2016), NIL and two parental cultivars were irrigated with cool water (16–19°C) and at a depth of about 25 cm from tillering stage (20 days after transplanting) to grain maturity. In the entire rice growth stage, atmospheric temperatures (T_a) were 13.6–23.7°C and 13.7–25.6°C, and ranges of T_a from booting to milky stage were 15.4–20.3°C and 16.1–20.6°C taken at Yunnan Meteorological Agency, where measured mean daily water temperatures from booting to milky stage were 18.3 ± 0.14°C and

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18.6 ± 0.66°C in the experimental period (2016 and 2017), respectively.

Plant sampling and determined indices. At the booting stage, flag leaves of ten representative plants for each genotype were sampled per replication, frozen in liquid nitrogen and stored at –80°C freezer. Soluble protein, soluble sugar, free amino acid, proline, catalase, peroxidase and nitrate reductase were measured in triplicate.

Determination of SP, PRO, SS and FAA. SP and PRO concentration was measured according to the method of Cao *et al*. (2017). SS content was determined using the anthrone colorimetric method (Zhang *et al*. 2010). FAA was assayed using the method described by Li *et al*. (2000).

Determination of CAT, POD and NR activities. CAT activity was determined using the method described by Gill and Tuteja (2010). One unit of CAT activity was defined as the amount of decreasing enzyme in absorbance of 0.01 per min, which was determined by an ultraviolet absorbance method in A_{240} (Popović *et al*. 2017). POD activity was measured by the guaiacol method (Li *et al*. 2000), and one unit activity of which corresponded to the amount of increasing enzyme that decomposes 0.01 of substrate per min. Nitrate reductase (NR) activity was determined according to the method described by Li *et al*. (2000).

Phenotypic data and genotyping analyses. The phenotypic data analysis was performed using Statistical Analysis System (SAS) software (Ver. 9.4, 2013), and phenotypic values were compared according to Duncan's test ($P < 0.05$). DNA was extracted from fresh leaves by CTAB method (Rogers & Bendich 1989). A total of 480 SSR markers distributed at regular intervals (around 3–5 cM) on rice all the chromosomes were used to examine polymorphism parents (Yang *et al*. 2018). PCR was performed using the procedure of Yang *et al*. (2018), and PCR products were separated by electrophoresis on an 8% acrylamide gels and stained with ethidium bromide.

Linkage map construction and QTL analyses. 180 SSR markers covering 1820.6 cM of the linkage map with an average interval of 15.67 cM was constructed (Figure 1) using MAP functionality in the IciMapping software (Ver. 4.0; Yang *et al*. 2018). QTL analysis was conducted by inclusive composite interval mapping (ICIM) using the same software (Meng *et al*. 2015), and set probability level (PIN) of 0.01, walking speed of 1cM. A logarithm of the odds (LOD) threshold was used as 1000 permutations to advocate QTL. The additive and epistatic QTL × environment interaction effects were analysed by mixed composite interval mapping (MCIM) with QTL Network 2.1 software, the threshold probability is $P < 0.005$ (Yang *et al*. 2007). QTL nomenclature followed the method of McCouch (2008).

RESULTS

Phenotypic variation of the parents and NILs. Numerous contrasting traits were observed for the parents (Table 1). LTH had higher SP, FAA, PRO, CAT and NR, but lower SS and POD than Towada. All traits of NIL exhibited transgressive segregation and continuous distribution, suggesting an involvement of polygenic inheritance (Table 1). The means comparison between six growing conditions showed that the N application rate had a significant effect on physiological indices except for SP (Table 1), but the mean values of studied traits for NIL between the same nitrogen rates were not significant in either year. According to results of skewness and kurtosis (Table 1), a coincidence with normal distribution was observed for SP, SS, FAA, PRO and NR. Furthermore, the positive or negative signs of distribution, POD showing a significant genetic variability compared with other traits.

QTLs for SP and SS. By using ICIM, six QTLs were detected for SP on chromosomes 1, 6, 7 and 10 (Table 2, Figure 1) and R^2 from 10.89 to 27.76%. Among them, *qSP-1*, *qSP-6a* and *qSP-6b* explained 16.34–27.76% of the total variation. The *qSP-1* (for N1) and *qSP-7* (for N2) were detected in both years (Table 2). Meanwhile, five QTLs for SS were identified on chromosomes 3, 5, 6 and 7 (Table 2, Figure 1), and R^2 from 16.89 to 28.48%, with alleles from LTH. The *qSS-6* (for N1), *qSS-7a* (for N2) and *qSS-7b* (for N3) were detected in both years. By using MCIM, 4 QTLs for SP were identified on chromosomes 1, 3 and 6, and the contribution of additive × environment interaction effect (H^2AE) ranged from 0.87 to 6.23% (Table 3), while the contribution of epistatic × environment interaction effect (H^2AAE) was 2.03% and 3.58%, respectively (Table 4). Three QTLs for SS were identified, and H^2AE was 3.78 to 6.25% (Table 3), while H^2AAE was 1.49% and 1.94%, respectively (Table 4).

QTLs for FAA and PRO. By using ICIM, FAA was controlled by four QTLs located on chromosomes 3, 6 and 10 (Table 2, Figure 1), and R^2 ranged from 13.56 to 29.14%. The largest-effect *qFAA-6* explained

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Table 1. The phenotypic value of soluble protein (SP), soluble sugar (SS), free amino acid (FAA), proline (PRO), catalase (CAT), peroxidase (POD) and nitrate reductase (NR) activity in the rice flag leaf of the parents and near-isogenic lines (NILs) grown at cold-water stress at three nitrogen levels

Traits	Environment (year/nitrogen)	Parents		NILs			
		LTH	Towada	mean ± SD	range	skewness	kurtosis
SP (%)	E1 (2016/N1)	1.35	0.95	1.21 ± 0.47^{a}	0.66–2.25	0.98	0.85
	E2 (2016/N2)	1.67	1.19	1.24 ± 0.50^{a}	0.60–1.89	0.93	1.11
	E3 (2016/N3)	1.17	1.12	1.18 ± 0.48^{a}	0.54–2.58	1.84	4.11
	E4 (2017/N1)	1.37	1.02	1.23 ± 0.49^{a}	0.62–2.28	0.98	0.86
	E5 (2017/N2)	1.54	1.26	1.32 ± 0.69^{a}	0.51–2.56	0.80	0.86
	E6 (2017/N3)	1.35	1.05	1.15 ± 0.57^{a}	0.16–3.27	1.56	3.19
SS (%)	E1 (2016/N1)	5.41	5.52	6.26 ± 1.89^{ab}	4.97–7.88	0.29	–1.47
	E2 (2016/N2)	6.12	7.89	7.39 ± 2.27^{a}	4.12–8.42	–1.28	–0.13
	E3 (2016/N3)	2.62	3.27	3.98 ± 1.12^{c}	1.49–5.39	–1.11	–0.43
	E4 (2017/N1)	5.48	5.52	6.27 ± 1.78^{ab}	4.94–7.36	0.28	–1.45
	E5 (2017/N2)	6.73	7.66	6.79 ± 2.32^{a}	4.15–7.57	0.60	–0.12
	E6 (2017/N3)	2.35	4.17	5.41 ± 1.52^{c}	1.59–6.29	0.72	0.69
FAA (μg/g FW)	E1 (2016/N1)	28.20	28.45	23.57 ± 9.35^{b}	9.12–57.43	0.74	0.29
	E2 (2016/N2)	39.28	31.78	43.67 ± 8.85^{a}	10.25–61.28	0.22	–0.50
	E3 (2016/N3)	21.77	25.67	24.52 ± 8.31^{b}	4.72–45.32	0.45	–0.39
	E4 (2017/N1)	33.43	25.68	26.95 ± 6.72^{b}	4.63–51.58	1.36	1.94
	E5 (2017/N2)	28.31	37.45	40.56 ± 9.74^{a}	2.29–76.54	0.39	–0.29
	E6 (2017/N3)	27.26	29.79	22.57 ± 9.57^{b}	8.22–46.24	0.77	0.28
PRO (%)	E1 (2016/N1)	8.27	7.98	9.69 ± 1.87^{a}	7.11–16.25	–0.35	5.29
	E2 (2016/N2)	7.43	6.22	9.76 ± 1.41^{a}	7.62–12.51	–0.28	2.39
	E3 (2016/N3)	5.76	6.47	7.29 ± 1.24^{b}	4.28–12.95	1.25	1.63
	E4 (2017/N1)	8.50	6.96	9.55 ± 1.61^{a}	7.26–16.13	1.51	3.32
	E5 (2017/N2)	7.33	6.35	9.72 ± 1.33^{a}	7.15–14.51	–0.26	2.34
	E6 (2017/N3)	6.55	7.12	6.61 ± 1.64^{b}	4.06–11.73	1.23	0.54
CAT (U/g·min FW)	E1 (2016/N1)	18.79	14.56	21.48 ± 3.37^{b}	6.24–24.84	–2.14	5.64
	E2 (2016/N2)	23.67	22.89	22.75 ± 2.86^{ab}	15.57–26.18	–1.22	1.04
	E3 (2016/N3)	24.34	22.56	23.26 ± 2.15^{a}	15.68–27.72	–1.87	2.05
	E4 (2017/N1)	24.43	25.66	20.11 ± 0.78^{b}	17.85–26.48	–2.62	3.17
	E5 (2017/N2)	23.71	22.84	22.75 ± 2.84^{ab}	15.47–26.85	–1.24	1.05
	E6 (2017/N3)	24.76	25.47	23.24 ± 1.13^{a}	16.25–29.46	–4.15	1.16
POD (U/g·min FW)	E1 (2016/N1)	54.34	25.08	41.13 ± 24.45^{b}	12.17–98.75	1.43	2.76
	E2 (2016/N2)	63.58	37.96	46.25 ± 19.70^{ab}	16.25–80.50	0.52	–0.14
	E3 (2016/N3)	48.49	58.71	53.21 ± 37.24^{a}	23.12–72.51	0.57	0.33
	E4 (2017/N1)	34.25	35.54	34.21 ± 9.21^{b}	11.27–86.37	0.79	0.94
	E5 (2017/N2)	62.57	33.49	45.15 ± 19.47^{ab}	15.52–82.58	0.55	–0.14
	E6 (2017/N3)	43.69	45.24	62.37 ± 21.42^{a}	16.51–76.25	1.65	4.39
NR (μg/(g·h))	E1 (2016/N1)	9.97	8.56	10.58 ± 5.36^{b}	4.06–16.32	–0.54	–1.63
	E2 (2016/N2)	15.89	9.29	13.79 ± 4.92^{a}	5.66–18.55	–1.16	0.87
	E3 (2016/N3)	14.70	11.69	13.32 ± 4.97^{a}	6.30–17.26	–0.98	–0.49
	E4 (2017/N1)	8.18	5.23	6.31 ± 2.79^{b}	2.79–20.92	1.92	6.52
	E5 (2017/N2)	11.26	9.97	12.14 ± 2.57^{a}	3.40–15.87	–1.47	3.18
	E6 (2017/N3)	12.79	8.81	10.87 ± 2.93^{b}	3.87–16.92	–0.64	0.53

SD – standard deviation; the values followed by a common letter in the same column are not significantly different at $P < 0.05$ (Duncan tested); N1 – 0 kg N/ha; N2 – 120 kg N/ha; N3 – 240 kg N/ha

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Table 2. QTLs associated with soluble protein (SP), soluble sugar (SS), free amino acid (FAA), proline (PRO), catalase (CAT), peroxidase (POD) and nitrate reductase (NR) activity in the rice flag leaf of the parents and near-isogenic lines (NILs) grown at cold-water stress at three nitrogen levels (inclusive composite interval mapping)

Traits	Environment (year/nitrogen)	Chr.	QTL	Marker interval	LOD	R^2/%	AE
SP	E1 (2016/ N1)	1	*qSP-1*	RM3359–RM5496	3.52	27.76	0.32
	E4 (2017/ N1)	1	*qSP-1*	RM3359–RM5496	3.27	18.75	0.68
	E5 (2017/ N2)	1	*qSP-1*	RM3359–RM5496	3.95	20.01	0.74
	E2 (2016/ N2)	6	*qSP-6a*	RM340–RM3628	4.16	16.88	0.57
	E3 (2016/ N3)	6	*qSP-6b*	RM3628–RM4924	3.11	16.34	0.83
	E2 (2016/ N2)	7	*qSP-7*	RM5711–RM6432	3.23	10.89	–0.23
	E5 (2017/ N2)	7	*qSP-7*	RM5711–RM6432	4.35	27.59	0.54
	E6 (2017/ N3)	10	*qSP-10a*	RM6144–RM8207	3.49	23.48	0.49
	E6 (2017/ N3)	10	*qSP-10b*	RM8207–RM7217	3.81	26.59	0.58
SS	E1 (2016/ N1)	3	*qSS-3*	RM6329–RM6147	4.10	23.47	1.47
	E2 (2016/ N2)	5	*qSS-5*	RM3796–RM3476	9.88	21.76	0.85
	E1 (2016/ N1)	6	*qSS-6*	RM340–RM3628	7.57	24.43	0.57
	E4 (2017/ N1)	6	*qSS-6*	RM340–RM3628	5.62	16.89	0.75
	E2 (2016/ N2)	7	*qSS-7a*	RM5606–RM7571	17.43	19.53	0.95
	E5 (2017/ N2)	7	*qSS-7a*	RM5606–RM7571	5.74	21.24	1.46
	E3 (2016/ N3)	7	*qSS-7b*	RM7571–RM5455	12.06	28.48	0.79
	E6 (2017/ N3)	7	*qSS-7b*	RM7571–RM5455	5.76	19.98	0.59
FAA	E1 (2016/ N1)	3	*qFAA-3*	RM569–RM6349	4.37	18.37	3.58
	E2 (2016/ N2)	6	*qFAA-6*	RM340–RM3628	6.95	24.25	4.97
	E5 (2017/ N2)	6	*qFAA-6*	RM340–RM3628	5.86	25.28	17.63
	E2 (2016/ N2)	10	*qFAA-10a*	RM6364–RM1375	5.65	29.14	6.79
	E3 (2016/ N3)	10	*qFAA-10b*	RM6144–RM8207	3.99	13.56	2.99
PRO	E1 (2016/ N1)	3	*qPRO-3*	RM1350–RM6329	5.92	13.28	–4.27
	E2 (2016/ N2)	6	*qPRO-6a*	RM340–RM3628	3.44	21.96	2.53
	E5 (2017/ N2)	6	*qPRO-6a*	RM340–RM3628	4.27	14.15	1.98
	E3 (2016/ N3)	6	*qPRO-6b*	RM3628–RM4924	5.38	15.92	2.42
	E6 (2017/ N3)	6	*qPRO-6b*	RM3628–RM4924	9.67	21.54	1.98
	E4 (2017/ N1)	7	*qPRO-7*	RM8249–RM134	6.81	8.46	–1.76
CAT	E4 (2017/ N1)	1	*qCAT-1a*	RM3252–RM7180	16.79	26.43	–2.68
	E3 (2016/ N3)	1	*qCAT-1b*	RM6340–RM490	4.64	19.72	–3.42
	E2 (2016/ N2)	4	*qCAT-4a*	RM3843–RM4244	12.94	13.42	–2.71
	E3 (2016/ N3)	4	*qCAT-4b*	RM255–RM3866	7.45	22.58	–5.63
	E1 (2016/ N1)	6	*qCAT-6a*	RM340–RM3628	14.32	26.77	–2.78
	E4 (2017/ N1)	6	*qCAT-6a*	RM340–RM3628	15.86	27.65	–2.95
	E5 (2017/ N2)	6	*qCAT-6b*	RM3628–RM4924	14.97	19.71	–3.47
POD	E1 (2016/ N1)	1	*qPOD-1*	RM3252–RM7180	5.87	14.83	2.84
	E4 (2017/ N1)	1	*qPOD-1*	RM3252–RM7180	6.23	17.98	3.48
	E3 (2016/ N3)	3	*qPOD-3*	RM3513–RM1350	4.86	15.85	3.65
	E5 (2017/ N2)	7	*qPOD-7a*	RM7571–RM5455	12.13	17.85	–2.95
	E5 (2017/ N2)	7	*qPOD-7b*	RM5455–RM5672	13.49	13.46	–3.79
NR	E2 (2016/ N2)	1	*qNR-1*	RM283–RM1141	10.89	22.61	4.81
	E2 (2016/ N2)	4	*qNR-4*	RM4244–RM255	5.92	13.85	–4.93
	E1 (2016/ N1)	6	*qNR-6a*	RM340–RM3628	3.79	16.82	–3.82
	E2 (2016/ N2)	6	*qNR-6a*	RM340–RM3628	4.71	17.57	–2.97
	E4 (2017/ N1)	6	*qNR-6a*	RM340–RM3628	5.37	23.73	–4.98
	E5 (2017/ N2)	6	*qNR-6b*	RM3628–RM4924	3.92	19.57	–3.67
	E3 (2016/ N3)	7	*qNR-7*	RM3753–RM7161	4.68	17.35	–4.53

Chr – chromosome on which the QTL was located; LOD – additive logarithm of odds value; R^2 – proportion of the total variance explained by each QTL; AE – additive effect, negative value indicates that the allele from Towada, and positive value indicates that the allele of cv. Lijiangxintuanheigu contributes to increase the value of the parameter; N1 – 0 kg N/ha; N2 – 120 kg N/ha; N3 – 240 kg N/ha

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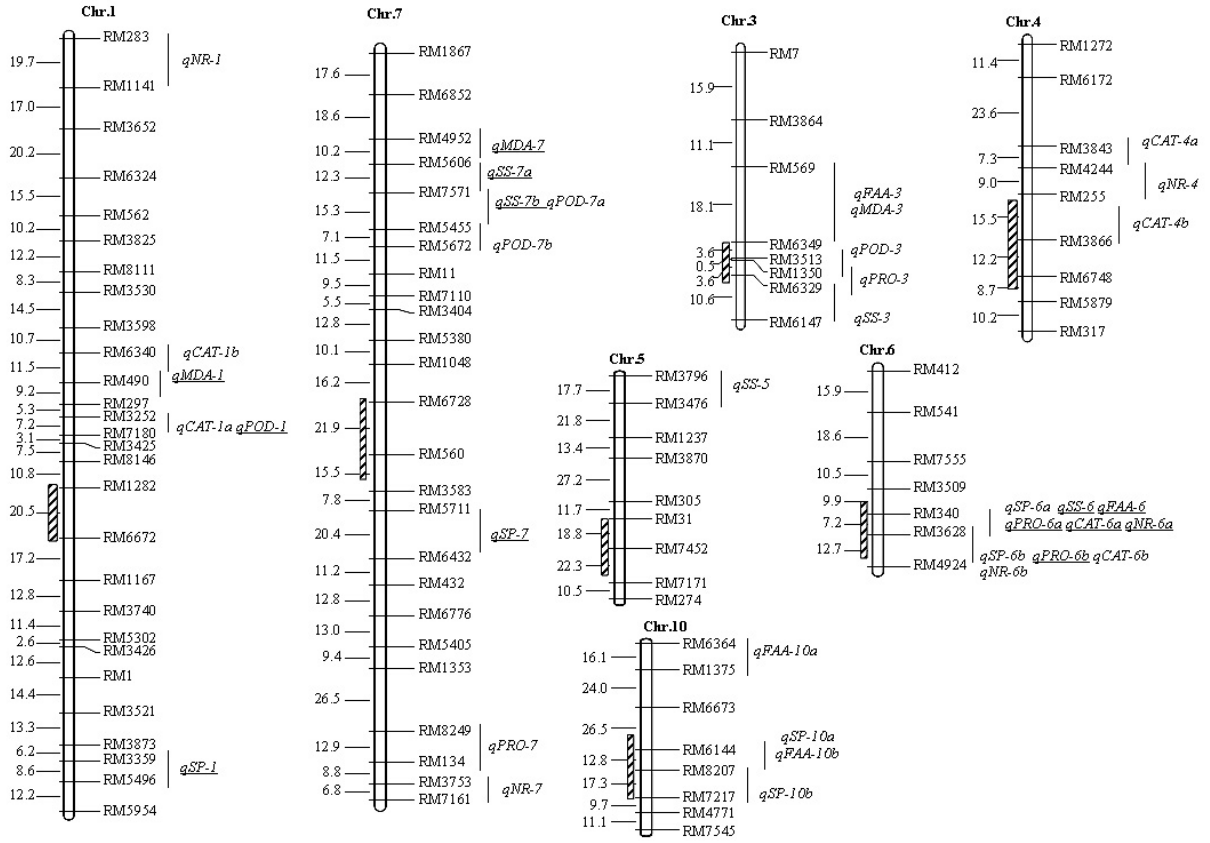

Figure 1. Chromosomal positions of QTLs for physiological indices of flag leaf at the booting stage in rice under cold--water stress combined with nitrogen fertilization

▨ represents the region of QTLs for spikelet fertility in previous studies; map distances (cM) are shown on the left; the marker and QTL names are shown to the right of the linkage group (chromosomes 1, 3, 4, 5, 6, 7 and 10); the underlined QTL is marked by both inclusive composite interval mapping and mixed composite interval mapping

24.25% or 25.28% of the total variation, with alleles from LTH. Four significant QTLs for PRO were detected on chromosomes 3, 6 and 7 (Table 2, Figure 1), which explained 8.46–21.54% of the phenotypic variance. The *qPRO-6a* (for N2) and *qPRO-6b* (for N3) alleles from LTH were identified in both years. By using MCIM, 2 QTLs for FAA were identified on chromosomes 4 and 6, and H^2AAE was 2.52% and 3.07%, respectively (Table 4). Meanwhile, 2 QTLs for PRO were identified, and H^2AE was 1.17 and 7.36% (Table 3), with H^2AAE being 2.17% and 2.49%, respectively (Table 4).

QTLs for CAT, POD and NR. By using ICIM, six QTLs for CAT were detected on chromosomes 1, 4 and 6 (Table 2, Figure 1) and R^2 varying from 13.42 to 27.65%, which were tending towards Towada. The large-effect *qCAT-6a* was detected in two consecutive years, and they explained 26.77% or 27.65% of the phenotypic variation, respectively. Four QTLs for CAT were located on chromosomes 1, 3 and 7 (Table 2, Figure 1), which accounted for 13.46–17.98% of the total variation, and the *qPOD-1* (for N1) was detected in two consecutive years. NR was controlled by five QTLs located on chromosome 1, 4, 6 and 7 (Table 2, Figure 1), and R^2 ranged from 13.73 to 23.73%. The *qNR-6a* was detected in both years and two nitrogen levels (for N1 and N2), its alleles were from Towada. By using MCIM, three QTLs were detected for CAT, POD and NR, respectively, and H^2AE ranged from 2.87 to 6.86% (Table 3), with H^2AAE varying from 0.97 to 2.52% (Table 4).

DISCUSSION

In this study, the coupling effect of chilling and moderate N application rate significantly increased the content of SP, SS, FAA, PRO, and enhanced CAT, POD and NR activity in the rice flag leaf. Moreover, high nitrogen reduced the accumulation of SP, SS, FAA, PRO and NR, however, it could maintain high

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activities of CAT and POD under a low temperature environment (Table 1). The changes of SS, PRO, POD and CAT activity could partly contribute to mitigating chilling stress (Oliver *et al*. 2005; Morsy *et al*. 2007; Gill & Tuteja 2010), and increasing PRO, FAA, POD and CAT activities also relied on the application of nitrogen (Waraich *et al*. 2012). Therefore, these results could provide a substantial basis for chilling resistance in rice.

By using ICIM, several QTLs were detected in both years, including *qSP-1*, *qSP-7*, *qSS-6*, *qSS-7a*, *qSS-7b*, *qFAA-6*, *qPRO-6a*, *qPRO-6b*, *qCAT-6a*, *qPOD-1* and *qNR-6a*, R^2 varied from 10.89 to 28.48% (Table 2, Figure 1). However, most of them were detected under one N level, except for *qSP-1* and *qNR-6a* which were detected across low N and moderate N. By using MCIM, 20 A-QTLs and 14 pairs of QTLs with epistatic × environment interaction effect were detected (Table 3, 4), and fourteen A-QTLs were detected by ICIM and MCIM (Table 3, Figure 1). Thus, this suggested that these traits were controlled by different genes under different N conditions. Furthermore, the positive or negative signs of additive effects from the parents (LTH and Towada) contribute to these traits, and transgressive selection was observed for several traits. Besides QTL for soluble protein and soluble sugar (http://www.Gramene.org/), other QTLs have never been reported before. Nevertheless, we found that some QTLs (*qPOD-3*, *qPRO-3*, *qSS-6*, *qNR-6a* and *qSP-10a*) were located in adjacent regions where the QTLs for spikelet sterility of the same donor parent (LTH) were identified on chromosomes 3, 6 and 10 (Shirasawa *et al*. 2012; Mitchell *et al*. 2016; Ulziibat *et al*. 2016; Yang *et al*. 2018) (Figure 1). Taken together, these results suggest that the traits (POD, soluble sugar, nitrate reductase and soluble protein) shared a similar genetic basis, their QTLs might be useful for cold-tolerant rice improvement.

In addition, co-localization regions of multiple QTL were observed, i.e. the interval of RM3252–RM7180

Table 3. Additive QTLs for environment interaction of soluble protein (SP), soluble sugar (SS), free amino acid (FAA), proline (PRO), catalase (CAT), peroxidase (POD) and nitrate reductase (NR) activity in the rice flag leaf of the parents and NIL grown at cold-water stress at three nitrogen levels (mixed composite interval mapping)

Traits	QTL	Marker interval	ADD	H^2A/%	2016			2017			H^2AE/%
					AE1	AE2	AE3	AE4	AE5	AE6	
SP	*qSP-1*	RM3359–RM5496	2.58	16.47	1.03	–2.31	2.99	0.57	0.33	0.59	0.87
	qSP-3	RM1350–RM6329	1.23	9.35	1.54	2.49	–2.02	0.82	1.44	2.41	2.59
	qSP-6a	RM340–RM3628	1.45	20.47	1.36	2.48	0.59	3.96	1.92	1.68	2.46
	qSP-6b	RM3628–RM4924	2.54	13.69	2.12	2.77	2.63	1.95	4.38	2.54	6.23
SS	*qSS-1*	RM3252–RM7180	2.42	11.44	1.49	2.23	1.45	2.58	1.74	–1.14	3.78
	qSS-6	RM340–RM3628	1.38	19.48	0.74	1.99	2.43	1.07	0.38	2.36	6.25
	qSS-7b	RM7571–RM5455	1.73	10.49	1.01	2.44	1.73	2.36	–1.74	–1.13	4.66
FAA	*qFAA-4*	RM4244–RM255	2.65	7.66	2.13	1.97	0.42	–2.18	–1.99	0.88	2.99
	qFAA-6	RM340–RM3628	2.73	12.47	–1.64	–1.55	2.19	2.74	1.73	2.97	3.69
PRO	*qPRO-4*	RM6172–RM3843	2.78	6.37	1.55	2.27	2.14	3.45	1.57	0.92	1.17
	qPRO-6a	RM340–RM3628	3.24	15.06	3.27	4.33	1.45	1.96	2.39	2.07	7.36
CAT	*qCAT-1a*	RM3252–RM7180	–2.67	7.79	1.68	–2.54	–2.12	0.85	1.24	1.72	3.92
	qCAT-6a	RM340–RM3628	–2.34	10.29	2.37	1.49	2.64	2.57	1.78	1.34	6.43
	qCAT-7	RM3404–RM5380	–1.87	9.38	4.52	3.46	1.07	–0.79	2.56	2.31	4.85
POD	*qPOD-1*	RM3252–RM7180	2.83	17.44	1.69	–0.57	2.38	1.54	2.48	1.05	3.74
	qPOD-3	RM1350–RM6329	1.68	6.94	1.04	1.63	3.47	2.01	0.92	1.36	4.25
	qPOD-7b	RM5455–RM5672	–2.47	14.89	2.68	–1.69	3.31	4.12	1.06	3.43	2.87
NR	*qNR-1*	RM283–RM1141	1.95	7.75	–2.59	2.44	1.39	1.53	–2.96	0.67	4.38
	qNR-5	RM1237–RM3870	2.17	9.44	–1.39	2.58	–1.93	1.85	1.45	1.03	6.86
	qNR-6a	RM340–RM3628	–2.76	12.23	3.17	2.63	2.34	1.98	1.08	2.46	3.69

ADD – additive effect; H^2A – contribution of additive effect; AE – additive × environment interaction effect; H^2AE – contribution of additive × environment interaction effect; N1 – 0 kg N/ha; N2 – 120 kg N/ha; N3 – 240 kg N/ha

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Table 4. Epistatic QTLs for environment interaction of soluble protein (SP), soluble sugar (SS), free amino acid (FAA), proline (PRO), catalase (CAT), peroxidase (POD) and nitrate reductase (NR) activity in rice flag leaf of the parents and NIL grown at cold-water stress at three nitrogen levels (mixed composite interval mapping)

Traits	QTL_i	QTL_i interval	QTL_j	QTL_j interval	AA	H^2AA/%	2016			2017			H^2AAE/%
							AAE1	AAE2	AAE3	AAE4	AAE5	AAE6	
SP	*qSP-1*	RM3252–RM7180	*qSP-6a*	RM3628–RM4924	1.34	2.57	2.23	1.46	0.99	1.58	1.27	1.09	3.58
	qSP-5	RM1237–RM3870	*qSP-10*	RM6364–RM1375	3.95	3.88	–0.82	–2.49	–1.46	3.21	–2.65	0.48	2.03
SS	*qSS-3*	RM1350–RM6329	*qSS-4*	RM4244–RM255	3.43	4.09	2.45	0.78	2.16	–0.57	1.82	0.96	1.49
	qSS-4	RM3843–RM4244	*qSS-7*	RM8249–RM134	1.47	2.35	1.27	2.02	1.49	3.05	0.78	0.36	1.94
FAA	*qFAA-1*	RM1–RM3521	*qFAA-4*	RM6172–RM3843	–2.01	4.28	0.58	1.84	–1.49	0.55	–1.47	1.22	2.52
	qFAA-3	RM6329–RM6147	*qFAA-10*	RM6673–RM6144	2.59	3.16	0.93	1.15	–1.26	–0.94	1.06	0.89	3.07
PRO	*qPRO-3*	RM7–RM3864	*qPRO-5*	RM3796–RM3476	–1.83	3.14	0.86	–1.26	2.47	1.37	0.35	0.14	2.49
	qPRO-4	RM5879–RM317	*qPRO-7*	RM7571–RM5455	6.99	5.02	1.55	–1.49	1.82	–2.17	1.46	1.45	2.17
CAT	*qCAT-1a*	RM3252–RM7180	*qCAT-6a*	RM340–RM3628	0.92	2.43	0.75	1.58	–0.39	2.29	0.78	1.22	1.76
	qCAT-3	RM1350–RM6329	*qCAT-7*	RM7110–RM3404	–3.78	4.49	0.52	0.78	0.15	0.09	–0.24	0.21	1.48
POD	*qPOD-1*	RM3252–RM7180	*qPOD-3*	RM3513–RM1350	1.39	2.77	0.56	0.69	1.06	0.44	1.28	2.03	2.52
	qPOD-4	RM255–RM3860	*qPOD-6*	RM541–RM7555	4.02	3.45	0.71	0.05	–1.12	0.96	1.24	–1.08	1.89
NR	*qNR-1*	RM1167–RM3740	*qNR-5*	RM3796–RM3476	–3.36	1.85	–0.74	–1.27	–2.59	–1.14	–0.97	–0.56	1.32
	qNR-5	RM3796–RM3476	*qNR-7*	RM5405–RM1353	–1.47	2.56	–0.92	–0.27	0.68	0.73	0.45	0.78	0.97

AA – epistatic effect; H^2AA – contribution of epistatic effect; AAE – epistatic × environment interaction effect; H^2AAE – contribution of epistatic × environment interaction effect; N1 – 0 kg N/ha; N2 – 120 kg N/ha; N3 – 240 kg N/ha; i, j – a pair of interaction loci; the underlined QTL is marked by both inclusive composite interval and mixed composite interval mapping

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(CAT and POD) on chromosome 1, RM1350–RM6329 (SP and PRO) on chromosome 3, RM4244–RM255 (FAA and NR) on chromosome 4, RM340–RM3628 (SP, SS, FAA, PRO, CAT and NR) and RM3628–4924 (SP, PRO, CAT and NR) on chromosome 6, RM7571–RM5455 (SS and POD) on chromosome 7, RM6144–RM8207 (SP and FAA) on chromosome 10 (Table 2, Figure 1). The QTLs for soluble sugar and protein were reported within the co-localized RM340–RM3628 region (Dumont *et al*. 2009), and higher sugars in the leaf/stem reduced sugars and starch content in floral organs, and driving sugar source-to-sink (stems/flowers) transport partition (Zhang *et al*. 2010) and osmotic adjustment (Theocharis *et al*. 2012). Likewise, proline could protect antioxidant enzymes, and it is known as a stabilizer of proteins and membranes, and an inducer of osmotic-stress genes (Szabados & Savoure 2010). The results revealed that nitrogen may have contributed to cold tolerance of rice by preventing cell membrane, osmotic adjustment and ROS scavenging system damage. Therefore, further studies are required to dissect the effect of unequivocally candidate genes for these co-location targeted regions more precisely, which should provide an important strategy to improve cold tolerance and nitrogen input in rice.

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