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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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in well-aerated greenhouse environments (Ray et al. 1999, Mano et al. 2006) and in upland field conditions (Y. Mano, unpublished).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 (Smirnoff 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...
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 BC$_2$F$_1$ 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 F$_1$ 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 BC$_2$F$_1$ population (Mano and Omori 2008) was used for the analysis. A single F$_1$ plant derived from a cross between Mi29 × *Z. nicaraguensis* was backcrossed to Mi29 and 42 BC$_2$F$_1$ plants were obtained. These 42 BC$_2$F$_1$ plants were backcrossed using Mi29 as the pollen parent to produce BC$_3$F$_1$ seeds. 214 BC$_3$F$_1$ individuals grown from 4–6 BC$_3$F$_1$ seeds from each of the 42 BC$_2$F$_1$ spikes were developed.

To generate the BC$_3$F$_1$ population, the same F$_1$ plant that was used for the development of the 214 BC$_2$F$_1$ population, was crossed to Mi29. A single BC$_3$F$_1$ plant was backcrossed to Mi29 three times to produce the BC$_4$F$_1$ seeds. During the backcross process, SSR markers bnlg1832 (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 BC$_3$F$_1$ plant used to develop the BC$_4$F$_1$ mapping population. The frequency of heterozygous genotypes (Mi29/Z. nicaraguensis; shadowed regions) was 0.225 (data not shown), which is higher compared to the expected value of 0.125 in the BC$_3$F$_1$ generation.

**Root anatomy**

A total of 123 BC$_3$F$_1$ 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 BC$_3$F$_1$ 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
High-density map around the aerenchyma locus in Zea

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 µM of fluorescently labeled forward and unlabeled reverse primers, 200 µM dNTPs, 3.5 mM MgCl₂ (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 µL. 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 BC₁F₁ 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 BC₁F₁ model using the QGene program (Nelson 1997). In the BC₄F₁ 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 BC₁F₁ model using the MAPMAKER/EXP 3.0.

QTL analysis

In the BC₄F₁ population, composite interval mapping (CIM) was applied to map the QTLs controlling aerenchyma formation in the BC₁F₁ 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 ± standard deviation) for Mi29 (n = 10) and 2.0 ± 0.1 for Z. nicaraguensis (n = 10) (Fig. 2). Scores of the BC₄F₁ 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 (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
× 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 bnlg1832 (bin 1.05–ume1228 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 BC2F1 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 bnlg1832–ume1228 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 BC2F1; data not shown).

Mapping QTL for aerenchyma formation in the BC2F1 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

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**Fig. 4.** Linkage map of the BC2F1 (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.
between the BC$_2$F$_1$ (bin 1.06, Mano and Omori 2008) and BC$_2$F$_1$ analyses (bin 1.05, this study) was about 20 cM based on the map distance of the BC$_2$F$_1$ population and about 8 cM based on that of the BC$_2$F$_1$ 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 BC$_2$F$_1$ 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, $\chi^2$ = 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, Illis 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 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 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 2000).

The high-density linkage map developed in this study, the same peak is 8–20 cM. Because we developed the 2 populations from the same single F$_1$ plant, the difference of the QTL positions between the BC$_2$F$_1$ and BC$_2$F$_1$ 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 BC$_2$F$_1$ 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 interspecies crosses in tomato, recombination between pairs of markers was less in the BC$_2$F$_2$ (developed from heterozygous chromosome segments flanked by homozygous regions) than in the BC$_1$ 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 BC$_2$F$_1$ plant (Fig. 1).
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 formation 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 suitable experimental materials (e.g. mutant and isogenic line). We will soon have available near isogenic lines of

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