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I Gopinath
College of Post Graduate Studies, CAU (I), Meghalaya, India

Corresponding Author:
I Gopinath
College of Post Graduate Studies, CAU (I), Meghalaya, India

QTL mapping for cold tolerance with specific reference to rice

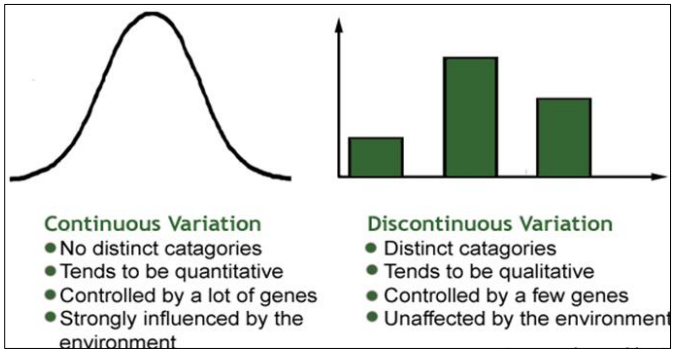
I Gopinath

Abstract
Rice is a staple food for more than half the world's population. It has highest global production next to wheat. With global climate change, most rice growing regions are experiencing extreme environmental fluctuations. Rice is susceptible to a variety of abiotic stresses including cold stress. In the temperate regions, rice growth is constrained by limited period that favours growth, where it needs optimum temperature between 25 ^{0}C to 35 ^{0}C. As the temperatures goes below 15°C, rice crop shows a wide range of chilling injury depending on the length of exposure and the developmental stage. Seedlings subjected to prolonged exposure (*i.e*. several days to weeks) can exhibit necrosis and mortality while shorter or intermittent exposure often leads to yellowing (chlorosis) and stunting, thus greatly reducing rice yields. Many QTLs related to cold tolerance at different stages have been identified by different researchers using mapping populations like recombinant inbred lines (RILs), doubled haploids (DH), F_2:F_3 lines, backcrosses and introgression lines. Therefore, the development of cold tolerant plants by the introduction of molecular breeding is assuredly a meaningful approach to hasten the breeding for improved plants. Intuitively, molecular breeding would be a faster way to mapping of beneficial QTL than through conventional breeding. The QTLs identified could be brought together by pyramiding into the breeders' material and thus reduce the negative effect of cold stress.

Keywords: Rice, quantitative trait loci, molecular breeding, cold tolerance, mapping populations, pyramiding

Introduction
The narrow genetic base of recent cultivars is a serious obstacle to maintain, sustain and improve crop productivity due to rapidly occurring vulnerability to potentially new biotic and abiotic stresses owing to uniformity. Plant germplasm resources, originated from a number of historical genetic events as a response to environmental stress and selection, are the important reservoirs of natural genetic variations that can be exploited to increase the genetic base of the cultivars. Gene bank collections are the important reservoirs of natural genetic variations originating from a number of historical genetic events as a response to environmental stresses (Hoisington *et al.*, 1999) [11]. Unlocking biodiversity held in gene banks and mobilizing useful variation to breeding programs are required for the genetic improvement of crops and to meet the overarching goal of diversification of the adapted gene pools. However, many agriculturally important traits such as productivity and quality, tolerance to environmental stresses, and some of forms of disease resistance are quantitative (also called polygenic, continuous, multifactorial, or complex traits) in nature. The genetic variation of a quantitative trait is controlled by the collective effects of numerous genes, known as quantitative trait loci (QTLs). Identification of QTLs of agronomic importance and its utilization in a crop improvement requires mapping of these QTLs in the genome of crop species using molecular markers (Sehgal *et at.*, 2016) [27]. Therefore, the basic concepts and a brief description of QTL mapping for cold tolerance with is an abiotic stress are focused in the following paragraphs.

What is a quantitative trait?
A quantitative trait is a measurable phenotype that depends on the cumulative actions of many genes and the environment.
Example: Plant height (measured on a ruler).
The traits showing continuous range of variations without natural discontinuities in a population and is more or less normally distributed, are called as quantitative characters or metric characters, because their study depends on measurements instead on counting (Falconer, 1985) [8].



Quantitative Trait Locus

A quantitative trait locus is the location of a gene, which is one of multiple such loci in the genome that affects a trait that is measured on a quantitative (linear) scale.

QTLs determine the genetic component of variation in quantitative traits.

QTL Mapping

Definition

The identification of QTLs based on conventional phenotypic evaluation is not possible. A major breakthrough in the characterization of quantitative traits that created opportunities to select QTLs was initiated by the development of DNA (or molecular) markers in 1980s. One of the main uses of DNA markers in agricultural research has been in the construction of linkage maps for the diverse crop species. Linkage maps have been utilized for identifying chromosomal simple traits and quantitative traits using QTL analysis. The process of construction of linkage maps and conducting QTL analysis to identify genomic regions associated with traits is known as QTL mapping (McCouch and Doerge, 1995) [21].

Principle of QTL mapping

QTL analysis is based on the principle of detecting an association between phenotype and the genotype of markers.

Markers are used to partition the mapping population into different genotypic groups based on the presence or absence of a particular marker locus and to determine whether significant differences exist between groups with respect to the trait being measured. Situations where a marker and a trait fail to segregate independently are said to display "linkage". QTL analysis, thus, depends on such linkages.

Traditional QTL Mapping (Linkage Mapping)

The general steps involved in a traditional QTL mapping experiment are as follows:

1. select two parental strains that have differences between them in the alleles that affect variation in a trait. The parents need to be different in the mean phenotypic value of the trait as different allelic combinations can yield the same phenotypic mean;
2. develop an appropriate mapping population by crossing the selected parents;
3. phenotype the mapping population for the trait(s) of interest (morphological characters, agronomic traits, disease and pest scores, drought resistance, etc.) under greenhouse, screen-house, and/or field conditions;
4. generate the molecular data on the population with adequate number of uniformly spaced polymorphic markers;
5. construct a genetic map; and
6. identify molecular markers linked to the trait(s) of interest using statistical programs.

Mapping Populations Used in QTL Mapping

Various types of mapping population may be produced from the heterozygous F1 hybrids (Fig. 1)

1. Double haploid lines (DHLs): plants are regenerated from pollen (which is haploid) of the F_1 plants and treated to restore diploid condition in which every locus is homozygous. Since the pollen population has been generated by meiosis, the DHLs represent a direct sample of the segregating gametes.
2. Backcross (BC) population: the F_1 plants are backcrossed to one of the parents.
3. F_2 population: F_1 plants are selfed.
4. $F_{2:3}/F_{2:4}$ lines: $F_{3/4}$ plants tracing back to the same F_2 plant, also called F_2families.
5. Recombinant inbred lines (RILs): inbred generation derived by selfing individual

F_2 plants and further single seed descent. A population of RILs represents an 'immortal' or permanent mapping population.

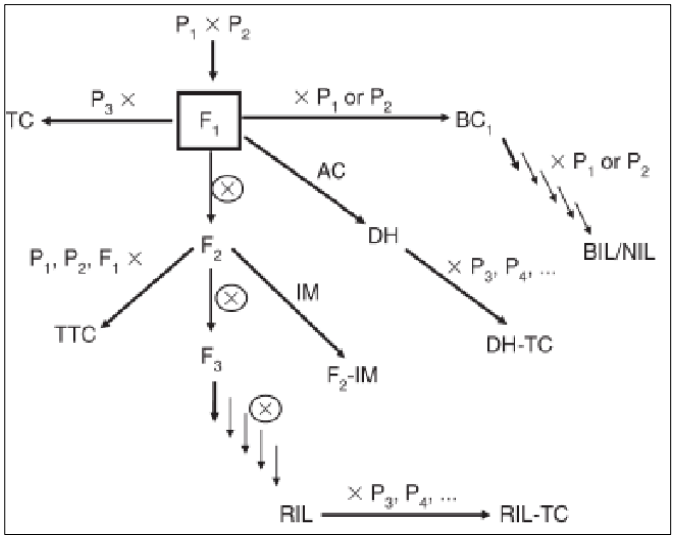


Fig 1: Schematic diagram of different biparental mapping populations.

Each of the above mapping populations possesses advantages and disadvantages. Hence, the choice of the type of mapping population depends on many factors such as the plant species, type of marker system used, and the trait to be mapped later on. F_2 populations, derived from F_1 hybrids, and backcross populations, derived by crossing the F_1 hybrid to one of the parents, are the simplest types of mapping populations developed for self-pollinating species. Their main advantages are that they are easy to construct and require only a short time to produce. However, such populations are not fixable due to their inherent heterozygous genetic constitution (Fig 2). This restricts their wide utility in QTL analysis. The length of time needed for producing RIL population is the major disadvantage, because usually six to eight generations are required. Development of a DH population takes much less time than RIL; however, the production of DH populations is only possible in species that are amenable to tissue culture (e.g., cereal species such as rice, barley, and wheat). The major advantages of RIL and DH populations are that they produce homozygous or 'true-breeding' lines that can be multiplied and reproduced without genetic change occurring. This allows for the conduct of replicated trials across different locations and years. Furthermore, seed from individual RIL or

DH lines may be transferred between different laboratories for further linkage analysis and the addition of markers to existing maps. Information provided by co-dominant markers is best exploited by an F_2 population, while information obtained by dominant marker systems can be maximized by using RILs or DHLs. Double haploids, F_2 or F_3 families, or RILs are advantageous if the trait to be mapped cannot be accurately measured on a single-plant basis but must be assessed in replicated field experiments.

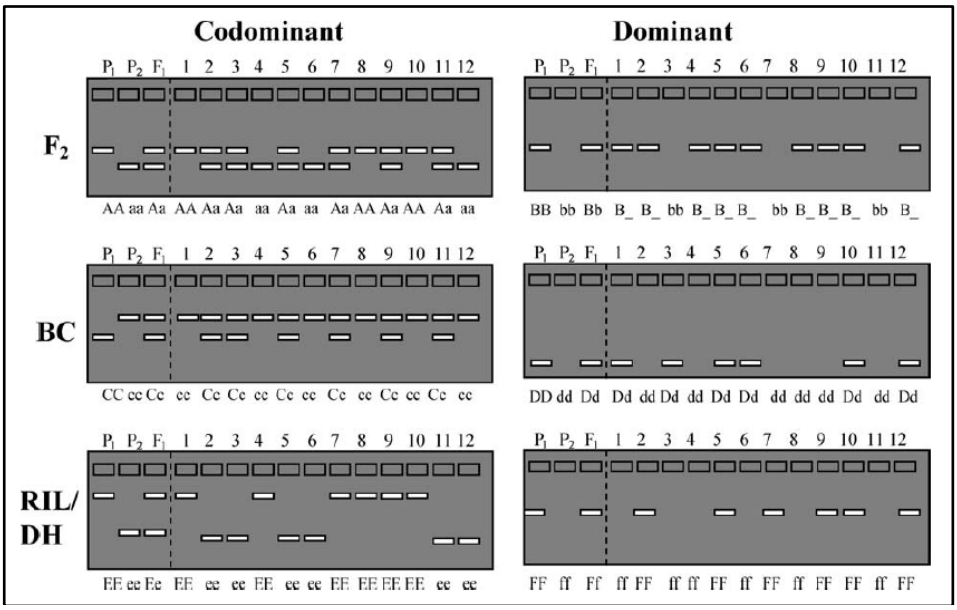


Fig 2: Hypothetical gel picture representing segregating codominant markers and dominant markers for typical mapping populations.

Construction of Genetic/Linkage Maps

A linkage map may be thought of as a 'road map' of the chromosomes derived from two different parents. Linkage maps indicate the position and relative genetic distances between markers along chromosomes. Construction of a linkage map, using genotyping data generated on any of the above-mentioned mapping populations, is an important step before initiating any QTL analysis. In a segregating mapping population, there is a mixture of parental and recombinant genotypes. The frequency of recombinant genotypes is used to calculate recombination fractions, which is then used to infer the genetic distance between markers (Fig. 3). By analysing the segregation of markers, the relative order and distances between markers can be determined; the lower the frequency of recombination between two markers, the closer they are situated on a chromosome (conversely, the higher the frequency of recombination between two markers, the further away they are situated on a chromosome).

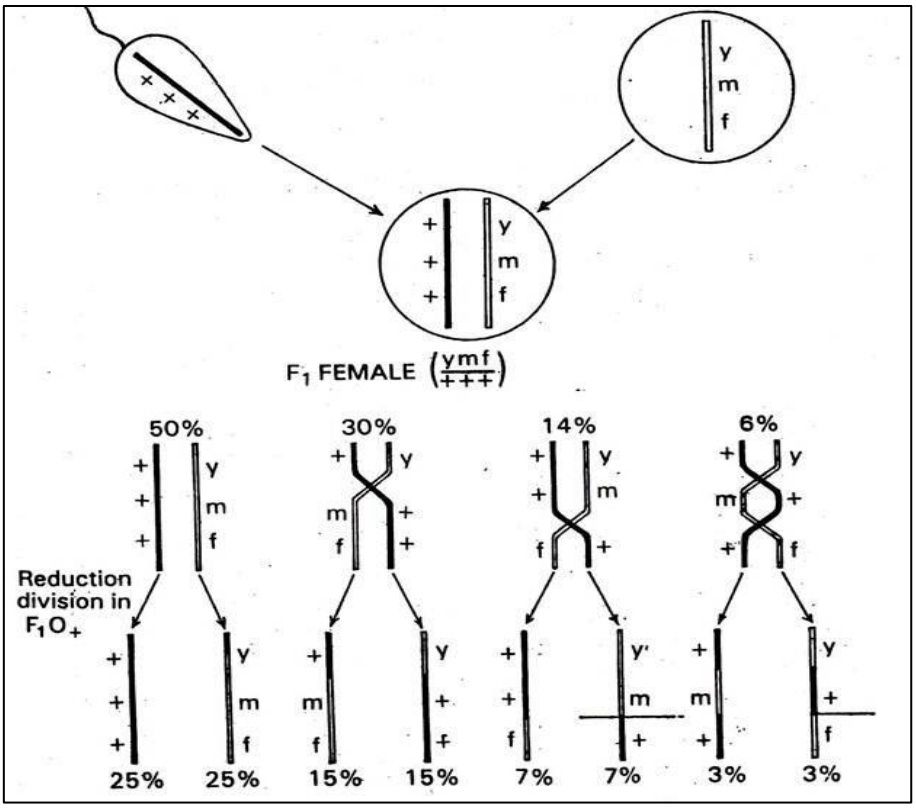


Fig 3: Diagram indicating crossover or recombination between homologous chromosomes

Two commonly used mapping functions that convert recombination frequency into centimorgan (cM) distance are,

1. Kosambi mapping function, which assumes that recombination events influence the occurrence of adjacent recombination events (Xu 2010).
   $Map\ units = -\frac{1}{2}\ln(1-2r)$; *where, r= recombination frequency.*
2. Haldane mapping function, which assumes no interference between crossover events (Xu 2010).
   $Map\ units = \frac{1}{4}\ln[(1+2r)/(1-2r)]$

Linkage between markers is usually calculated with an odds ratio (i.e., the ratio of linkage versus no linkage). This ratio is more conveniently expressed as the logarithm of the ratio and is called a logarithm of odds (LOD) value or LOD score (Risch 1992) [26]. LOD values of >3 are typically used to construct linkage maps. LOD values may be lowered in order to detect linkage over a greater distance or to place additional markers within maps constructed at higher LOD values (Collard *et al.*, 2005) [5]. Linked markers are grouped together into linkage groups, which represent chromosomal segments or entire chromosomes (Fig. 4).

$$LOD = Log10\ [\frac{L(r)}{L(r=0.5)}]$$

It is important to note that distance on a linkage map is not directly related to the physical distance of DNA between genetic markers, but depends on the genome size of the plant species. Furthermore, the relationship between genetic and physical distance varies along a chromosome. For example, there are recombination 'hot spots' and 'cold spots,' which are chromosomal regions in which recombination occurs more frequently or less frequently, respectively. Commonly used software programs for constructing linkage maps include Mapmaker/EXP, MapManager QTX and THREaD Mapper Studio which are freely available from the Internet. JoinMap is another commonly used program for constructing linkage maps (Fig. 5).

Phenotyping of mapping population and sample size

Data is pooled over locations and replications to obtain single quantitative trait value for the line over multiple locations for better understanding of QTL x environment interaction.

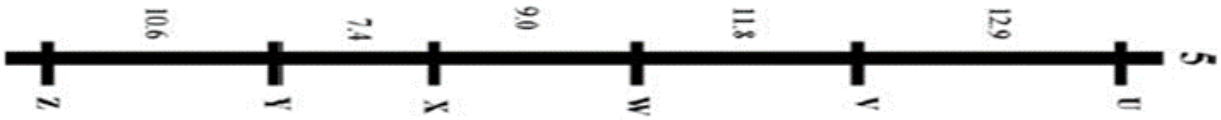


Fig 4: Linkage map based construction based on a small recombinant inbred population.

Detection of QTLs

Four widely used methods for detecting QTLs are single-marker analysis, interval mapping by maximum likelihood, interval mapping by regression, and composite interval mapping.

1. **Single-Marker Analysis (Point Analysis):** The traditional method to detect a QTL in the vicinity of a marker is studying single-genetic markers one at a time. The phenotypic means for progeny of each marker class are compared (e.g., means of the marker classes AA, Aa, aa). The difference between two means provides an estimate of the phenotypic effect of substituting an A allele by an a allele at the QTL. To test whether the inferred phenotypic effect is significantly different from zero, a simple statistical test, such ast-test or F-test, is used. A significant value indicates that a QTL is located in the vicinity of the marker. Single-point analysis does not require a complete molecular linkage map. The farther a QTL is from the marker, the less likely it is to be detected statistically due to crossover events between the marker and the gene.
2. **Interval Mapping by Maximum Likelihood:** QTL interval mapping is the most common method of QTL analysis. The principle behind interval mapping is to test a model for the presence of a QTL at many positions between two mapped marker loci. This model is a fit, and its goodness is tested using the method of maximum likelihood. For example, if it is assumed that a QTL is located between two markers, the 2-loci marker genotypes (i.e., AABB, AAbb, aaBB, aabb for DH progeny) each contain mixtures of QTL genotypes. Maximum likelihood involves searching for QTL parameters that give the best approximation for quantitative trait distributions that are observed for each marker class. Models are evaluated by computing the likelihood of the observed distributions with and without fitting a QTL effect. The map position of a QTL is determined as the maximum likelihood from the distribution of likelihood values (LOD scores: ratio of likelihood that the effect occurs by linkage to likelihood that the effect occurs by chance), calculated for each locus.
3. **Interval Mapping by Regression:** Interval mapping by regression was developed primarily as a simplification of the maximum likelihood method. It is essentially the same as the method of basic QTL analysis (regression on coded marker genotypes) except that phenotypes are regressed on QTL genotypes. Since the QTL genotypes are unknown, they are replaced by probabilities estimated from the nearest flanking markers.
4. **Composite Interval Mapping:** One of the factors that weakens interval mapping is fitting the model for a QTL at only one location. There are two problems with this approach: (a) the effects of additional QTL will contribute to sampling variance and (b) if two QTLs are linked, their combined effects will cause biased estimates. The method of composite interval mapping (CIM) was proposed as solution. CIM will perform the analysis in the usual way, except that the variance from other QTLs is accounted for by including partial regression coefficients from markers ('cofactors') in other regions of the genome. CIM gives more power and precision than simple interval mapping (SIM) because the effects of other QTLs are not present as residual variance. CIM can remove the bias that can be caused by QTLs that are linked to the position being tested.

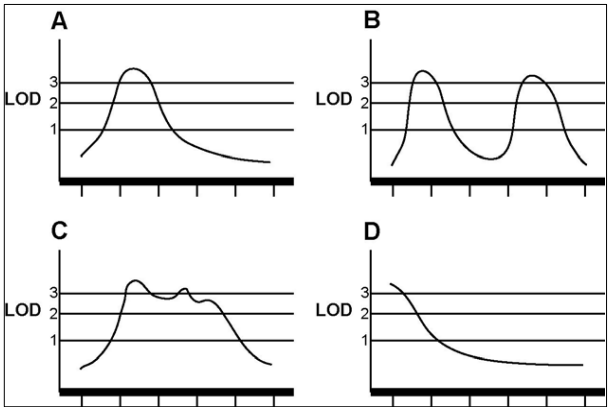


Fig 5: Hypothetical output showing a LOD on chromosome using different interval mappings.

QTL Mapping Softwares

There are over 100 genetic analysis software packages (linkage analysis and QTL mapping). Here, we list some features of the most commonly used software packages.

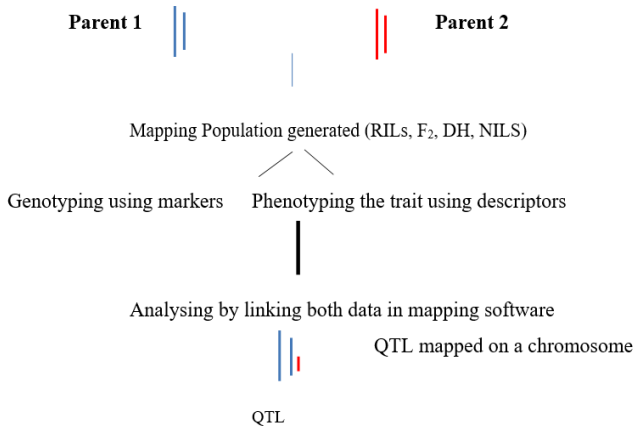
MapMaker/QTL: A user-friendly, freely distributed software program runs on almost all platforms. It analyzes F2 or backcross data using standard interval mapping.

MQTL: MQTL is a computer program for CIM in multiple environments. It can also perform SIM. Currently, MQTL is restricted to the analysis of data from homozygous progeny (double haploids, or RILs). Progeny types with more than two marker classes (e.g., F2) are not handled.

PLABQTL: PLABQTL is a freely distributed computer program for CIM and SIM of QTL. Its main purpose is to localize and characterize QTL in mapping populations derived from a bi parental cross by selfing or production of double haploids. Currently, this program is the easiest software for composite interval mapping.

QTL Cartographer: QTL Cartographer is a QTL software written for either UNIX, Macintosh, or Windows. It performs single-marker regression, interval mapping, and composite interval mapping. It permits analysis from F2 or backcross populations. It displays map positions of QTLs using the GNUPLOT software.

Summary of QTL mapping



Mapping in context for cold tolerance in rice

Rice is a staple food for more than half the world's population. It has highest global production next to wheat. Rice is one of the most important cereal crops, it has highest global production (494.4 million tons) but second to wheat (729.5million tons) (www.fao.org). It is the number one crop of India and belongs to the family poaceae. Rice accounts for about 42 per cent of total food grain production and >55 per cent of diet in India. It is widely grown in India due to its wide adaptability. In India, rice is grown in an area of 42.7 m ha producing 112.73 mt with a productivity of 2480 kg per ha.

Low temperature/Cold Stress

With global climate change, most rice growing regions are experiencing extreme environmental fluctuations. Rice is susceptible to a variety of abiotic stresses including cold stress. In the temperate regions, rice growth is constrained by limited period that favours growth, where it needs optimum temperature between 25ºC to 35ºC. At temperatures below 15°C, rice plants exhibit a wide range of chilling injury depending on the length of exposure and the developmental stage (Coly and Toll, 1979). Seedlings subjected to prolonged exposure (*i.e*. several days to weeks) can exhibit necrosis and mortality while shorter or intermittent exposure often leads to yellowing (chlorosis) and stunting, thus greatly reducing rice yields. The cold tolerance is developmentally regulated and growth stage specific. However, identifying QTLs associated with cold tolerance (CT) and elucidating their genetic relationship are the prerequisite for developing rice varieties with cold tolerance. (Fig. 6)

Symptoms caused by low temperature on rice are as follows,

- Delayed and lower percentage of germination
- Necrosis and mortality
- Growth retardation
- Sterility of the spikelets
- Affect seedling establishment
- Chlorosis
- Decreased tillering
- Incomplete panicle exsertion

Coly and Toll (1979) reported that the rice plant is affected by low temperature during seedling stage. Only water temperature has it's effect on rice growth in early stages. From flag leaf differentiation up to the final stage of reduction division of the pollen mother cell.

Pandey *et al.* (1993) [22] reported that four cold-tolerant rice varieties, *viz*. Khonorullo, Namyi, Abor b and Meghalaya-1 were crossed with two cold-susceptible ones, *viz*. Pusa 33 and Subhadra (DR92), in all possible combinations. F1 hybrids of all the crosses showed complete panicle excertion indicating dominance of cold tolerance trait.

The *indica* cultivars with their origin from tropical environments are much susceptible to cold stress when compared to *japonica* cultivars which originated from temperate climates. Therefore, there is enormous scope for improvement of *indica* type cultivars that could be done by crossing *indica* genotypes with *japonica* ones.

Bertin *et al.* (1996) [3] screened rice varieties for chilling tolerance during germination and vegetative growth using different techniques. Effects of temperature ranging from 10 to 25ºC were investigated during germination the screening was found to be most effective at 10ºc. However time of data collection has to be considered in order to discriminate slow germinating varieties from chilling sensitive varieties.

Rajinder *et al.* (1999) [25] reported that the occurrence of low night temperatures during reproductive development is one of the most limiting factor for rice yields. The results of the economic analysis reveal that new cold tolerant varieties would lead to significant increase in financial benefit in cold condition.

Purohith *et al.* (2009) [24] reported that early heading coupled with high yielding potentiality is a desired objective in rice breeding programme for boro culture in eastern India. Spikelet number per panicle is a major target trait for improving rice. Development of new plant types and hybrids are two major approaches for improving yield potential of irrigated rice.

Variabilities/traits to evaluate cold stress

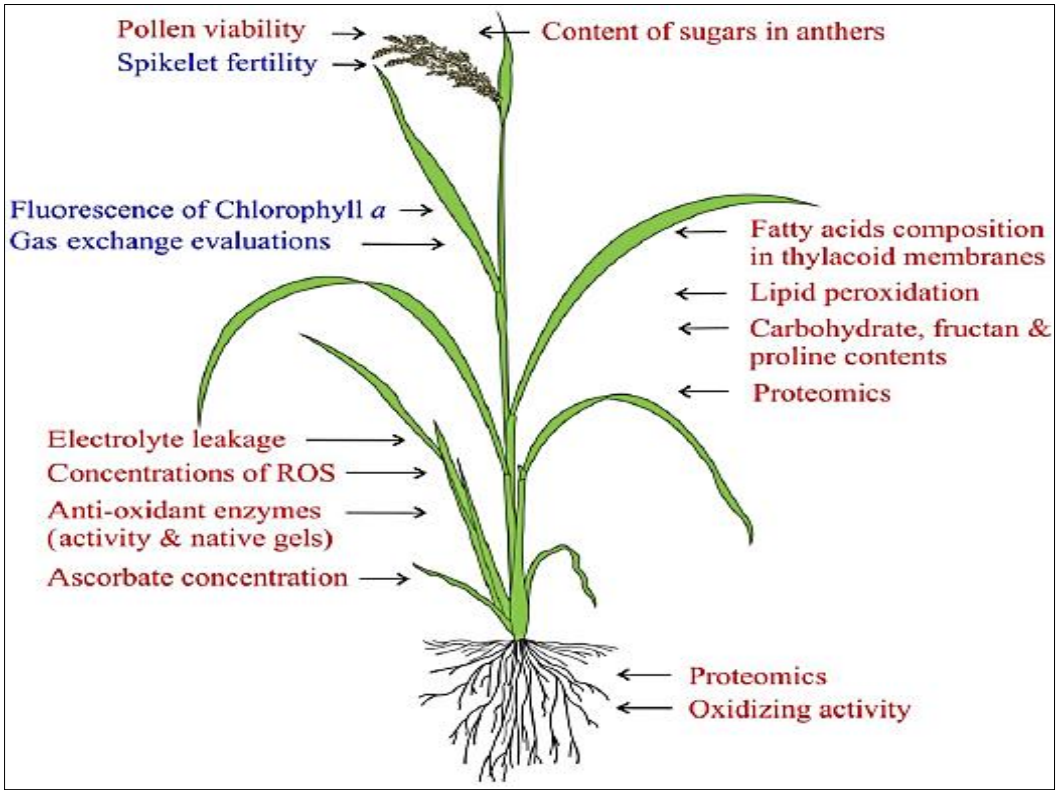


Fig 6: Traits to be evaluated for cold tolerance studies in rice

Table 1: Methods and traits evaluated in different stages of plant development for cold-tolerance selection in rice

Growth stage	Methodology of screening	Evaluated trait	Reference
Germination	10, 15, 20, and 25°C for 3 to 30 days (depending on the temperature)	Germination rate (radicle protrusion)	Bertin *et al.* (1996) [3]
	15°C for 10 days	Coleoptile length	Hou *et al.* (2003) [12]
	13°C to 15°C for 7 days	Percentage of germination	Lee (2001) [18]
Vegetative	10°C for 3, 6, and 9 days	Survival rate 10 days after the end of the cold treatment	Bertin *et al.* (1996) [3]
	Cool-air treatment at 12°/10°C (day/night) for 10 days at 3-leaf stage	Growth and discoloration	Lee (2001) [18]
	4°C for 6 days in the dark	Survival rate after 14 days of recovery	Koseki *et al.* (2010) [16]
Reproductive	12°C at the young microspore stage for 3–5 days	% of fertility	Bertin *et al.* (1996) [3]
	Cool water (20 cm depth) at 19.4°C from the primordial stage to the completion of heading	% of fertility	Kuroki *et al.* (2007)
	17°C at the booting stage for 10 days	% of fertility	Suh *et al.* (2010); Jena *et al.* (2010)

QTLs for cold tolerance

Cold tolerance and heat tolerance are complex traits that are controlled by quantitative trait loci (QTL). Many QTLs related to cold tolerance at different stages such as germination, seedling, vegetative, reproductive and grain maturity have been identified by different researchers using recombinant inbred lines (RILs) (Andava and Tai, 2006; Jiang *et al.*, 2008; Suh, *et al.*, 2010) [14, 13, 30], doubled haploid (DH) (Chen, *et al.*, 2006; Lou, *et al.*, 2007) [4], F2-:F3lines (Han, *et al.*, 2006), backcross and introgression lines.

Andaya *et al.* (2003) [2] reported that the booting stage is more sensitive to low temperature stress. The map with a total length of 1,276.8 cM and an average density of one marker every 7.1 cM was developed from 181 loci produced by 175 microsatellite markers. QTLs on chromosomes 1, 2, 3, 5, 6, 7, 9 and 12 were identified to confer cold tolerance at the booting stage.

Kim *et al.* (2003) [15] reported that to the RAPD analysis for the cold tolerance of 94 F2 plants from a cross between Dular (indica, cold sensitive cultivar) and Toyohatamochi (japonica,

cold-tolerant cultivar), OPT8511 was confirmed to have strong association with cold tolerance of rice. This marker could be of use in markerassisted selection for cold tolerance in rice.

Shi-quan *et al.* (2004) [29] reported that the genetic analysis showed that cold tolerance at booting stage of near-isogenic lines (NILs) of Kunming xiaobaigu was controlled by a gene with large phenotypic variance. This gene explained 10.50% of phenotypic variance and 5.10% of phenotypic variance of fully filled grains, and was tentatively designated as Ctb(t).

Table 2: QTLs for cold tolerance at different growth stages in rice identified on 12 chromosomes

Trait	Parents	Mapping population	No. of QTL identified	Salient features	References
Low temperature germinability	Italica Livorno (T) & Hayamasari (S)	122 RILs	3	QTL identified on chromosome 3 and 4. A major QTL, qLTG3–1 identified on chromosome 3, accounts for 35.0% phenotypic variation with a LOD score of 15.7.	Fujino *et al.* (2004) [9]
Survival percentage of seedling	M-202 (T) & IR50 (S)	191 RILs	15	QTL identified on chromosome 1, 3, 4, 6, 8, 10, 11, 12. The major QTL, *qCTS12a* identified on chromosome 12, accounts for 40.6% phenotypic variation with a LOD score of 20.34.	Andaya and Mackill (2003) [2]
Spikelet sterility	ZL1929-4 (T) & Towada (S)	F_2 with 2,810 plants	1	A major QTL, *qCTB7*, identified on chromosome 7 accounts for 9 and 21% phenotypic variation with a LOD score of 7.74 and 11.2 in F2 and F3 generations, respectively.	Zhou *et al.* (2010) [37]
Per cent seed set	IR66160-121- 4-4-2 (T) & Geumobyeo (S)	153 F_8 RILs	3	QTL identified on chromosome 3, 7, and 9 contributing 7.4 to 9.4% phenotypic variation with a LOD score ranging from 2.5 to 4.9.	Suh *et al.* (2010) [13, 30]
Per cent Ratooning Germinability (PRG) and Overwintering Germi-nability (POG)	89-1(Gr 89-1) and Shuhui 527	312 RILs in F_9	5	QTL identified on chromosome 3, 3, 7, 11 and 11 contributing 6.7 to 17.8% phenotypic variation with a LOD score ranging from 2.7 to 5.8.	Zhang *et al.* (2012) [33]
Leaf yellowing and leaf rolling	Lijiangxintuanheigu (japonica) and Sanhuangzhan-2 (indica)	204 RILs	9	Four QTL on chromosomes 1, 6, 9 and 12 were detected using leaf Yellowing. And 4 QTL on chromosomes 7, 8, 9, 11 and 12 were detected using leaf rolling and per cent seedling survival, two major qCTS-9 and qCTS-12 contributing 15.8% and 14.7% phenotypic variation with a LOD score 8.0 and7.7.	Zhang *et al.* (2014) [34]
Root conductivity (RC)	DX as a cold-tolerant donor and Nanjing 11 (NJ) recurrent parent	151 BC_2 F_1	2	QTL identified on chromosome 10. QTL, named qRC10-1 and qRC10-2 contributing 9.4% to 32.1% phenotypic variation with a LOD score ranging from 3.1 to 6.1.	Xiao *et al.* (2014) [31]
Low-temperature vigor of germination	Milyang 23 (indica) and Jileng 1 (japonica) rice	200 F_2:F_3	20	QTL were mapped on chromosomes 1, 2, 3, 5, 6, 7, 8 and 11. Contributing 5.3 to 22.9% phenotypic variation with a LOD score ranging from 2.12 to 4.26.	Han *et al.* (2006) [10]

Major QTL for cold tolerance at seedling stage

Table 3: Some of the QTLs governing the cold tolerance mapped across 12 chromosomes of rice

Sl. No.	QTL name	Marker Name	Chr. No.	LOD Score	Source
1	*qCTS-1-c*	RM315-RM472	1	8.03	Lou *et al.* (2007) [20]
2	*qCTS1-1*	RM1282-RM3426	1	19.2	Liu *et al.* (2013) [19]
3	*qCTS-2*	RM561-RM341	2	15.09	Lou *et al.* (2007) [20]
4	*qCTS-3*	RM200-RM85	3	5.28	Andaya and Mackill (2003) [2]
5	*qCTS-4-2*	RM255-RM348	4	4.79	Andaya and Mackill (2003) [2]
6	*qSPA-5*	RM 161	5	5.3	Park *et al.* (2013) [23]
7	*qCTS-6*	RM161-RM340	6	3.1	Zhang *et al.* (2013) [35]
8	*qCTS-7*	RM336-RM10	7	3.33	Zhang *et al.* (2005) [36]
9	*qCTS-8-1*	RM284-RM230	8	5.68	Andaya and Mackill (2003) [2]
10	*qCTS-9*	RM6854-RM566	9	6.28	Zhang *et al.* (2014) [34]
11	*qRC10-1*	RM1108-RM171	10	5.2	Xiao *et al.* (2014) [34]
12	*qSTC-11*	RM202-RM209	11	19.9	Zhang *et al.* (2005) [36]
13	*qCTS-12*	RM27628-RM397	12	5.3	Zhang *et al.* (2013) [35]

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