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Genotype × environment interaction in QTL analysis of an intervarietal almond cross by means of genetic markers

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Abstract Besides QTL location and the estimation of gene effects, QTL analysis based on genetic markers could be used to comprehensively investigate quantitative trait-related phenomena such as pleiotropy, gene interactions, heterosis, and genotype-by-environment interaction (G × E). Given that the G × E interaction is of great relevance in tree improvement, the objective of the research presented here was to study the effect of years on QTL detection for 15 quantitative traits by means of isozymatic markers in a large progeny group of an intervarietal cross of almond. At least 17 putative QTLs were detected, 3 of which had alleles with opposite effects to those predicted from the parental genotypes. Only 3 QTLs behaved homogeneously over the years. Three possible causes are discussed in relation to this lack of stability: the power of the test statistic being used, the low contribution of the QTL to the genetic variation of the trait, and a differential gene expression dependent on the year (G × E). Most cases showing lack of stability involved traits whose heritability estimates change drastically from year to year and/or whose correlation coefficients between years are low, suggesting the presence of G × E as the most likely cause. A marker-assisted selection scheme to improve late flowering and short flowering duration is suggested for an early and wide screening of the progeny.

Key words Genetic markers · QTL · G × E interaction · Almond · Flowering time · Yield · Tree breeding

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Introduction

The analysis of genes controlling the expression of quantitative traits (QTL) is being extensively carried out in crop species. Such research has two main objectives: (1) to assist selection during the breeding programs, and (2) the genetic dissection of quantitative traits into individual factors that could be further isolated and used in transfection or transformation experiments. Several quantitative traits have been studied by means of molecular markers: cold tolerance (Vallejos and Tanksley 1983), water use efficiency (Martin et al. 1989), insect resistance (Nienhius et al. 1987), fruit weight and quality parameters as well as some vegetative growth traits in tomato (Osborn et al. 1987; Weller et al. 1988; Tanksley and Hewitt 1988; Paterson et al. 1988, 1991; Bretó et al. 1994), and traits related to yield and vegetative development in maize (Kahler and Wehrhahn 1986; Beavis et al. 1991; Edwards et al. 1992), in rye (Plaschke et al. 1993), in perennial ryegrass (Humphreys 1992), etc. In all of these traits, genes controlling the character of interest have been detected, which makes the above-mentioned objectives of QTL research feasible. Thus, great efforts have been focused on constructing saturated linkage maps and on locating QTLs affecting important agronomic traits. Some annual crops have been extensively studied (tomato, corn, etc.) and, in spite of their inherent complexities, perennial crops (apple, orange, etc.) will be the next target species.

Tree breeding programs are very different from those of annual crops due to the larger size of the plant and the existence of a long juvenile period. In most fruit trees, the long juvenile phase of seedling trees (up to 10 years) causes difficulties in the early assessment of fruit and tree characteristics. In a typical breeding program most of the 40 000 seedlings generated may be screened for disease resistance each year, and up to 1000 trees selected on the basis of habit in the second year are retained on rootstocks for assessment of fruit characters 4–5 years after germination (King et al. 1991). This

process is costly in terms of land and other resources. Furthermore, in perennial crops, which are not usually cultured under a controlled environment like a greenhouse, the "stability" or performance at a given site across years is very important. The genotype × environment (G × E) interaction or differential genotypic expression across environments reduces association between phenotypic and genotypic values and may cause selections from one environment to perform poorly in another, thereby forcing plant breeders to examine genotypic adaptation. In addition, the sampling problem associated with yearly variation suggests a necessary testing for many crop cycles. To save time, breeders opt to substitute temporal with spatial variation, assuming that testing over a wide geographic range can ensure a parallel degree of temporal buffering capacity in their germ plasm. Therefore, perennial crop improvement is mainly limited to clonal selection or selection in a first generation progeny of a cross. Doubtless, QTL analysis of tree fruits, or woody plants in general, opens new possibilities for their improvement, and they are the species of economic interest where the help of biotechnology is most needed.

Important aspects of QTL analysis have been previously studied, such as the predictive value of QTLs within a pedigree and across evolutionary distance (Paterson et al. 1991), the effects of different experimental designs, heritabilities of the quantitative trait, and types of gene action on QTL detection (Carbonell et al. 1993), and the efficiency of generations for estimating marker-associated QTLs (Moreno-González 1993). Therefore, there is a considerable body of knowledge that can be used to thoroughly investigate well-known phenomena related to quantitative traits, other than QTL location and estimation of gene effects, such as pleiotropy, gene interactions, heterosis, and genotype-by-environment (G × E) interaction.

Recently, Dicenta et al. (1993a, b) found a large year-to-year variation in the heritability estimates of some almond traits related to flowering and fruiting. Heritability is a parameter specific to a population under a given set of experimental conditions; hence, the environment may have a marked influence on its estimates. In fact, Aastveit and Aastveit (1993) also found that if a G × E interaction exists, great differences are expected to occur in the estimates of genetic correlation coefficients obtained in different environments.

QTL analysis based on genetic markers could be used as an approach to study G × E interaction and, even more, to gain stability by designing a marker-assisted selection (MAS) scheme that takes into account all of the QTLs involved in the trait for the different environments. Paterson et al. (1991) tried to scope the predictive value of QTLs across environments, but the design they used does not enable environmental and laboratory effects to be distinguished due to confounding. The objective of the investigation presented here was to study the effect of years on QTL detection for 15 quantitative traits by means of isozymatic markers in a progeny of an intervarietal cross of almond.

Materials and methods

The plant material consisted of 123 seedlings established in 1986 that had been derived by hand pollination of emasculated flowers of Spanish almond variety 'Ramillete' with pollen of Italian variety 'Tuono'.

Fifteen quantitative characters were evaluated in 1989 and 1990, and 6 of these were also evaluated in 1991 (see Table 1). Self-compatibility was also determined in 53 seedlings by means of microscopic observation of the pollen-tube growth in the pistil (Dicenta and García 1993). The flowering and maturity times were recorded as the number of days elaspsed after 1st of January: initial flowering was determined when 5% of the flowers were opened; maximum flowering, 50%; final flowering, 95%. The duration of flowering was calculated as the difference between final and initial flowering times. Flower and yield intensities were visually evaluated from 0 (none) to 5 (maximum). Maturation date was determined when 95% of the fruit of a tree appeared with the mesocarp fully opened. The duration of maturation was the difference between the maturation and final flowering times. Fifty fruits per plant were measured for the fruit-related traits. The average in-shell weight in grams was calculated and, after removal of the shell (endocarp), the average weight of a kernel was obtained. The percentage of kernel was calculated as the ratio of kernel weight to in-shell weight. The percentage of empty nuts (failures) and double kernel was also recorded. Color intensity of the tegument was measured subjectively (from 1 = very pale brown to 5 = very dark brown). Rugosity was evaluated, with the seeds being classified into three groups.

One branch per seedling was taken and kept on ice from Murcia (CEBAS-CSIC) to Valencia (IVIA), where completely mature leaves were excised and kept under −75 °C until the analysis was performed several months later. Leaves were homogenized in the extraction buffer described by Arús et al. (1993). Crude extracts were electrophoresed in 14% horizontal starch gels following methods described in Bretó et al. (1993). The almond cultivars 'Ramillete' and 'Tuono' were used as controls in every analysis.

Goodness of fit between observed and expected genotype frequencies of individual genes and tests of independence between pairs of segregating loci were based on the chi-square statistic. The recombination fraction of linked loci was estimated using the maximum likelihood methods described by Allard (1956).

Table 1 Trait coding and parental mean values over years

Traits	Code	Mean values		
		Years	Ramillete	Tuono
Initial flowering	IF	3	39.3	54.7
Maximum flowering	MF	3	46.0	59.0
Final flowering	FF	3	52.0	64.7
Duration of flowering	DF	3	12.7	10.0
Flower intensity	FI	3	4.3	3.3
Maturation date	MD	2	223.5	214.5
Duration of maturation	DM	2	170.5	150.0
Yield intensity	YI	3	4.3	3.0
Percentage of failures	NF	2	2.0	0.6
In-shell weight	SW	2	3.4	2.8
Kernell weight	KW	2	1.1	1.0
Percentage of kernel	PK	2	30.6	35.7
Number of double kernel	ND	2	0.4	3.0
Rugosity of the seed	RS	2	1.0	2.5
Color of the tegument	CT	2	2.5	1.5
Self-compatibility	SC	1	No	Yes

The association of isozymatic markers and quantitative traits was studied by chi-squared contingency analysis for traits recorded as categorical variables or by one-way ANOVA and *t* contrasts of means for the others. Epistatic effects were tested by two-way ANOVA for those markers that resulted in QTL detection with any other isozymatic locus, for each year. For those traits that were shown to be associated to common marker locus, their correlation coefficients were also calculated taking the year into consideration to investigate the existence of pleiotropic effects. If the traits were recorded as categorical variables (density of flowering, color intensity, etc.), the Spearman's rank correlation was used.

Table 2 Single locus segregation analysis

Locus	Parental phenotypes	Expected ratio	Offspring phenotypes			χ^2
			aa	ab	bb	
Pgm-2	bb × ab	1:1	–	51	72	3.59
Pgi-2	bb × ab	1:1	–	56	67	0.98
Prx$_c$-2	ab × aa	1:1	72	51	–	3.59
Got-1	ab × ab	1:2:1	37	55	31	1.96
Got-2	ab × bb	1:1	–	64	59	0.20

Results

Isozymatic patterns of the four enzymatic systems assayed in the 'Ramillete' × 'Tuono' progeny (GOT, glutamate oxalacetate transaminase; PGI, phosphoglucose isomerase, PGM, phosphoglucomutase; PRX, peroxidase) are shown in Fig. 1. Segregation data (Table 2) were in agreement with the existence of five polymorphic loci, *Got-1*, *Got-2*, *Pgi-2*, *Pgm-2* and *Prx$_c$-2*. No significant departure from the expected was found, although some excess of homozy-gotes for both *Pgm-2* and *Prx$_c$-2* was found. Of the ten possible gene pairs six were examined for independence. As expected, only *Pgi-2* and *Pgm-2* showed linkage, with a recombination fraction equal to 0.1639 ± 0.0335.

For those quantitative traits measured as categorical variables, significant differences were obtained; these were mainly related to *Prx$_c$-2* (Table 3). No constancy through the years was found. To investigate the cause of this lack of constancy, homogeneity through the years per trait disregarding the marker locus genotype was studied by calculating the Spearman's correlation coefficients between pairs of years for each trait. No significant values were obtained, which suggests the existence of a G × E interaction. As an example, the highest value (0.35 ± 0.0001) was found for flower intensity between years 1989 and 1991. In general, the years 1990 and 1991 were the most different.

The probability values for genotypic differences at the marker locus of the one-way ANOVA for each trait and year are presented in Table 4. The significant differences found for 1989 usually agree with those for 1990, but they do not agree between 1991 and the other two years. The three flowering time traits are related to *Got-2* for 1989 and 1990, while 1 or several QTLs responsible for these traits and also for flowering duration are associated with *Prx$_c$-2* but not with *Got-2* for 1991. The direction of the effects of the QTL alleles is

Table 3 Probabilities of the chi-square statistic calculated to compare the distributions of traits with respect to the marker genotypes

Marker	Year	Traits				
		FI	YI	RS	CT	SC
Pgm-2	1989	0.27	0.68	0.75	0.02*	0.28
	1990	0.47	0.49	0.64	0.11	
	1991	0.28	0.93			
Pgi-2	1989	0.79	0.57	0.20	0.07	0.96
	1990	0.81	0.40	0.93	0.13	
	1991	0.25	0.35			
Prx$_c$-2	1989	0.58	0.10	0.02*	0.13	0.23
	1990	0.15	0.03*	0.27	0.03*	
	1991	0.69	0.74			
Got-1	1989	0.34	0.97	0.46	0.36	0.89
	1990	0.91	0.68	0.14	0.15	
	1991	0.62	0.30			
Got-2	1989	0.77	0.49	0.31	0.04*	0.11
	1990	0.80	0.21	0.18	0.23	
	1991	0.54	0.73			

* Probability < 0.05

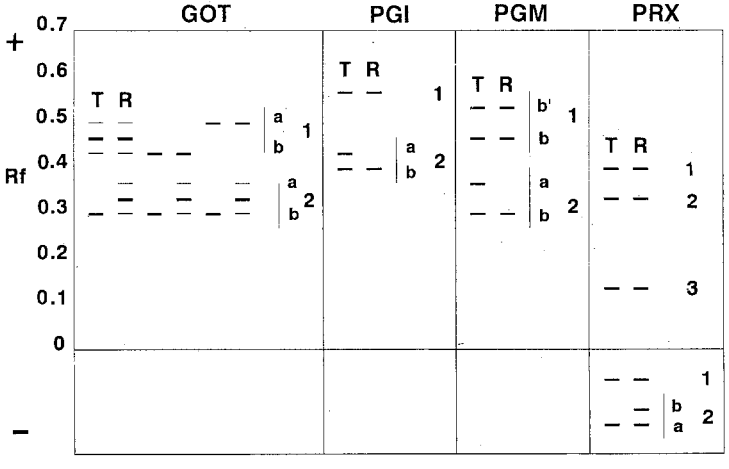

Fig. 1 Enzymatic patterns found in the segregant analysis of progeny derived from the almond cross 'Ramillete' (*R*) × 'Tuono' (*T*). Five polymorphic loci were found: *Got-1*, *Got-2*, *Pgi-2*, *Pgm-2*, and *Prx$_c$-2*. No variation was observed for PGM zone 1, PGI zone 1, PRX$_a$ zones 1, 2, and 3, and PRX$_c$ zone 1

Table 4 Probabilities of the F statistic obtained from the one-way ANOVA to test the association of the quantitative trait with a marker

Marker	Year	Traits										
		IF	MF	FF	DF	MD	DM	NF	SW	KW	PK	ND
Pgm-2	1989	0.07	0.32	0.73	0.23	0.16	0.12	0.18	0.17	0.03*	0.89	0.23
	1990	0.05*	0.10	0.51	0.06	0.57	0.45	0.61	0.28	0.02*	0.68	0.12
	1991	0.58	0.11	0.25	0.60							
Pgi-2	1989	0.08	0.06	0.72	0.23	0.21	0.16	0.87	0.05*	0.04*	0.39	0.73
	1990	0.27	0.20	0.48	0.52	0.85	0.92	0.82	0.03*	0.27	0.13	0.74
	1991	0.25	0.15	0.28	0.51							
Prx_c-2	1989	0.79	0.33	0.51	0.25	0.21	0.13	0.49	0.72	0.40	0.56	0.21
	1990	0.36	0.33	0.13	0.51	0.34	0.70	0.23	0.08	0.02*	0.01**	0.81
	1991	0.00**	0.01**	0.02*	0.04*							
Got-1	1989	0.21	0.34	0.07	0.36	0.89	0.51	0.58	0.64	0.20	0.81	0.18
	1990	0.18	0.08	0.00**	0.07	0.95	0.34	0.60	0.92	0.27	0.99	0.03*
	1991	0.05*	0.23	0.11	0.11							
Got-2	1989	0.04*	0.06	0.00**	0.08	0.48	0.05*	0.58	0.41	0.86	0.25	0.30
	1990	0.00**	0.00**	0.00**	0.91	0.22	0.03*	0.39	0.70	0.90	0.35	0.88
	1991	0.51	0.84	0.96	0.22							

*,** Probability < 0.05 and 0.01, respectively

obtained by comparing the mean value of the trait within each marker genotypic class (Table 5) for a given year. It is noteworthy that all QTLs found linked to *Got-2* show an inverse direction of their effect from the predicted with respect to the values of 'Ramillete' and 'Tuono'.

Only one significant epistatic interaction was obtained, that between *Got-2* with *Prx_c-2* for flowering duration in 1991, making the duration of flowering for those plants with the 'Ramillete' genotype at both loci last the longest.

High values of the correlation coefficient were obtained among initial, maximum, and final flowering times for every year (data not shown), and these are in agreement with previous results of Dicenta and García (1992). The highest values were obtained in 1991, where *Prx_c-2* was found to be associated to these traits. A high correlation coefficient was obtained between duration of

Table 5 Trait means for each marker genotypic class for significant associations trait-marker. Upper values refer to 1989, followed by 1990 and 1991 values

Trait	*Pgm-2*		*Pgi-2*		*Prx_c-2*		*Got-1*			*Got-2*	
	bb	ab	bb	ab	ab	aa	aa	bb	ab	ab	bb
IF	47.6	48.4			47.8	48.0	47.3	47.1	48.3	48.2	47.1*
	44.7	45.5*			44.7	45.1	44.6	44.3	45.3	45.5	44.1*
	51.5	52.0			49.9	52.5*	51.7	48.7	52.1*	50.9	51.6
MF					51.2	51.6				51.9	50.7
					48.8	49.3				49.9	47.8*
					53.7	55.8*				54.7	54.9
FF					60.9	60.5	59.7	59.6	61.5	61.5	59.4*
					56.6	57.2	56.6	55.7	57.8*	57.4	56.1*
					59.0	60.5*	60.2	58.4	60.0	59.7	59.7
DF					13.1	12.5					
					11.9	12.1					
					9.1	8.0*					
DM										157.2	160.5*
										154.6	157.5*
SW			4.12	3.57*							
			3.13	2.82*							
KW	1.48	1.36*			1.46	1.41					
	1.04	0.97*			1.06	0.98*					
PK					41.4	39.7					
					39.1	33.7*					
ND							6.6	10.3	4.7		
							5.1	8.3	3.2*		

* Years showing significant differences at $P < 0.05$ (from Table 4)

flowering and final flowering time for 1989 only, where a certain association between both traits and *Got-2* was found. A high correlation coefficient was also found between duration of flowering and initial flowering time for 1991 (and not for 1989), which was the year where both characters were observed to be associated to *Prx$_c$-2*.

Discussion

The results of our linkage analysis agree with those of Arús et al. (1993), although our estimated distance between *Pgi-2* and *Pgm-2* is approximately 3 times larger. Given that both experiments involved the same heterozygous parental line ('Tuono'), this difference in recombination fraction is more likely due to the environmental conditions under which the progeny was grown (south versus north Spain) than to the cross itself. The great variability and plasticity of *Prunus dulcis* Mill. is well known. Its hybrid origin and its sexual reproduction by seeds has allowed it to adapt to very different environmental conditions such as hot and arid south Mediterranean areas, cold and mountainous areas from center and southwestern Asia, or certain preandine areas from Argentina. Recombination through hybridization is one of the main sources of genetic variability; it shows a quantitative genetic control, displays continuous variation, and is subject to large environmental fluctuations (Fatmi et al. 1993). Therefore, the plasticity in genetic recombination of this species could have played an important role in its adaptative success.

At least 17 putative QTLs have been detected. Only 3 of them behave homogeneously through the years. Three factors may be involved in this lack of stability: (1) the test statistic used to detect the association being dependent on the character definition (categorical vs. continuous variables), (2) the contribution of the QTL to the genetic variability of the character, and (3) a differential gene expression depending on the year, i.e. due to genotype × environment (G × E) interaction. The consistency through years improved when the traits were recorded in such a way that the use of ANOVA was possible, mainly for 1989 and 1990 (see Table 4). The first two factors are closely related; in fact, power studies by Carbonell et al. (1993) have shown that the power to detect a given QTL is related to its contribution to the heritability of the trait. Thus, with respect to moderate heritabilities (see Dicenta et al. 1993a, b for values of the heritabilities), if the specific contribution of the QTL to the total genotypic variation is low, it may remain undetected. This could explain cases like RS (Table 3) and PK (Table 4). If heritability is low and the statistical test is not very powerful, it is a matter of chance that the association is detected or not. This could have happened in cases like the QTLs detected for CT (Table 3). It is important to point out that the strength of the association between a marker locus and a trait is related to both the distance between the marker and the QTL and the gene effects of the QTL. Given that the statistical test based on the "one marker at a time" methodology does not allow these causes to be distinguished (they are confounded), it is possible that the association between *Pgi-2* and KW found in 1989 (Table 4) is due to the linkage between *Pgi-2* and *Pgm-2* (the latter being associated with KW in 1989 and 1990).

Most cases in which there is a lack of stability in QTL detection involve traits whose heritability estimates change drastically from one year to another, like YI (Table 3) and ND and DF (Table 4), and/or whose correlation coefficients between years are low, like YI (from −0.14 to 0.28), KW (0.36), and DF (from 0.00 to 0.24). This suggests that there are important differences in the number and/or the relative contributions of the genes controlling the quantitative trait that are dependent on the year (G × E interaction).

It is noteworthy that the variation for 3 traits related to yield (YI, KW, and PK) in 1990 was associated to segregation at *Prx$_c$-2*. Temperatures records showed that the winter of that year (from December 1989 to February 1990) was the warmest, without any below 0 °C temperatures, followed by that of 1989. Therefore, it is not a matter of adverse conditions, such as in those cases described by Vallejos and Tanksley (1983) and Beavis et al. (1991), but a matter of G × E interaction playing an important role in the determination of the phenotypic value of the plant. G × E interaction must be considered part of the complexity of the quantitative trait itself.

Most of the associations found in 1990 are also found in 1989; however, none of the associations found in 1991 are found in 1990 or 1989 (see Table 4). The coldest winter to which the experimental plot was subjected was that from December 1990 to February 1991, during which period temperatures of −3 °C were registered. Similarly, the lowest values of the correlation coefficients per trait among years were between 1991 and 1989 or 1990 (data not shown). Winter temperature regimes affect flowering times and the duration of flowering. The way these traits become affected must be by a change in gene expression: 1 or several QTLs linked to *Got-2* are involved in IF, MF, and FF in 1989 and 1990, while other (or others) QTLs linked to *Prx$_c$-2* are specially relevant for those traits in 1991. This differential gene expression may be just a difference in the level of gene expression at both QTLs in such a way that the final result is a change in their genic effects (losing or gaining importance in their contribution to the genotypic value). This also may explain the epistatic effects detected between *Prx$_c$-2* and *Got-2* for DF in 1991 but not detected in the other years; that is, a genotype at 2 epistatic QTL-by-environment (year) interaction.

Given that a common marker was associated with several traits, correlation coefficients among those traits per year were studied. High correlation coefficients (about 0.7) were found among IF, MF, and FF for the 3 years; DF and FF for 1989 (0.71) and DF and IF for 1991 (−0.73). On the basis of data from correlation analyses

and the simultaneous change of association of IF, MF, and FF with other marker loci being dependent on the year, these traits must involve many QTL in common (QTLs with pleiotropic effects).

Although the differences are not significant in all years, the direction is the same most of the time (Table 5). Some directions inverse to those predicted were found; those involving all QTLs linked to *Got-2* and that involved in percentage of kernel linked to Prx_c-*2*. Other authors have reported similar findings in QTL analyses of F_2 populations (Bretó et al. 1994; de Vicente and Tanksley 1993) in which these QTLs were directly related to the appearance of transgressive individuals, thereby underlining their importance in plant breeding programs.

The main limiting factors of high almond yield in Spain are low temperatures at the initial flowering and developing stages of the fruit that causes plant damage and a lack of bee activity, bees being the main factors of pollination. Therefore, within the Spanish almond breeding program, improvement in traits like flowering time, duration of flowering and fruit maturation, and self-compatibility are clearly objectives to be worked upon. The results obtained suggest the following marker-assisted-selection (MAS) scheme within the 'Ramillete' × 'Tuono' progeny for late flowering and short flowering duration: (*Pgm-2*) ab, (Prx_c-*2*) aa, (*Got-1*) ab and (*Got-2*) ab, although higher values of KW, SW, PK and ND, and short duration of maturation is associated to (*Pgm-2*) bb, (*Pgi-2*) bb, (Prx_c-*2*) ab, (*Got-1*) bb and (*Got-2*) ab.

Variation in gene expression is mainly due to development, age, tissue, and environmental conditions (soil, pathogens, antipest or disease treatments, stress conditions, etc.). Therefore, it seems reasonable to deal with G × E interaction in terms of the different genes involved (or genes involved differently) in a quantitative trait depending on the weather conditions of the year. And what is most important, if QTL analysis is to be used for MAS during the breeding programs, the stability of the QTLs detected through years should also be considered, at least for perennial plant improvement. This kind of G × E interaction is well known in quantitative genetics where the importance of measuring is stressed in order to determine the optimum breeding strategy. Nowadays, it is possible not only to measure but also to uncover the differential gene expression involved in G × E interaction using the methodology developed for QTL analysis. Epistatic interactions have been shown to be very important in the design of the marker-assisted selection scheme (Bretó et al. 1994). Due to the continuously changing environmental conditions, we consider QTL analysis of the G × E interaction also essential to establish the MAS scheme and to dissect the quantitative trait itself.

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