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ORIGINAL PAPER

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Inheritance of plant regeneration from maize (*Zea mays* L.) shoot meristem cultures derived from germinated seeds and the identification of associated RAPD and SSR markers

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Abstract The inheritance of shoot regeneration through shoot-tip meristem culture derived from maize seedling was evaluated, and the markers (RAPD and SSR) associated with this regeneration character were identified both in a group of North American maize inbreds and a crossing population. A discrete distribution of percent regeneration and no. of shoots per explant was observed in the inbred group and the F_2 population. The results suggested that this regenerable trait was controlled by several major genes. Five RAPD markers were identified to be relevant to percent regeneration in maize shoot-tip culture system. One RAPD marker and three SSR markers were associated with no. of shoot per explant and its relevant traits. Of them marker BC603-1600 explained 18% of the variation for no. of shoot per explant and 16% of the variation for callus size. The BC603-1600 was sequenced and assigned in linkage group 7 based on a NCBI blast search. The information provided here should benefit to determine the genetic mechanisms involved in the maize regeneration response related to shoot meristem culture pathway and benefit to select high regenerable germplasm by using marker assisted selection.

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Introduction

Since the first success with plant regeneration from maize immature embryos (Green and Phillips 1975), the regeneration have been achieved from a numbers of different organs (Harms et al. 1976; Chang 1983; Rhodes et al. 1986; Wang 1987; Pescitelli et al. 1990; Büter et al. 1991; Zhong et al. 1992). Environmental factors, such as culture media and growth condition, strongly affect the establishment of regenerable tissue culture systems (Armstrong et al. 1992). Genetic studies of regeneration response of different genotypes have been performed with immature embryo culture and anther culture (Willman et al. 1989). Additive effects were confirmed to be more important than dominant gene effects for the number of plants regenerated, while cytoplasmic and maternal effects on regeneration were significant in backcross generations (Willman et al. 1989; Hodges 1986). In addition, relatively few genes (or block of genes) were found to be involved in dominating plant regeneration capacity (Hodges 1986; Willman et al. 1989).

To assist breeding for enhanced level of regeneration based on immature embryo and anther cultures systems restriction fragment length polymorphism (RFLP) markers linked to regeneration have been identified in maize (Armstrong et al. 1992; Wan et al. 1992; Bentolila et al. 1992; Beaumont et al. 1995). The region tagged by RFLP marker c595 on the long arm of chromosome 9 was found to highly associate with somatic embryo formation (Armstrong et al. 1992). In contrast, at least six chromosomal regions appeared to be associated with the ability of inducing embryo-like structures from maize microspores. Although RFLP marker-based selection are considered to be the most useful type of descriptors due to the high degree of polymorphism that is detected by this technique in corn the labor-intensive and time-consuming nature of the RFLP assay may not make this feasible (Vuylsteke et al. 2000).

Recently, PCR-based molecular markers, such as random amplified polymorphic DNA (RAPD) makers and simple sequence repeat (SSR) markers were appar-

ently emphasized in marker associated selection and genetic diversity analyses because they possess the potential of reducing time, effort and expense required for molecular mapping. RAPD markers have been used for a variety of purposes including the construction of genetic linkage maps (Reiter et al. 1992), gene tagging, identification of cultivars (Nybom 1994), assessment of genetic variation in populations and species (Nesbitt et al. 1995) and to identify the desired genotypes during selection. However, there is some loss of information when RAPD markers are used because they are dominant rather than co-dominant (Sun et al. 2001). SSR marker is ubiquitous in eukaryotic genomes and its utility has greatly been facilitated by recent advances in PCR technology. The high level of polymorphism, relative to RFLPs and RAPDs, combined with a high interspersion rate make them an abundant source of genetic markers (Gupta et al. 1999). SSR loci are highly polymorphism in corn and SSR analyses possesses potential advantages of reliability, reproducibility, discrimination, standardization and cost-effectiveness over RFLP analyses (Senior and Heun 1993; Smith et al. 1997).

A multi-shoot culture system has been developed in maize based on seedling apical cultures (Zhong et al. 1992; Li et al. 2002), characterized with simplicity and feasibility to a broad range of genotypes. There is a potential to use it as an effective target for maize transformation (Zhong et al. 1996). However, according to our earlier report (Li et al. 2002), The capacity of plant regeneration through this system was still genetically determined, indicating significant difference among genotypes (from 0% to 100% of regeneration). The genetic knowledge of the regeneration trait based on multi-shoot culture and the discovery of molecular markers associated with it will have great advantages for efficiently developing elite inbred with increased regeneration capacity by increasing the precision with which genotypes can be selected. But no information exists so far regarding the inheritance of tissue culture response from maize shoot meristem cultures as well as the association with molecular markers.

The objectives of the presented study were to assess the inheritance of plant regeneration from maize shoot meristem culture and to identify molecular markers associated with plant regeneration based in this tissue culture system.

Materials and methods

Plant materials and tissue culture procedure

Forty-five typical corn inbreds from University of Guelph and a F_2 population of 150 individuals developed from a cross between CG-37 (high regeneration response) and CG-44 (poor regeneration response) were used in this study. The seeds were surface sterilized in 70% (v/v) ethanol for 5 min, soaked in 50% commercial bleach (5.5% sodium hypochlorite) with 0.1% Tween 20 for 25 min and rinsed five times with sterile, distilled water. Following surface sterilization, the seeds were germinated in Petri plates (100 mm×25 mm) on MS0 medium consisting of MS (Murashige and Skoog 1962) basal medium, MS vitamins, 0.7% Phytagar (Gibco Labs, Grand Island, NY) in the dark, at 25°C for seven days. Coleoptilar nodes of 3–5 mm long, containing a meristematic region, which was identified by the swelling that occurs at the junction of the mesocotyl and epicotyl were removed from seedlings that were 4–5 cm long. The explants were cultured on multi-shoot induction medium (MSI). The medium consisted of MS basal medium and MS vitamins with 4 g/l proline, 2 mg/l BA, 1 mg/l 2,4-D, 3% sucrose and 0.7% Phytagar. Three weeks later, cultures were transferred onto MSE medium (MS basal medium plus MS vitamins and 3% sucrose, 0.5 mg/l BA, 0.7% Phytagar) to allow somatic embryo-like structure maturation and shoot elongation. Two weeks later, the multi-shoot clumps were transferred onto MSR medium which was identical to MSE medium except that the BA was replaced by 1 mg/l indole-3-butyric acid (IBA) to enhance the development of a root system (Li et al. 2002). Two to four weeks afterward, shoots with a large root mass were transplanted into pots containing Turface (Applied Industrial Materials Corp., Buffalo Grove, IL) and fertilized daily as described by Earl and Tollenaar (1997).

PCR procedure

DNA was extracted from 100 mg of leaf tissue using high throughput method of QIAGEN Dneasy 96 Plant Test Kit. Twenty eight 10-mer RAPD primers from UBC (University of British Columbia, Canada) were used. The amplifications were performed in a 20 μl reaction volume containing 0.2 mM of dNTP, 2.5 mM $MgCl_2$, 1.0 U Taq polymerase, 15 pmol of primer and 20 ng of template DNA. The PCR program consisted of 30 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 3 min. The PCR products were electrophoresed through 1.2% agarose gel, stained with ethidium bromide and visualized with ultraviolet light. The presence or absence of clear DNA fragments was scored for each sample.

Thirty-four pairs of corn SSR primers were used to genotype F_2 population. PCR amplification was performed in a 20 μl reaction volume, containing 0.2 mM of dNTP, 2.0 mM $MgCl_2$, 1.5 U Taq polymerase, 15 pmol of each primer and 30 ng of template DNA. The mixture was overlaid with mineral oil and subjected to PCR on a PTC-100TM thermal cycler (MJ Research Inc) using a 'touch-down' PCR program consisting of 18 cycles of denaturation at 95°C for 1 min and extension at 72°C for 2 min. The annealing temperature (1 min) was progressively decreased by 1°C every third cycle from 65°C to 55°C. The PCR reaction continued for 20 additional cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min, following a 10 min final extension at 72°C. The PCR products were analyzed on a 4% Metaphor agarose gel, stained with ethidium bromide, visualized with ultraviolet light and photographed.

Data analysis

Twenty typical genotypes were separated into high and low regeneration groups. A t-test was used to determine markers that were significantly correlated with the regeneration ability for the seedling apical meristem. Single marker analysis was carried out using a standard one-way ANOVA (PROC.GLM.SAS) for F_2 population data. GT(Genotype by Trait) biplot methodology (Yan 2001) was also employed to analyze the interaction between molecular markers and regeneration-relevant traits in F_2 generation, been based on the formula: $T_{ij}-T_j/S_j=\lambda_1\zeta_{i1}\tau_{j1}+\lambda_2\zeta_{i2}\tau_{j2}+\varepsilon_{ij}$, where T_{ij} is the average value of genotype i for trait or marker j; T_j is the average value of trait or marker j over all genotypes, Sj is the standard deviation of trait or marker j among the genotype average; ζ_{i1} and ζ_{i2} are the PC1 (first principle component) and PC2 (second principle component) scores, respectively, for genotype i; τ_{j1} and τ_{j2} are the PC1 and PC2 scores, respectively, for trait or marker j; and ε_{ij} is the residual of the model associated with the genotype i in trait

or marker j. Broad-sense heritability was calculated according to the formula: $h^2=[V_{F2}-(V_{P1}+V_{P2}+V_{F1})/3]/V_{F2}$. V_{F2} is the phenotypic variance of F_2 population; V_{P1} and V_{P2} are the error variance of parents; V_{F1} is the error variance of F_1 population.

Results

Inheritance of plant regeneration

Forty-five North America corn genotypes were screened for plant regeneration in a modified shoot-tip meristem culture procedure (Li et al. 2002). A large diversity in the percentage of regeneration was observed among the North American inbreds tested. The histogram of the percent regeneration seemed to be divided into discrete categories, including four response groups centered around 10%, 40%, 75% and 100% of regeneration (Fig. 1). The finding indicate that the frequency of plant regeneration from seedling meristem may be controlled by a few genes rather than many genes.

Figure 2 revealed a F_2 frequency distribution of no. of multi-shoots per explant from a crossing between a high regeneration responder CG-37 and low regeneration responder CG-44. In Fig. 2, F_1 hybrids expressed inter-parent phenotype, close to a semi-dominant pattern. A transgressive variation in F_2 was noticed, including non-responders, individuals that responded like the highly

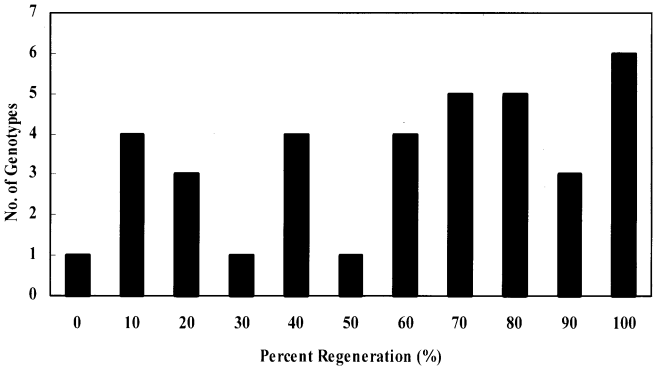

Fig. 1 The histogram of percent regeneration from shoot apical meristem of twenty maize inbreds collected from North American

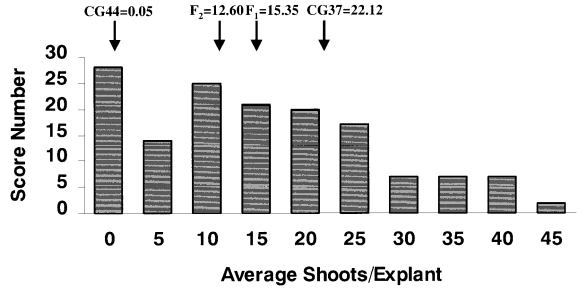

Fig. 2 Frequency distribution of no. of shoots per explant in F_2 generation (CG-37×CG-44). The arrows showed the average shoots number per explant for CG-37 (female parent), CG-44 (male parent) and F1 generation

Table 1 RAPD markers significantly associated with regeneration or lack of regeneration in a collection of North American maize inbreds

Inbreds	Regeneration >25%							Regeneration <25%							Marker Frequency		Differency
	C88	C74	C69	C99	C59	C62	C103	C102	C97	C94	C90	C95	C33	C89	C91	C39	C96	C64	C36	C44a	>25%	<25%	
% Regeneration	100	100	100	86	85	83	82	76	74	70	63	60	49	46	48	44	25	20	12	0.04			
RAPD Marker																							
BC625–2000	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	0	0	0.93	0.25	0.68**
BC646–1800	1	0	0	0	0	1	1	0	1	0	1	1	1	0	1	1	0	0	0	0	0.56	0	0.56*
BC302–500	0	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	1	1	1	1	0.25	1.00	–0.75**
BC603–250	1	0	1	1	0	0	0	0	1	0	0	0	0	1	1	0	1	1	1	1	0.31	1.00	–0.69**
BC631–800	0	0	0	1	1	0	0	0	0	0	0	1	1	1	0	0	1	1	0	1	0.25	0.75	–0.50*
Marker Index[b]	0.7	0.2	0.2	–0.5	–0.2	0.7	1	0.2	0.2	0.5	1	0.7	0.7	–0.2	0.7	1	–1	–0.5	–1	–1			

** P<0.01, * P<0.05; [a] C=CG; [b] Marker Index=[Markers associated with regeneration]–[Markers associated with a lack of regeneration]/Total numbers

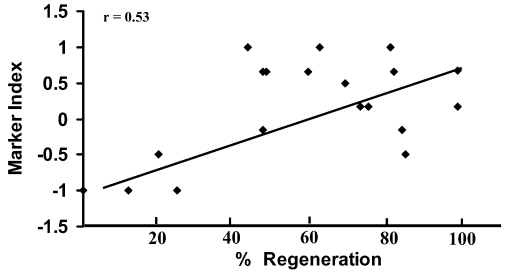

Fig. 3 Correlation of marker index and percent regeneration for twenty maize inbreds collected from North American

regenerable parent (CG-37) and individuals that produced twice as many shoots per explant as CG-37. The broad-sense heritability (h^2) of this trait was calculated as 68.57% ($P<0.05$). These results further suggest that the regeneration efficiency in this system is controlled by a few genes.

Identification of RAPD markers associated with percent regeneration through 20 North American corn inbreds

Twenty eight RAPD primers chosen generated 161 RAPD fragments based on 20 corn inbreds with an average of six bands per primer. The size of amplified products ranged from 200 to 2000 base pairs. The correlation analysis showed that five out of 121 markers tested were significantly associated with the percentage of explants that produce multi-shoots (Table 1). Two markers (625-2000 and 646-1800) were associated with regeneration, whereas, three others (303-500, 603-250 and 631-800) were associated with a lack of regeneration. Generally, a higher marker index represented a higher percentage of plant regeneration (Fig. 3).

Identification of RAPD and SSR markers associated with regeneration in F2 population

A significant correlation ($r=0.68$, $P<0.01$) between no. of multi-shoots per explant and callus size (cm^2) was observed. Therefore, callus size and no. of multi-shoots per explant were facilitated to evaluate the association with RAPD and SSR markers. Five RAPD primers from Table 1 were used to screen the F_2 population (CG37×CG-44). QTL analysis showed that one RAPD marker (BC603-1600) was significantly associated with

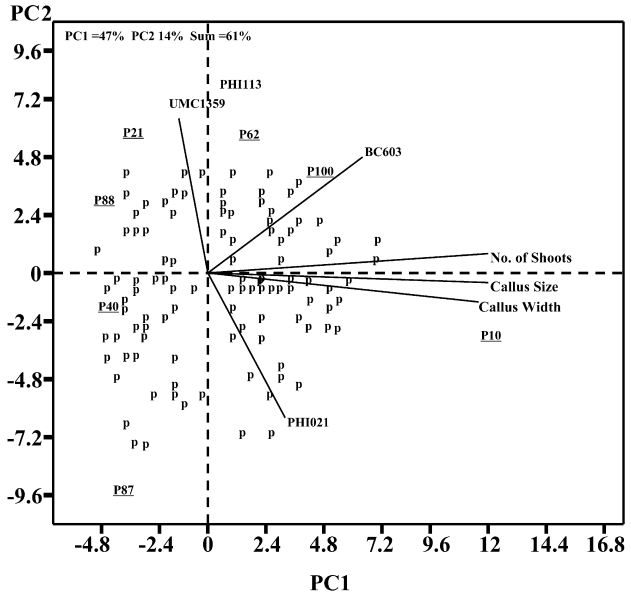

Fig. 4 GT biplot analysis for relationship among selected markers and regeneration-relevant traits. PC1: first principle component; PC2: second principle component; P and p representing an individual in F_2 generation

no. of shoots per explant and callus size based on F_2 data. This QTL explained 16–18% of the phenotypic variance for callus size and no. of multi-shoots per explant, respectively (Table 2).

According to the results of corn marker screening project in University of Guelph (unpublished data), 33 pairs of SSR primers that contributed a high polymorphism were used for screening F_2 population. One SSR marker, umc1359 which located on chromosome 8, was associated with no. of multi-shoots per explant ($R^2=0.05$, $P<0.05$) (Table 2). Two SSR markers, Phi021 and Phi113 located on chromosome 4 and 5, were associated with callus size ($R^2=0.04$ and 0.05, respectively; $P<0.05$) (Table 2).

GT biplot analysis for 150 F_2 individuals against one RAPD marker, three SSR markers and three traits that associated with regeneration explained 61% the total variation of the standardized data (Fig. 4). The largest variation explained by the biplot came from no. of shoot and its relevant traits, as indicated by the relative length of their vectors. The correlation coefficient between any two traits or markers is approximated by the cosine of the angle between their vectors. Thus, $\cos 180°=-1$, $\cos 0°=1$ and $\cos 90°=0$. In Fig. 4, a stronger positive association

Table 2 Marker contribution for regeneration efficiency in F_2 population

Traits	Markers	Chromosome	R^2	P
# Shoots/Explant	BC603–1600	7 s	0.18	0.001
	UMC1359$(TC)_{12}$	8 s	0.05	0.04
Callus Size (cm^2)	BC603–1600	7 s	0.16	0.001
	Phi 021$(AG)_n$	4 s	0.04	0.04
	Phi 113$(GTCT)_n$	5 s	0.05	0.03

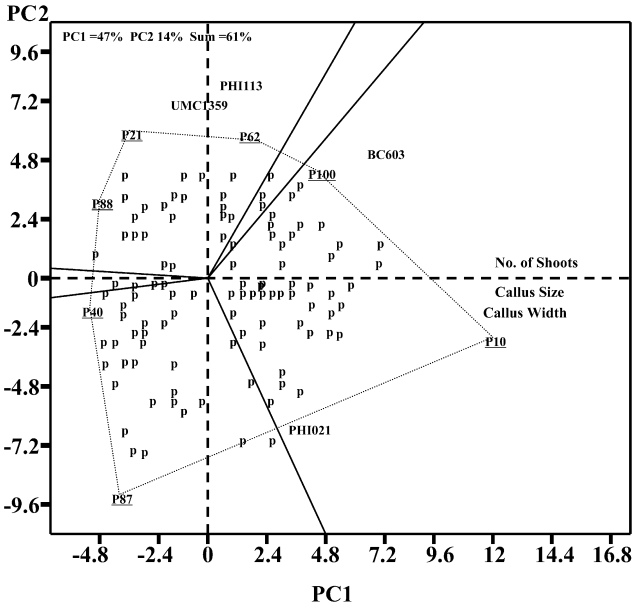

Fig. 5 GT biplot analysis for best individuals in each given marker groups. PC1: first principle component; PC2: second principle component. P and p: representing an individual in F_2 population

between regeneration-relevant traits and RAPD marker BC603-1600 existed rather than the other markers. Furthermore, the performance of different individuals in each group of markers was evaluated. With individual P10, P87, P88, P40, P21, P62, P100 as the corner or vertex individuals, marker BC603-1600 and traits relevant to regeneration fell in the sector in which P10 was the vertex individual. This means that P10 was the best individual for BC603-1600 and its associated regeneration-relevant traits. P21 and P62 were the best for marker Phi113 and umc1359. No marker fell into sectors with P87 and P88 as vectors, indicating that these individuals were not the best in any of the markers. They are the poorest individuals for any markers (Fig. 5).

Discussion

Plant regeneration capacity is a critical factor for effective practice of corn engineering. As far as we know, the description of a genetic component of regenerable traits expressed in a corn shoot-tip culture procedure has not been reported in the literature, neither genetic marker associated with these traits.

Two F_2 populations were developed from two typical North American inbreds, in which one parent (CG-37) possesses 100% regeneration ability and average 30 shoots per explant, and another parent only possesses 0.04% regeneration ability and 1 shoot per explant averagely. Theoretically, a maximum variation for regeneration traits will be provided from this F_2 population. Each of the populations contained more than 150 individuals and was analyzed for the frequency distribution of no. of multi-shoots induced on shoot meristem tissues. Results from both populations exhibited a similar distribution pattern (data not shown). Along with evidence of a relatively higher heritability in this study, the trait is likely to be a discrete category. However, X^2 tests, including one gene and two gene models, did not support the hypothesis of one gene or two gene segregation (data not shown). Thus, our results suggest that the no. of multi-shoots per explant is controlled by several major genes. This correspond well to the postulation made from maize anther culture, maize immature embryo culture, rice immature embryo culture and wheat immature embryo culture (Bentolila et al. 1992; Hodges 1986; Willman et al. 1989; Peng and Hodges 1989; Lazar et al. 1984).

In present study, five RAPD markers were identified to be associated with percent regeneration trait based on elite maize inbreds. Relatively high reproducibility for RAPD markers was observed throughout recent experiments, which was not identical with certain reports that described the poor reliability and repeatability of RAPD markers (Nagaoka and Ogihara 1997; Ellsworth et al. 1993). In our study, the RAPD markers identified to be relevant to percent regeneration from corn inbreds germplasm were different with the markers that associated with the no. of multi-shoot per explant and callus size in F_2 generation. There could be number of reasons for this phenomenon, including that the two traits (percent regeneration (%) and no. of regenerated shoots) could be controlled by different genetic factors; could have different genetic background and diversity between the F_2 population and the 20 corn inbreds. The surveying of molecular markers associated with percent regeneration in F_2 generation was impossible because the data in F2 was generated according to individual basis. This problem can be solved by the establishment of high generation inbred lines.

In F_2 population, one RAPD marker and three SSR markers that were confirmed to associate either with no. of multi-shoots per explant or callus size were located on four different genetic regions. The most prominent QTL effect for no. of multi-shoots per explant and callus size was pointed on linkage group 7. They explained 18% and 16% of the total variation, respectively. The DNA sequence of BC603-1600 marker had more than 90 percentage of homology with partial sequence of subfamily z1B-1 of 19-KD zein gene family in maize according to NCBI blast search result. Z1B-1 has been mapped in linkage group 7 (http://www.agron.Missouri.edu/mnl/77/42song.html) (Fig. 6). Zein genes in maize endosperm control the synthesis of storage proteins which are rich in the amino acids proline and glycine (Song and Messing 2002). According to our previous study (Li et al. 2002) proline has a positive impact on plant regeneration from shoot-tip meristem culture. However, we need more evidences to explain the mechanism of BC603-1600 on regeneration response. Other markers, UMC1359, Phi021 and Phi1134, were located on linkage group 8, 4 and 5, respectively (Fig. 6), explaining 5%, 4% and 5% of total variation, respectively

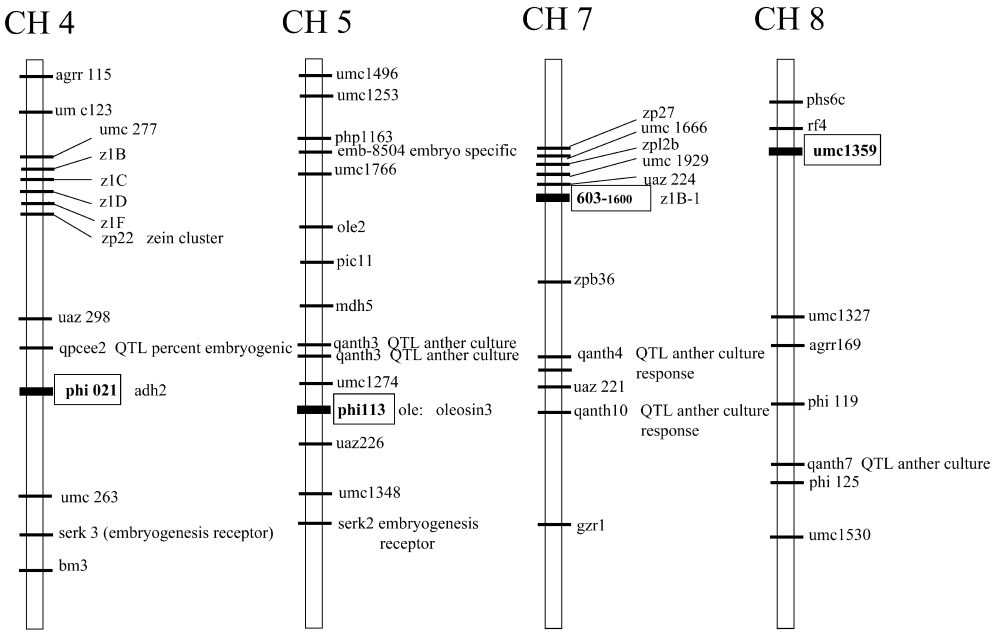

Fig. 6 Partial maize linkage groups containing molecular markers associated with plant regeneration via shoot apical culture, anther culture and immature embryo culture. Modified from NCBI gene database

(Table 2). All markers together accounted for 23% of the total phenotypic variation for no. of multi-shoot per explant and for 25% of the total phenotypic variation for callus size observed among the F_2 population.

The linkage group map in Fig. 6 was created based on NCBI DNA database. In addition of the RAPD and SSR markers that was identified to associate with regeneration capacity of maize shoot meristem culture in the present study there also had several molecular loci that was mapped by previous researchers and was relevant to anther culture response and embryogenesis response based on immature embryo culture (Fig. 6). In linkage group 4, 5 and 7, QTL percent embryogenesis loci and QTL anther culture response loci (qanth4, qanth10, qanth3 and qanth6) were relatively closer to the multi-shoot regeneration-relevant loci Phi021, 603-1600 and Phi113 (Fig. 6). Further attempt is necessary to determine the genetic distance between shoot regeneration-relevant loci and anther/embryo regeneration-relevant loci.

This study demonstrated that GT biplot by Yan (2001) is an excellent tool for visualizing genotype by marker and trait data. It effectively reveals the interrelationships among traits and molecular markers, along with the different genotypes. In GT biplot, the variation of marker or trait is represented by the length of relative length of their vectors and the correlation between different marker or trait is calculated by the cosine. Therefore, a quick view of the variation and association between various characters become possible. Moreover, the performance of different genotype or individual in a given marker or trait can be visual on biplot graph, so that it is easier to group genotypes into different categories and to find the best genotype in a given marker or trait.

Molecular marker assisted selection (MAS) have been widely used in plant breeding program. Selecting high regenerable lines could be high input of labor and energy through conventional breeding methodology due to the complex of tissue culture. With the technique and procedure of using molecular markers, the development of high regenerable lines and agronomical improved lines will be feasible in a much shorter time and less expense than by traditional breeding. The information provided here should benefit to determine the genetic mechanisms involved in the maize regeneration response related to shoot meristem culture pathway and benefit to select high regenerable germplasm by using marker assisted selection although needing a large population due to the relatively low resolution from recently identified markers. Further studies may be attempted to identify more molecular markers responsible for plant regeneration based on this tissue culture system to map the QTL in maize linkage groups.

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