High-density linkage map around the root aerenchyma locus *Qaer1.06* in the backcross populations of maize Mi29 × teosinte “*Zea nicaraguensis*”

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The teosinte *Zea nicaraguensis* forms constitutive aerenchyma in the root cortex as observed in flooding tolerant wetland plants. We have previously identified a quantitative trait locus (QTL) controlling aerenchyma formation under non-flooding conditions on chromosome 1 (*Qaer1.06*) using 214 individuals of a maize Mi29 × *Z. nicaraguensis* BC₂F₁ population. The present studies objective was to increase the marker density around the *Qaer1.06* locus, which is essential for the development of near-isogenic lines possessing the aerenchyma-forming gene with only a small region of the *Z. nicaraguensis* chromosome segment. A survey of 62 SSR and 38 insertion/deletion (INDEL) markers identified a total of 36 useful markers in the region of interest. These were selected for the construction of a linkage map in a 214 individual BC₂F₁ and a 123 individual BC₄F₁ population from a cross between Mi29 × *Z. nicaraguensis*. Using the BC₄F₁ population, we performed QTL mapping and the results indicated that a QTL for aerenchyma formation under non-flooding condition was located on chromosome 1 (bin 1.05) at a position that is near to *Qaer1.06*. The markers obtained here should be useful for the development of high quality near-isogenic lines possessing the *Qaer1.05-6*.

Key Words: aerenchyma, flooding, near-isogenic line, maize, quantitative trait locus, teosinte.

Introduction

Aerenchymatous root in plants perform an important role for adaptation in oxygen-deficient flooded or waterlogged soils because oxygen is provided to the root tip by diffusion through the aerenchyma. Maize (*Zea mays* L. ssp. *mays*) forms lysigenous aerenchyma in the cortical cells of roots following hypoxia or low oxygen levels during flooding, and physiological and molecular mechanisms associated to this character have been widely investigated (Jackson *et al.* 1985a, He *et al.* 1996, Saab and Sachs 1996, Gunawardena *et al.* 2001, Subbaiah and Sachs 2003). This flooding-inducible aerenchyma formation is also observed in upland crops such as barley (Pang *et al.* 2004), wheat (Huang *et al.* 1994, Watkin *et al.* 1998) and triticale (Watkin *et al.* 1998), and several reports suggest that flooding tolerant varieties possess larger inducible aerenchyma compared to intolerant varieties (Boru *et al.* 2003, Setter and Waters 2003, Pang *et al.* 2004, Zaidi *et al.* 2007). From an evaluation of over 4,000 accessions, Takeda (1989) has identified and selected barley genotypes that exhibit a superior tolerance to flooded conditions and the tolerant genotypes abundantly developed aerenchymatous adventitious roots (Stanca *et al.* 2003).

The teosintes represent a wild relative of maize typically found in regions of Mexico, Guatemala, Honduras and

Nicaragua that receive frequent levels of precipitation could be a valuable germplasm resource for developing flooding tolerant maize (Bird 2000, Iltis and Benz 2000, Mano and Omori 2007). The study of the teosintes also will enhance the understanding of the mechanism of aerenchyma formation because hybridization between non aerenchyma-forming maize and aerenchyma-forming teosinte are easily generated.

Among the several teosintes classified by Doebley and Iltis (1980), Iltis and Doebley (1980) and Iltis and Benz (2000), constitutive aerenchyma that develop in the absence of hypoxia or low oxygen is expressed in *Z. luxurians* and *Z. nicaraguensis* in well-aerated greenhouse environments (Ray *et al.* 1999, Mano *et al.* 2006) and in upland field conditions (Y. Mano, unpublished). A similar situation is known with flooding tolerant rice (Jackson *et al.* 1985b, Colmer 2003) and other wetland species (Smirnov and Crawford 1983, Justin and Armstrong 1987). Constitutive aerenchyma is typically in these species; however, during flooding conditions, the degree of aerenchyma in rice and teosinte is increased and additional complete (functional) aerenchyma channels can be developed more rapidly compared to inducible aerenchyma-forming upland plants. Therefore, constitutive aerenchyma in plants is an advantageous consequence for adaptation to temporary flooding conditions (Colmer and Voesenek 2009).

In prior studies, we have focused on the presence of constitutive aerenchyma formation in teosinte *Z. nicaraguensis*. In quantitative trait locus (QTL) analyses to determine the

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capacity of constitutive aerenchyma formation, using teosintes *Z. nicaraguensis* and *Z. luxurians*, we have recently identified QTLs associated to aerenchyma formation (Mano *et al.* 2007, 2008). Therefore, the transfer of aerenchyma formation gene(s) from teosinte to maize can be achieved via molecular marker-assisted backcrossing program. Once the experimental material (e.g. isogenic line) is obtained, transcriptome analyses will be undertaken to reveal the mechanism of aerenchyma formation. This approach has been applied to identify candidate gene associated with maize root responses towards environmental stimuli (Hochholdinger and Tuberosa 2009).

In a previous study using a 214 BC₂F₁ individual population of maize Mi29 × *Z. nicaraguensis*, a QTL for aerenchyma formation under non-flooding conditions was located to chromosome 1 (bin 1.06) and designated as *Qaer1.06*. The objectives of this study were (1) to increase molecular markers around the aerenchyma locus *Qaer1.06*; (2) to verify an effect of the *Qaer1.06* in the genetic background of maize Mi29; and (3) to search for an additional QTL for aerenchyma formation other than the *Qaer1.06*. These results will be essential to determine a precise position of the *Qaer1.06*, develop high-quality near-isogenic line possessing the *Qaer1.06* useful for gene isolation as well as the development of flooding tolerant maize by marker-assisted selection.

Materials and Methods

Plant materials

Maize inbred Mi29, a line that does not form constitutive aerenchyma, and the teosinte *Z. nicaraguensis* (CIMMYT 13451) were crossed to form the initial F₁ hybrid and populations. Because *Z. nicaraguensis* is an outcrossing species, it was self-pollinated twice prior to crossing to Mi29, in order to form a more stable aerenchyma-forming S2 generation for use in the genetic analysis.

For the marker survey and construction of a high-density linkage map, an Mi29 × *Z. nicaraguensis* 214 individual BC₂F₁ population (Mano and Omori 2008) was used for the analysis. A single F₁ plant derived from a cross between Mi29 × *Z. nicaraguensis* was backcrossed to Mi29 and 42 BC₁F₁ plants were obtained. These 42 BC₁F₁ plants were backcrossed using Mi29 as the pollen parent to produce BC₂F₁ seeds. 214 BC₂F₁ individuals grown from 4–6 BC₂F₁ seeds from each of the 42 BC₂F₁ spikes were developed.

To generate the BC₄F₁ population, the same F₁ plant that was used for the development of the 214 BC₂F₁ population, was crossed to Mi29. A single BC₁F₁ plant was backcrossed to Mi29 three times to produce the BC₄F₁ seeds. During the backcross process, SSR markers bnl1832 (bin1.05), umc1128 (1.07) and their flanking markers that bracket 45 cM around the *Qaer1.06* region were used in the selection process. Figure 1 shows the graphical genotype of a BC₃F₁ plant used to develop the BC₄F₁ mapping population. The frequency of heterozygous genotypes (Mi29/*Z. nicaraguensis*; shadowed regions) was 0.225 (data not shown), which is

Fig. 1. Graphical genotype of a single BC₃F₁ plant used to develop the BC₄F₁ mapping population of the cross between Mi29 × *Z. nicaraguensis*. Shadowed regions indicate the fragments of heterozygous genotypes (Mi29/*Z. nicaraguensis*). Map distance is based on the study of Mano and Omori (2008).

higher compared to the expected value of 0.125 in the BC₃F₁ generation.

Root anatomy

A total of 123 BC₄F₁ individuals were grown in a greenhouse maintained at a temperature of 30°C day/25°C night with natural light at 13–14 hours day length. The degree of constitutive aerenchyma in the root cortex of six-leaf stage seedlings that grew under non-flooding condition was visually scored: 0 (no aerenchyma), 0.5 (partial formation), 1 (radial formation) and 2 (radial formation extended toward epidermis) as described in Mano *et al.* (2006). In addition, because the variation for the trait in the backcrossed population was not large, we used a more discrete scoring rating (i.e. 0.25, 0.75 and 1.5) to generate more accurate trait evaluation data. For the QTL analysis, we averaged evaluation scores at 10 and 15 cm from the root tips of two adventitious roots per individual for a total of 4 positions per individual.

Survey of useful markers around the *Qaer1.06*

Approximately 1–4 µg of DNA was isolated from 50 mg of fresh leaf tissue using the method described by Komatsuda *et al.* (1998). Based on maize GDB (<http://www.maizegdb.org/>), we selected 62 SSR markers and 38 insertion/deletion (INDEL) markers in the vicinity of the *Qaer1.06* region from bin 1.05 to 1.07.

To detect polymorphisms in the SSR and INDEL markers, 48 BC₂F₁ individuals were tested for each primer pair because the *Z. nicaraguensis* used in this experiment was not a pure line and amplicon size could differ within *Z. nicaraguensis*. The SSR analysis was performed as

described by Mano *et al.* (2005).

For the INDEL marker analysis, all the forward primers were 5-end labeled with fluorescence dye FAM or HEX. Reaction mixtures contained 20 ng of genomic DNA, 0.25 μ M of fluorescently labeled forward and unlabeled reverse primers, 200 μ M dNTPs, 3.5 mM $MgCl_2$ (final concentration), 0.25 units of Taq DNA polymerase (Qiagen GmbH, Hilden, Germany) and its corresponding reaction buffer with Q-solution for a total volume of 10 μ L. The amplification conditions described in the MaizeGDB were followed: 3 min at 94°C followed by 30 cycles of 30 s at 94°C, 45 s at 60°C and 90 s at 72°C, and a final extension at 72°C for 10 min. PCR products were separated on a 20 cm, 5% denaturing polyacrylamide gel at 360 V for 1 h 15 min and then scanned in the Molecular Imager (Bio Rad, Hercules, CA).

Map construction

In the BC_2F_1 population, markers were grouped according to a two-point analysis with $LOD > 3.0$ with a recombination fraction of 0.25. The order of the markers was determined by the “three-point” and “order” command, first at $LOD > 3.0$, then at $LOD > 2.0$ using MAPMAKER/EXP 3.0 (Lander *et al.* 1987). Map distance (Haldane units) was computed in the BC_2F_1 model using the QGene program (Nelson 1997). In the BC_4F_1 population, the same procedure was applied for the construction of a linkage map with the single exception that the map distance was computed in the BC_1F_1 model using the MAPMAKER/EXP 3.0.

QTL analysis

In the BC_4F_1 population, composite interval mapping (CIM) was applied to map the QTLs controlling aerenchyma formation in the BC_1F_1 model using the software package Windows QTL Cartographer Version 2.5 (Wang *et al.* 2006). CIM was run with 2 cM walk speed applying the default parameters (model 6; 5 for control markers, 10 cM for window size and forward regression method) in the program. The experiment-wise significance threshold level was defined as the 50th highest LOD value of 2.0 by running 1,000 permutations (Churchill and Doerge 1994), corresponding to an experiment-wise Type-I error rate of 0.05.

Results

Aerenchyma formation

The average aerenchyma scores were 0.0 ± 0.0 (mean $\pm$ standard deviation) for Mi29 ($n = 10$) and 2.0 ± 0.1 for *Z. nicaraguensis* ($n = 10$) (Fig. 2). Scores of the BC_4F_1 individuals had a continuous distribution (average 0.2 ± 0.2 , Fig. 3) and a segregant with high degree of aerenchyma that was equivalent to *Z. nicaraguensis* was not observed (Fig. 2C and Fig. 2D).

Survey of useful markers around the *Qaer1.06*

Of the 62 SSR tested, 24 (38.7%) showed clear polymorphism between Mi29 and *Z. nicaraguensis*. Another 19

Fig. 2. Cross sections of adventitious roots at 10-15 cm from the root tip that emerged from the second node in six-leaf stage seedlings in non-flooding condition. Presence of aerenchyma is shown in (A) *Z. nicaraguensis* (score 2), (C) BC_4F_1 #10 (score 0.5) and (D) BC_4F_1 #16 (score 1) and the lack of aerenchyma in (B) Mi29 (score 0).

Fig. 3. Frequency distribution for aerenchyma formation in the Mi29 $\times$ *Z. nicaraguensis* BC_4F_1 population. Average score of two adventitious roots at 10 cm and 15 cm from the root tip (four positions in total) were used to determine the value for each BC_4F_1 individual.

(30.6%) showed only a monomorphic fragment and the remaining 19 (30.6%) gave no or only weak amplifications. For the 38 INDEL marker analyses, 12 (31.6%) showed clear polymorphism between the parents, 14 (36.8%) showed only a monomorphic fragment and 12 (31.6%) did not show any clear amplifications. In the marker survey, a total of 36 useful markers (24 SSRs and 12 INDELs) were identified and utilized for further mapping analyses.

Map construction

We constructed a linkage map of the 214 individual Mi29

Fig. 4. Linkage map of the BC₄F₁ (A), BC₂F₁ (B; this study) and BC₂F₁ (C; Mano and Omori 2008) populations of the cross between *Mi29* × *Z. nicaraguensis*. Short arms of the chromosome are on top. The scale is in centimorgans (Haldane units). Closed arrowheads indicate the position of the peak LOD. Bin numbers, traditional methods for referring to the location/position of markers, are in parentheses before marker names.

× *Z. nicaraguensis* BC₂F₁ population for the *Qaer1.06* region of chromosome 1 by using the 36 SSR and INDEL markers together with the previous mapped 6 SSR markers. The target region of the *Qaer1.06*, from bnlgl1832 (bin 1.05)–umc1128 (1.07) covered 46.9 cM at an average interval of 1.1 cM per marker (Fig. 4B). The map distance of 46.9 cM was very close to that using only the 6 SSR markers of the same mapping population at 46.5 cM (Fig. 4C, Mano and Omori 2008). These results suggested the absence of double recombination and the genotyping of the markers was accurate.

In the BC₄F₁ population, we also constructed a linkage map using the newly identified 36 markers and previous mapped 6 SSRs. The order of markers corresponded well

across the two maps. The map distance of 17.6 cM from bnlgl1832–umc1128 was shorter (37.5%) and the recombination was strongly suppressed compared to the BC₂F₁ map of 46.9 cM (Fig. 4A and Fig. 4B). The suppression was also found in the region for bin 1.07 to 1.12 (50.4 cM in BC₂F₁ vs. 27.1 cM in BC₄F₁; data not shown).

Mapping QTL for aerenchyma formation in the BC₄F₁ population

Through CIM analysis, a QTL for aerenchyma formation was identified at umc1297–IDP5918 (bin 1.05, LOD = 8.2, effect = 0.25), which explained 25% of the total phenotypic variance (Table 1). *Z. nicaraguensis*, with high degree of aerenchyma, contributed to the QTL. Distance of LOD peak

Table 1. Chromosome locations and mode of gene action of QTLs for root aerenchyma formation estimated by composite interval mapping (CIM) in the BC₄F₁ population of the cross between Mi29 × *Z. nicaraguensis*

QTL	Chr	Bin	Position ^a	Marker interval	LOD ^c	Effect ^d	Dir ^e	Var ^f
<i>Qaer1.05-6</i>	1	1.05	45 (36–47) ^b	umc1297–IDP5918	8.2	0.25	N	0.25
<i>Qaer8.05</i>	8	8.05	0 (0–12)	umc1712–bnlg162	2.6	0.17	N	0.07
							Total	0.31

^a QTL position in cM from the top of the segregating markers. bnlg1484 (bin 1.03) for chromosome 1 and umc1712 (8.05) for chromosome 8.

^b 2-LOD support interval.

^c Significant thresholds level (LOD=2.0) was determined by 1,000 permutation test.

^d Heterozygous (Mi29/*Z. nicaraguensis*)–homozygous for Mi29 alleles.

^e Parent contributing higher-value allele, where N=*Z. nicaraguensis*.

^f Proportion of phenotypic variance explained.

between the BC₂F₁ (bin 1.06, Mano and Omori 2008) and BC₄F₁ analyses (bin 1.05, this study) was about 20 cM based on the map distance of the BC₂F₁ population and about 8 cM based on that of the BC₄F₁ population, respectively (Fig. 4). We hereby designate the QTL as “*Qaer1.05-6*”. In addition to chromosome 1, small regions on chromosomes 3, 4, 7 and 8 were segregated in the BC₄F₁ population (Fig. 1). QTL analysis in these regions identified a minor and new QTL at the interval of umc1712–bnlg162 on chromosome 8 (bin 8.05, LOD=2.6, $r^2=0.07$) (Table 1).

Discussion

In 1989, a new species of teosinte *Z. nicaraguensis* was discovered in far northwest Nicaragua in an area that is frequently flooded for many weeks (Bird 2000, Iltis and Benz 2000). Using the unique germplasm *Z. nicaraguensis*, we have analyzed several flooding tolerance-related traits including the capacity to form constitutive aerenchyma and the ability to grow flooding-induced adventitious roots at the soil surface (Mano and Omori 2007, Kindiger and Mano 2009). In this study, using the BC₂F₁ and BC₄F₁ populations from the Mi29 × *Z. nicaraguensis* cross, we constructed a high-density linkage map bracketing the region at the aerenchyma locus *Qaer1.05-6*. In addition, we have confirmed a positive effect of the *Qaer1.05-6* on aerenchyma formation in the genetic background of individuals of a genotype that is approximately 80% Mi29 (Fig. 1). The high-density linkage map and BC₄F₁ segregants developed in this study can allow the detailed mapping of the *Qaer1.05-6* and the generation of near-isogenic lines possessing an extremely small chromosome segment of *Z. nicaraguensis*.

The map length around the *Qaer1.05-6* in the evaluated Mi29 × *Z. nicaraguensis* BC₄F₁ population of 17.6 cM was much shorter (37.5%) than BC₂F₁ population map (Fig. 4), suggesting the presence of recombination suppression in the BC₄F₁ population. This observation was also confirmed in the next generation of BC₅F₁ map of 19.1 cM at the same interval (Y. Mano, unpublished). The recombination suppression in the BC₄F₁ generation was chromosome 1 specific because the suppression was not observed (rather extended) at the remaining regions on chromosomes 3, 4 and 8 in the BC₄F₁ map (data not shown). In an earlier study utilizing

interspecies crosses in tomato, recombination between pairs of markers was less in the BC₂F₂ (developed from heterozygous chromosome segments flanked by homozygous regions) than in the BC₁ generation (developed from intact chromosomes) (Paterson *et al.* 1988, 1990). The authors suggested that chiasmata formation may be more frequent in homozygous regions, at the expense of recombination in heterozygous segments. In our study, above explanation may not be generally applicable since the chromosome segment of *Z. nicaraguensis* is relatively large on chromosome 1 in the BC₃F₁ plant (Fig. 1).

Using the BC₂F₁ population from Mi29 × *Z. nicaraguensis* cross, the peak of the QTL was located at the bin 1.06 (Mano and Omori 2008). In this study using the BC₄F₁ population of the same cross combination, the position of the QTL was shifted to the direction of the short arm compared to the BC₂F₁ population and the distance between the LOD peak is 8–20 cM. Because we developed the 2 populations from the same single F₁ plant, the difference of the QTL positions between the BC₂F₁ and BC₄F₁ populations is not due to the fact that the *Z. nicaraguensis* is not a pure line or different genetic composition. The difference is also not due to different marker density because when the QTL mapping in the BC₂F₁ population was re-calculated using the high-density linkage map developed in this study, the same peak was observed (data not shown). We will soon identify this QTL position more precisely using the developmental lines possessing a series of chromosome segments at the bin 1.05–7 region together with high-density linkage map.

Other than *Qaer1.05-6*, several QTLs controlling root aerenchyma formation with minor effects have been found in the teosintes. In maize × *Z. nicaraguensis* mapping populations, QTLs other than *Qaer1.05-6* were located on chromosome 1 (*Qaer1.01*, *Qaer1.02-3*, *Qaer1.11*), chromosome 5 (*Qaer5.01*, *Qaer5.09*, *Qaer5.09n*) and chromosome 8 (*Qaer8.06-7*) (Mano *et al.* 2007, Mano and Omori 2008). In this study, minor QTL was identified on chromosome 8 at bin 8.05 (*Qaer8.05*). The mode of gene action of the *Qaer8.06-7* was overdominance with negative effects (Mano *et al.* 2007), whereas that in the *Qaer8.05* was positive action when in the heterozygous genotype (Table 1). This suggests the two are different loci and the *Qaer8.05* is a newly identified QTL. As well as the ability to grow

flooding-induced adventitious roots at the soil surface (Mano *et al.* 2005), the capacity to form root aerenchyma is complex trait and is controlled by multiple genes. Nevertheless, we have acquired much QTL information regarding aerenchyma formation and it may now be possible to transfer and pyramid the QTLs from teosinte into maize via marker-assisted selection.

Inducible aerenchyma formation in plant roots is promoted by accumulation of endogenous ethylene (summarized by Evans 2003, Shiono *et al.* 2008) and many ethylene or hypoxia-induced genes have been reported (Saab and Sachs 1996). Recently, Mühlenbock *et al.* (2007) reported, in *Arabidopsis* hypocotyls, that the balanced activities of *LESION SIMULATING DISEASE1 (LSD1)*, *ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1)* and *PHYTOALEXIN DEFICIENT4 (PAD4)* regulate lysigenous aerenchyma formation in response to hypoxia. However, gene isolation with regard to inducible and/or constitutive root aerenchyma forming process has not been reported in crops because lack of suitable experimental materials (e.g. mutant and isogenic line). We will soon have available near isogenic lines of Mi29 possessing QTL for aerenchyma formation with only an extremely small chromosome segment of *Z. nicaraguensis*.

Laser microdissection has been used to analyze specific cells of interest (e.g. cortical tissue) from heterogeneous tissues (Nakazono *et al.* 2003, Ohtsu *et al.* 2007b). In addition, Margulies *et al.* (2005) reported a high parallel sequencing system (454 sequencing) that achieves 100-fold increase in throughput compared to current sequencing methods, and it has been applied for SNP discovery (Barbazuk *et al.* 2007) and global gene expression analysis in maize (Ohtsu *et al.* 2007a). In addition to map-based cloning, it will be possible to isolate constitutive aerenchyma-forming gene(s) from cortical tissue in roots of the developed isogenic line by applying a combination of laser microdissection and 454 transcriptome sequencing analysis.

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