

Identification of QTL controlling adventitious root formation during flooding conditions in teosinte (*Zea mays* ssp. *huehuetenangensis*) seedlings

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Summary

Adventitious root formation (ARF) at the soil surface is one of the most important adaptations to soil flooding or waterlogging. Quantitative trait loci (QTL) controlling ARF under flooding condition were identified in a 94 F₂ individual population by crossing maize (*Zea mays* L., B64) × teosinte (*Z. mays* ssp. *huehuetenangensis*). A base-map was constructed using 66 SSR and 42 AFLP markers, covering 1,378 cM throughout all ten maize chromosomes. The ARF capacity for seedlings was determined by evaluating the degree of root formation at the soil surface following flooding for 2 weeks. ARF showed continuous variation in the F₂ population. Interval mapping and composite interval mapping analyses revealed that the QTL for ARF was located on chromosome 8 (bin 8.05). Utilising a selective genotyping strategy with an additional 186 F₂ population derived from the same cross combination and 32 AFLP primer combinations, regions on chromosomes 4 (bin 4.07) and 8 (bin 8.03) were found to be associated with ARF. *Z. mays* ssp. *huehuetenangensis* contributed all of the QTL detected in this study. Results of the study suggest a potential for transferring waterlogging tolerance to maize from *Z. mays* ssp. *huehuetenangensis*.

Introduction

In humid temperate and subtropical regions throughout the world, excessive rainfall causes flooded or waterlogged soils, which result in a reduction of crop productivity. In Japan, maize is grown on upland rice paddy fields in order to maximise land productivity. In these instances, drainage in the rice paddies is often poor and waterlogging may occur from late spring to early summer during the rainy season. This often causes severe damage to maize seedlings due to poor adaptation to waterlogging. In order to increase crop productivity in the waterlogged soils, development of waterlogging-tolerant lines is required.

Wild species have often provided good genetic resources for introducing superior levels of biotic or

abiotic stress tolerance (Harlan, 1976). In the tribe Triticeae, wild barley *Hordeum marinum*, which is known to occupy areas likely to experience periods of waterlogging, exhibits superior tolerance when compared to cultivated barley (McDonald et al., 2001). Recent studies have documented variation in the anaerobic responses of maize to flooding (Sachs et al., 1996); however, few have identified the genetic basis of tolerance. Teosintes, considered to be the closest relatives of maize, include several subspecies of *Zea mays* (*Z. mays* ssp. *mexicana*, *Z. mays* ssp. *parviglumis* and *Z. mays* ssp. *huehuetenangensis*), and, in section Luxuriantes, *Z. luxurians*, *Z. diploperennis*, *Z. perennis* and a new species found on the Pacific coast of Nicaragua, *Z. nicaraguensis* (Iltis & Doebley, 1980; Iltis & Benz, 2000). Occasionally,

teosinte has been studied as a germplasm resource for maize improvement (Reeves, 1950; Cohen & Galinat, 1984) and teosintes from regions that receive frequent rainfall in Mexico, Guatemala, Honduras and Nicaragua could provide a superior genetic resource for the development of a waterlogging-tolerant maize.

In maize, several morphological responses during flooding have been reported (Subbaiah & Sachs, 2003). During flooding, maize can form aerenchyma cells in the roots but fail to do so in non-flooding conditions. At the opposite extreme, *Z. luxurians* is reported to possess aerenchyma cells in a non-flooded environment (Ray et al., 1999). Since the time required for a plant to develop aerenchyma cells may require weeks or months (Das & Jat, 1977; Burdick, 1989), it could be advantageous for a plant to possess aerenchyma cells in a non-stressed, non-flooding condition. However, alternative mechanisms may exist that allow a plant to circumvent the often deleterious effects of flooding or waterlogging.

Adventitious (nodal) root formation (ARF) at the soil surface can provide an alternative for some teosintes to address soil flooding or waterlogging (Bird, 2000). It has also been reported that some maize lines can express ARF during flooding (Jat et al., 1975; Lizaso et al., 2001). In a review of ARF in teosinte, it was found that *Z. luxurians* and *Z. mays* ssp. *huehuetenangensis* exhibit a higher capacity for ARF than some maize inbreds (Mano et al., 2005). We have also observed in greenhouse experiments that *Z. mays* ssp. *huehuetenangensis* seedlings exhibit extremely high adaptability to flooding by developing adventitious roots. In that experiment, 2-week old seedlings were grown and flooded with 12 cm of standing water. Following this treatment, the *Z. mays* ssp. *huehuetenangensis* seedlings formed a large number of adventitious roots above the soil surface (Y. Mano unpublished data). As a consequence, the adventitious roots of this teosinte can obtain oxygen and this characteristic may play an important role in their adaptation to flooding conditions.

Little is known regarding the genetics or mechanisms of flood-induced adventitious rooting. In this study, we report on the identification of QTL controlling ARF using segregating F₂ populations derived from crosses between maize 'B64' and teosinte '*Z. mays* ssp. *huehuetenangensis*'. These markers may help to develop waterlogging-tolerant elite maize lines through molecular marker assisted selection.

Materials and methods

Plant materials

Maize (*Zea mays* L.) inbred line B64 exhibits an extremely low capacity for ARF at the soil surface during flooding conditions; while some forms of teosinte (*Z. luxurians* and *Zea mays* ssp. *huehuetenangensis*) exhibit an extremely high tendency for ARF (Mano et al., 2005). In this study, B64 and *Zea mays* ssp. *huehuetenangensis* ($2n = 2X = 20$) were used as parents to develop populations in which QTL controlling ARF could be identified. The maize inbred line B64 (Accession No. 00094105) was obtained from the Gene Bank, National Institute of Agrobiological Sciences, Tsukuba, Japan and *Z. mays* ssp. *huehuetenangensis* (PI 441934, G-120 of Iltis & Doebley, ~2 km NE of San Antonio, western Guatemala) from North Central Regional Plant Introduction Station USDA, ARS, NCRPIS Iowa State University Ames, Iowa.

For this research, two greenhouse experiments were conducted for flooding tolerance. Experiment 1: Maize inbred line B64 was used as the female parent in a cross with *Z. mays* ssp. *huehuetenangensis*. A total of 94 F₂ plants derived from a single F₁ plant were evaluated. Experiment 2: Selective genotype analysis (Darvasi & Soller, 1992; Lander & Botstein, 1989) was performed on an additional 186 F₂ plants derived from a different F₁ plant generated by crossing B64 with *Z. mays* ssp. *huehuetenangensis*.

Flooding treatment

Experimental seedling tests were carried out in a phytotron (Experiment 1, 30 °C day/25 °C night with natural light of 11 h per day) and a greenhouse (Experiment 2, non-controlled condition with natural light of 13 h day length and minimum temperature above 20 °C). The F₂ population and their parents were grown in pots 11 cm in diameter and 15 cm in depth filled with granular soil (Kureha Chemical Industry, Tokyo Japan; 0.6 g N, 2.9 g P, 0.9 g K in each pot, one plant per pot). The seedlings were grown to the four-leaf stage (2 weeks after sowing), and were then flooded with water until the water level was 3 cm above the soil surface. Two weeks after initiation of flooding treatment, plant height was measured and the degree of ARF above the soil surface per plant was scored visually 0 (none), 1 (low), 2 (medium) and 3 (high). These values corresponded to an approximate total of all surface root lengths of ~5 cm, ~30 cm,

~60 cm and over 60 cm, respectively. Using this method, a correlation coefficient between replication in 27 maize lines was as high as 0.749 (Mano et al., 2005); thus phenotype of ARF was evaluated in the F₂ generation.

Soil redox potential measurements

The effect of waterlogging on soil conditions was analyzed by measuring soil redox potential (Eh). Eh was measured 5 cm below the soil surface every 2 or 3 days by using platinum tipped electrodes, a millivolt meter (Model EHS-120, Fujiwara Scientific Company, Tokyo, Japan). Eh was recorded in two experiments with three replications in each experiment.

Map construction and QTL analysis (Experiment 1)

An SSR-AFLP based map was constructed using 413-point markers for the 94-plant F₂ population derived from the B64 × *Z. mays* ssp. *huehuetenangensis* cross (Mano et al., 2004). This analysis provided an average density of 3.5 cM/marker, with considerable marker clustering in certain regions. A subset of 108 markers (66 SSRs and 42 AFLPs) were selected to provide a density of 5 to 10 cM intervals without clustering markers, and the “base-map” was constructed using MAPMAKER/EXP 3.0 (Lander et al., 1987).

QTL mapping for ARF in the F₂ population was performed using the interval mapping (IM) and composite interval mapping (CIM) methods implemented by the software package QTL Cartographer Version 1.14 (Basten et al., 2000). CIM was run with the default setting for model 6 in the program (5 background markers and a window size of 10 cM). Permutation tests were performed to estimate average log-likelihood (LOD) thresholds. After 1,000 permutations, average LOD thresholds of 4.17 (IM) and 4.79 (CIM) were chosen to declare a putative QTL significant, which maintained the chromosome-wide Type-I error rate of 0.05 (Churchill & Doerge, 1994).

Selective genotyping (Experiment 2)

AFLP analysis

To confirm the linkage of QTL to ARF expression in Experiment 1, we used an additional 186-plant F₂ population of the B64 × *Z. mays* ssp. *huehuetenangensis* cross. A selective genotyping strategy with AFLP

markers was applied using 15 F₂ plants representing each extreme from the ‘none’ and ‘high’ ARF classifications.

The AFLP analysis essentially followed the methods described by Vos et al. (1995), with some modifications. DNA restriction and ligation were performed using the fluorescent dye-based AFLP Plant Mapping Kit from Perkin Elmer Applied Biosystems (Foster City, CA). Two pre-selective amplification steps were conducted (Schiemann et al., 1999). A total of 32 primer combinations of 4 *Eco*RI primers [(5'-GACTGCGTACCAATTC-3') plus AAC (e02), AAG (e03), ACA (e05), ACC (e06)], and 8 *Mse*I primers [(5'-GATGAGTCCTGAGTAA-3') plus CAA (m17), CAC (m18), CAG (m19), CAT (m20), CTA (m29), CTC (m30), CTG (m31) and CTT (m32)] were used to evaluate AFLP fragments associated with ARF. To obtain good separation of amplified AFLP fragments, buffer gradient electrophoresis was performed with 1 × TBE in the cathode buffer (–) and 1 × TBE plus 0.5 M sodium acetate in the anode buffer (+) (Qi & Lindhout, 1997).

SSR analysis

Based on the data in the MaizeGDB (<http://www.agron.missouri.edu/ssr.html>), a total of 30 SSR primer pairs adjacent to the AFLP markers related to ARF were selected to construct an SSR-based linkage map around QTL for ARF using the 186-plant F₂ population. Sequences of all SSR markers were obtained from the MaizeGDB. All the forward primers were 5-end labeled with fluorescence dye FAM, HEX, ROX or TET. Reaction mixtures contained 20 ng of genomic DNA, 0.25 μM of fluorescently labeled forward and unlabeled reverse primers, 0.2 mM dNTPs, 3.5 mM MgCl₂ (final concentration), 0.15 units of Taq DNA polymerase (Qiagen GmbH, Hilden, Germany) and its corresponding reaction buffer in a total volume of 10 μL. The amplification conditions were identical to the “touchdown” profiles of Mellersch & Sampson (1993). PCR products were separated on a 20 cm, 5% denaturing polyacrylamide gel at 360 V for 1 h 30 min. After separation, the gel was scanned in the Molecular Imager (Bio Rad, Hercules, CA). QTL mapping for ARF was performed as described in Experiment 1. After 1,000 permutations, average LOD thresholds of 2.95 (IM) and 3.13 (CIM) were chosen to declare a putative QTL significant (Type-I error rate of 0.05).

Results

Soil redox potential measurements

Soil Eh measurements obtained immediately following flooding treatment were 389 ± 6 mV (mean \pm standard error) in Experiment 1 and 433 ± 9 mV in Experiment 2, indicating aerated conditions. Soil Eh measurements taken 2 weeks following flooding were 28 ± 37 mV and 134 ± 34 mV, in Experiment 1 and Experiment 2, respectively, suggesting O₂-deficient conditions. There is little effect of Fe²⁺ and/or H₂S toxicity on the seedlings because Fe³⁺ changes to Fe²⁺ and SO₄²⁻ changes to H₂S when soil Eh readings are below $-100 \sim -200$ mV.

Phenotypic variations

In *Z. mays* ssp. *huehuetenangensis*, root apices were observed at the soil surface 1 week following flooding treatment. Over time, the roots continued to elongate horizontally over the soil-water interface. Maize inbred line B64 exhibited no or a minor tendency for ARF during flooding treatment. Development of adventitious roots in *Z. mays* ssp. *huehuetenangensis* was accompanied by an increase in the damage and death of seminal roots.

Two weeks following flooding treatment, the degree of ARF in the parents and F₂ was scored. ARF in the parental plants indicated a wide level of variation (Figure 1), and scored an average of 0.4 ± 0.2 (mean \pm standard error) and 0.3 ± 0.3 for B64 and 2.4 ± 0.2 and 2.3 ± 0.2 for *Z. mays* ssp. *huehuetenangensis* in the environments utilised for Experiments

1 and 2, respectively. ARF indicated a continuous distribution in the F₂ populations (Figure 2). None of parents or F₂ populations showed leaf injury or chlorosis following exposure to flooding for 2 weeks.

Mapping QTL for ARF in the F₂ population (Experiment 1)

The base-map was constructed using 66 SSR and 42 AFLP markers, covering 1,378 cM at an average interval of 14.1 cM/marker throughout all ten maize chromosomes (Figure 3). By comparing published maize SSR maps (e.g. Sharopova et al., 2002), the coverage of this map is satisfactory for QTL analysis. Using QTL Cartographer ver. 1.14, single QTL was mapped on chromosome 8 (bin 8.05, umc1777-bnlg240 interval) with LOD scores of 7.0 for IM and 6.5 for CIM, respectively (Figure 3, Table 1). The allele of *Z. mays* ssp. *huehuetenangensis* at the locus increased ARF. In the IM analysis, one additional QTL below the threshold of significance was detected on chromosome 5 (bin 5.03; umc1048-bnlg150 interval, LOD = 3.0, $a = 0.36$, $d = -0.49$, $r^2 = 0.14$) and *Z. mays* ssp. *huehuetenangensis* contributed the QTL.

Selective genotyping (Experiment 2)

AFLP analysis

When comparisons were made between 15 F₂ plants obtained from the 186-individual F₂ population with high ARF scores and 15 with low ARF scores, a total of ten AFLP markers were found to be associated with ARF following a Chi-square test (at the 5% level)

Table 1. Chromosome locations and the mode of gene action of QTL for ARF in the two F₂ populations of B64 \times *Z. mays* ssp. *huehuetenangensis*

Chromosome (bin)	Interval	IM					CIM				
		LOD ¹	A ²	D ³	r ² ⁴	Pa ⁵	LOD ¹	A ²	D ³	r ² ⁴	Pa ⁵
Experiment 1 (F ₂)											
8 (8.05)	umc1777-bnlg240	7.0	0.81	-0.22	0.30	hueh.	6.5	0.77	-0.22	0.25	hueh.
Experiment 2 (sel. F ₂)											
4 (4.07)	umc1869-bnlg1189	4.1	0.44	-0.05	0.12	hueh.	3.9	0.39	-0.05	0.09	hueh.
8 (8.03)	phi115-phi014	4.0	0.41	0.03	0.10	hueh.	5.1	0.41	0.04	0.10	hueh.

¹LOD score.

²Additive effect.

³Dominance effect.

⁴Proportion of the phenotypic variance explained.

⁵Letter suffix indicates parent contributing higher-value allele, where hueh. = '*Z. mays* ssp. *huehuetenangensis*'.

B64

*Z. mays ssp. huehuetenangensis*

Figure 1. Two-week old flooded seedlings. Top: B64 (without adventitious root), bottom: *Z. mays ssp. huehuetenangensis* (with adventitious roots).

using 32 AFLP primer combinations. According to the map based on the same cross (Mano et al., 2004), these AFLP markers were located to five chromosome regions: chromosomes 1 (bin 1.06; significant markers e03m18-100, e03m32-220), 3 (3.03; e05m30-90), 4 (4.06; e05m30-125, e06m20-205), 5 (5.03; e02m29-380, e02m29-90, e03m31-220, e05m20-227) and 8 (8.03; e05m29-330).

SSR analysis

A partial linkage map of the 186-plant F_2 population was constructed for these five regions using 30 SSR markers. The map covered 554 cM at an average interval of 22.1 cM/marker. Of the five identified QTL regions, two that are located to chromosomes 4 (bin 4.07, umc1869-bnlg1189 interval) and 8 (bin 8.03, phi115-phi014 interval) were significantly associated

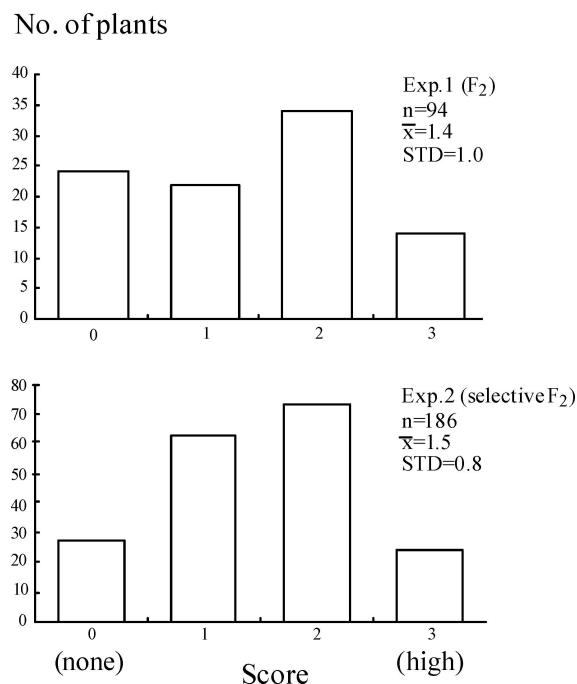


Figure 2. Frequency distributions for ARF for the B64 × *Z. mays* ssp. *huehuetenangensis* population used in Experiment 1 (F₂) and Experiment 2 (selective F₂).

with ARF by both IM and CIM (Figure 3). *Z. mays* ssp. *huehuetenangensis* contributed all of the QTL detected in the analyses (Table 1). The remaining three regions on chromosomes 1, 3 and 5 were below the threshold of significance (data not shown). Of these, the region on chromosome 5 (bin 5.03) was also detected in the Experiment 1 with an LOD of 3.0 (below the threshold of significance), suggesting the presence of minor QTL at the region.

Figure 4 shows LOD curves from the QTL analysis of ARF on chromosome 8 in the two experiments. Experiment 1 had a QTL with the maximum LOD value at the umc1777 locus, whereas Experiment 2 had the maximum LOD value at the phi115 locus, with an interval between markers of about 30 cM.

Discussion

No previous studies have mapped genes controlling ARF. This study for the first time identifies and maps QTL for ARF using molecular markers. Two experiments using QTL analysis of the total maize genome revealed that the regions on chromosomes 4, 5 (below

the threshold of significance) and 8 were found to be associated with ARF and the alleles of *Z. mays* ssp. *huehuetenangensis* at these loci increased ARF (Figure 3, Table 1). In the two experiments, the QTL associated with chromosome 8 were indicated to be separated by about 30 cM (Figure 4), suggesting the presence of two QTL. Though statistically they may appear to be different loci, it may be possible that the two QTL's represent the same locus because their mode of gene action was similar (Table 1), and the peak position of the LOD score can be altered in a QTL with moderate (minor) effects due to environmental interaction or statistical error. Nevertheless, we do not exclude the other possibility that the QTL on chromosome 8 may be different from each other: *Z. mays* ssp. *huehuetenangensis* is not a pure line or genetic composition of ARF in the donor plants could be different between the two experiments.

Tuberosa et al. (2002) reported QTL for root traits in hydroponics using maize Lo964 × Lo1016 cross. In total, 11 QTL for primary root length, seven for primary root diameter, nine for primary root weight and ten for the weight of the seminal roots were detected. All the three regions on chromosomes 4, 5 and 8, which harbored QTL for ARF found in this study also harbored QTL for primary root length in hydroponics. In addition, several QTL controlling root traits in maize have been investigated (Tuberosa et al., 2003) and one of the QTL for root pulling force was located on chromosome 4 (bin 4.06-7). This position of the QTL overlaps the proposed QTL for ARF. At present, the relationship between QTL controlling ARF and several root traits is not clear and further studies are necessary.

Morphological responses, including a decline in relative growth rate, leaf senescence, adventitious root formation and aerenchyma development, have been reported in plants exposed to waterlogging conditions (Krizek, 1982). Of these, ARF could be one of the most important traits for the breeding of waterlogging-tolerant maize because adventitious roots may directly obtain oxygen from their surroundings. The *Z. mays* ssp. *huehuetenangensis* accession used in this study showed extremely high adaptability to severe flooding in greenhouse experiments by forming a large number of adventitious roots at the soil-water interface. Other teosintes such as *Z. nicaraguensis* (Bird, 2000), *Z. luxurians* and *Z. mays* ssp. *mexicana* developed ARF following 14 days of flooding in additional greenhouse experiments (Y. Mano, unpublished). Consequently, teosintes that are indigenous to regions with frequent

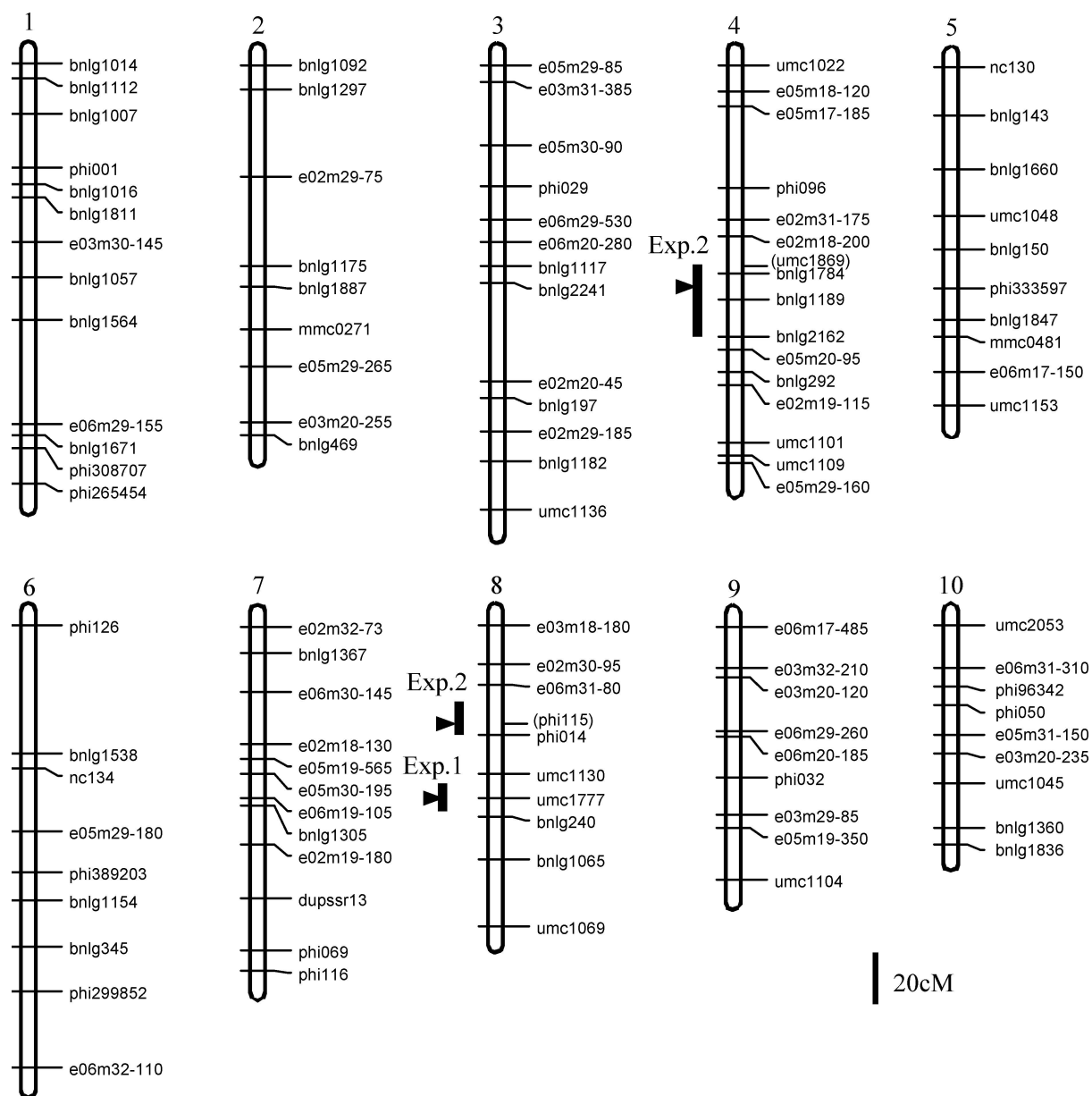


Figure 3. Chromosome locations of the QTL for ARF in the B64 × *Z. mays* ssp. *huehuetenagensis* populations found in the CIM. Short arms of the chromosome are on the top. The scale represents centi Morgan (Haldane units). Bars to the left of the chromosomes indicate 1-LOD likelihood intervals, and arrowheads indicate the position of the peak LOD in the interval. Markers in parentheses are SSR markers, which were mapped only in Experiment 2 and placed at approximate positions on the map.

precipitation in a seasonally wet habitat likely possess adaptation to frequent flooding. One extreme example of this characteristic would appear to be *Z. nicaraguensis*, which is adapted to northwest coastal Nicaragua and can tolerate frequent flooding during a 6-month rainy season (Bird, 2000; Iltis & Benz, 2000).

In previous studies, adventitious roots produced during waterlogging were reported to be beneficial for plant recovery (Jackson & Campbell, 1976; Kramer, 1951), except for sunflower (Wample & Reid, 1978). In addition, ARF on the soil surface during flooding conditions has been reported for other upland monocot crops such as barley (Arikado & Adachi, 1955) and

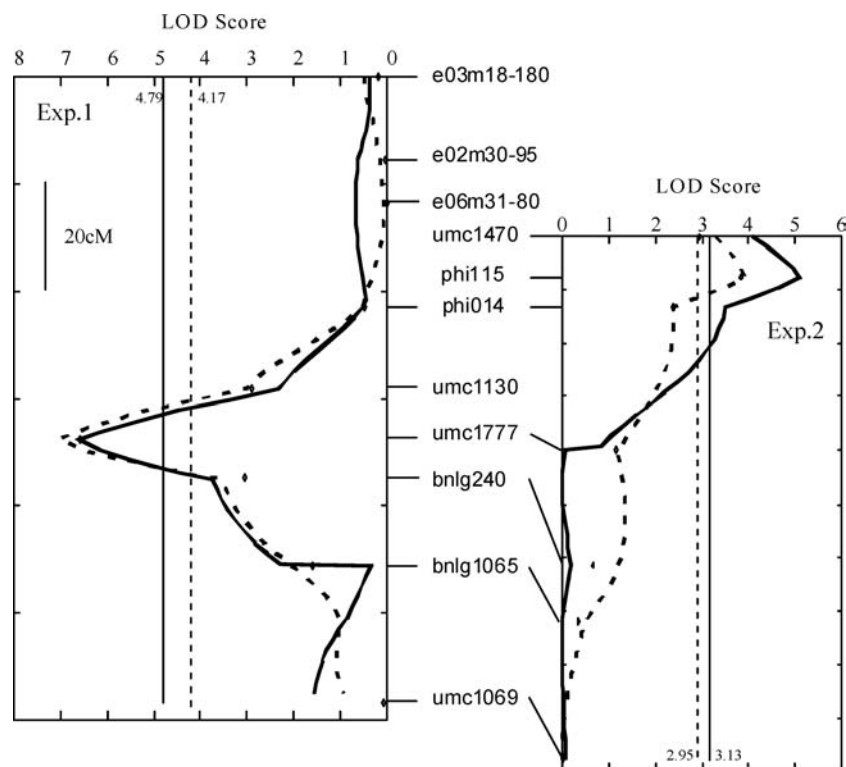


Figure 4. LOD scores from the QTL analysis of ARF on chromosome 8 in the B64 \times *Z. mays* ssp. *huehuetenangensis* population used in Experiment 1 (F_2) and Experiment 2 (selective F_2). Short arms of the chromosome are on the top. The scale represents centi Morgan (Haldane units). Dotted and solid lines indicate the LOD curves of the IM and CIM, respectively. Horizontal lines correspond to a 0.05 significant level.

Italian ryegrass (Tase & Kobayashi, 1992). In Italian ryegrass, the degree of rooting after flooding was closely correlated to plant growth, suggesting ARF is one of the morphologically adaptive traits that a plant can utilise to avoid anoxia (Tase & Kobayashi, 1992). These reports suggest that ARF is a useful breeding character for selection and development of waterlogging-tolerant crops.

In our experiments, flooding to 3 cm above the soil was not overly severe for either teosinte or the maize parents, or their F_2 populations since none of the entries exhibited leaf injury or damage by chlorosis. Also, no significant correlations were observed between ARF and plant growth under flooding condition in the two experiments (data not shown). If the experimental flooding conditions had been too severe, seedlings of all genotypes likely would have succumbed to damage, even if they possessed advantageous QTL for ARF. Consequently, the experimental conditions used in this research were suitable for observing segregation of ARF because plant damage due

to flooding did not mask ARF in the lines with ARF QTL.

Through QTL analysis of ARF using several mapping populations of different species, including *Z. nicaraguensis*, *Z. luxurians* and *Z. mays* ssp. *mexicana*, it should be possible to classify QTL for ARF into QTL common to a number of species and species-specific QTL. Functional analysis of QTL common to different species would be an efficient first step toward generating a better understanding of ARF, and provide a wide use of the genes.

In conclusion, the present study identifies regions of the *Z. mays* ssp. *huehuetenangensis* chromosomes that are involved in the control of ARF. QTL on chromosome 8 was strongly associated to ARF and likely provides an important role in ARF. Transfer of ARF alleles on chromosome 8, 4 and 5 from *Z. mays* ssp. *huehuetenangensis* to maize is now in progress using marker-assisted selection together with phenotypic selection. By this process, it should be possible to determine a detailed map position of ARF on chromosome

8, to reveal the relationship between ARF and several root traits by using near-isogenic lines and to develop waterlogging-tolerant elite maize lines.

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