

REVIEW

DNA Marker-Assisted Selection Approach for Developing Flooding-Tolerant Maize

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Abstract

Flooding due to worldwide climate change can drastically affect crop production. To overcome the detrimental effects of flooding during maize growth, we have been developing flooding-tolerant maize via DNA marker-assisted selection using a flooding-tolerant teosinte, *Zea nicaraguensis*, as a donor parent. Over the last decade, quantitative trait locus (QTL) information on flooding-tolerance-related traits in *Zea* species has been obtained at the NARO Institute of Livestock and Grassland Science, and near-isogenic lines containing one or more QTLs have been developed for several flooding-tolerance-related traits, such as the capacity to form constitutive aerenchyma, tolerance to flooding under reducing soil conditions, and ability to form adventitious roots at the soil surface. In field trials, we have been accumulating data demonstrating the effectiveness of teosinte-derived QTLs on flooding tolerance, and are preparing to release a flooding-tolerant F₁ maize hybrid within a few years. In addition, we have just started a project to clone *Qft-rd4.07-4.11* by using next-generation sequencing, which would make it possible to extend the use of this QTL to other upland crops.

Discipline: Plant breeding

Additional key words: aerenchyma, QTL, reducing soil conditions, waterlogging, wild *Zea*

Introduction

The risk of flooding due to worldwide climate change has increased in the regions of Southeast Asia, Peninsular India, eastern Africa and the northern half of the Andes (Hirabayashi et al. 2013). Flooding can drastically affect crop production, and about 15% of the total maize (*Zea mays* L. subsp. *mays*) growing area in Southeast Asia is affected by floods and waterlogging (Rathore et al. 2000). In order to increase land productivity and food self-sufficiency in Japan, maize must be grown in non-cultivated upland rice paddy fields that are frequently flooded or waterlogged during the rainy season. To overcome the detrimental effects of flooding on maize production, we have been developing flooding-tolerant maize via DNA marker-assisted selection.

We used a wild relative of maize, the teosinte *Zea nicaraguensis* Iltis & Benz (Poaceae), as a flooding-tolerant germplasm resource because of its unique habitat. This teosinte grows in lowland areas of Nicaragua that are frequently flooded during a six-month rainy season (Bird

2000). In our studies, *Z. nicaraguensis* showed high tolerance to flooding (Fig.1) and possessed unique morphological and physiological characteristics related to flooding tolerance (Mano & Omori 2007). These traits include the capacity to form constitutive aerenchyma (Mano et al. 2006b, Mano et al. 2007), tolerance to flooding under reducing soil conditions (Mano & Omori 2013a), a barrier to radial oxygen loss (ROL barrier, Abiko et al. 2012a), and the ability to form adventitious roots at the soil surface during flooding (Mano et al. 2009) (Fig. 2).

Given the generally low repeatability of flooding tolerance experiments on upland crops, particularly under field conditions, the results regarding varietal ranking obtained by different researchers are inconsistent (Kawaguchi 2011, Mano & Oyanagi 2009), and thus we used the following strategy described in Mano & Omori (2015): (1) conduct quantitative trait locus (QTL) mapping analyses separately for each flooding-tolerance-related trait in *Z. nicaraguensis*, (2) develop near-isogenic lines (NILs) of maize containing QTLs for flooding-tolerance-related traits, (3) evaluate flooding tolerance in each NIL in

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Fig. 1. Response to flooding under field conditions in maize Mi29 (left) and teosinte *Zea nicaraguensis* (right). Photo courtesy of Dr. K. Kawaguchi, Dr. A. Oyanagi, and Dr. F. Abe

greenhouse experiments and under field conditions, and (4) pyramid useful flooding-tolerance-related QTLs. We have previously reviewed the breeding of flooding tolerance in crops (Mano & Omori 2007, Mano & Oyanagi 2009), and therefore encourage the reader to refer to these reviews for additional details on traits related to flooding tolerance. Here, we focus on the current status of our program for developing flooding-tolerant cultivars in maize at the NARO Institute of Livestock and Grassland Science in Japan. We also present recent advances in molecular studies on gene expression and the identification of candidate genes for flooding tolerance in maize reported by other researchers, along with a summary of our own progress toward gene isolation.

Development of near-isogenic lines

1. QTL mapping

Mano et al. (2006a) first reported QTL mapping for flooding tolerance in maize and identified a QTL on chromosome 1 by using a partial linkage map. Since then, QTL mapping studies encompassing the entire maize genome have been reported by several researchers (Osman et al. 2013, Qiu et al. 2007, Zhang et al. 2013). Although reliable DNA marker information has been obtained in these

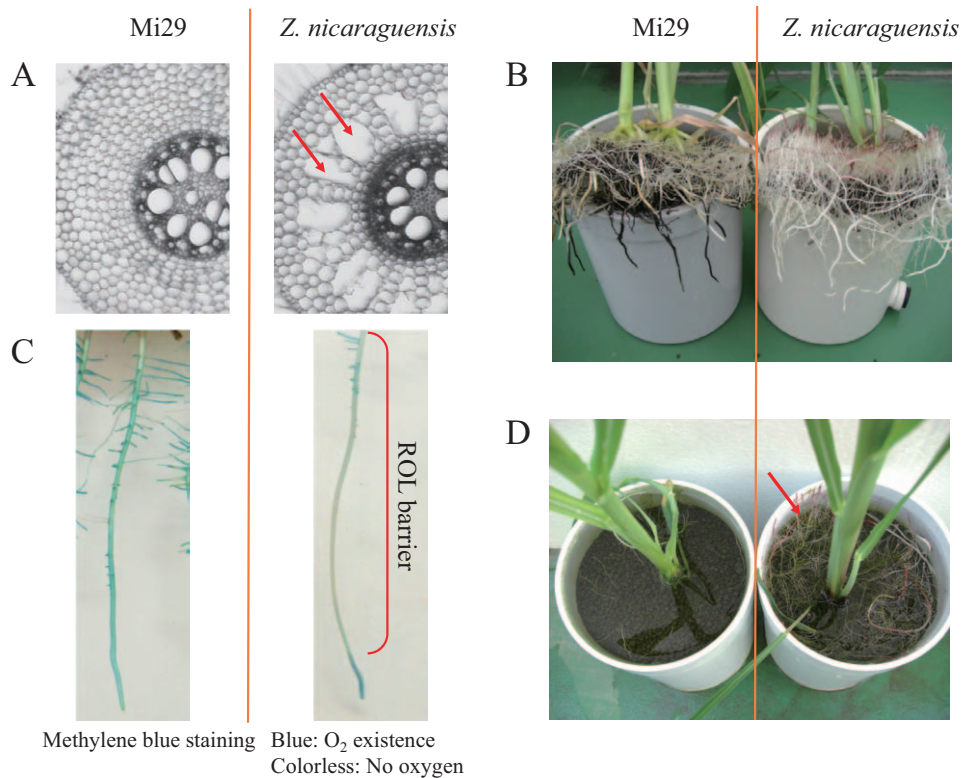


Fig. 2. Unique characteristics related to flooding tolerance in *Z. nicaraguensis*

(A) Capacity to form constitutive aerenchyma (arrows), (B) Tolerance to flooding under reducing soil conditions, (C) Barrier to radial oxygen loss (ROL barrier), and (D) Ability to form adventitious roots at the soil surface (arrow). Photo (C) courtesy of K. Watanabe and Prof. M. Nakazono (Nagoya Univ.). Photo (D) reproduced from Mano et al. (2009).

Table 1. Locations of QTLs for flooding tolerance-related traits in *Zea nicaraguensis* detected in mapping populations from three cross combinations

Traits	QTL	Chromosome	bin	LOD	Var ^a	Population ^b	References
Constitutive aerenchyma	<i>Qaer1.02-1.03</i>	1	1.02-1.03	4.1	0.11	2	Mano et al. 2007
	<i>Qaer1.05-1.06</i>	1	1.05-1.06	8.2-8.5	0.17-0.25	1	Mano and Omori 2008, 2009
	<i>Qaer1.06-1.07</i>	1	1.06-1.07	3.1-5.3	0.08-0.17	2, 3	Mano et al. 2007, 2012
	<i>Qaer1.11</i>	1	1.11	5.7	0.12	1	Mano and Omori 2008
	<i>Qaer5.09n</i>	5	5.09	2.9	0.06	1	Mano and Omori 2008
	<i>Qaer7.01</i>	7	7.01	3.7	0.12	3	Mano et al. 2012
	<i>Qaer8.05</i>	8	8.05	2.6	0.07	1	Mano and Omori 2009
Tolerance under reducing conditions	<i>Qft-rd4.07-4.11</i>	4	4.07-4.11	4.6-19.1	0.13-0.42	1	Mano and Omori (2013a)
Aboveground adventitious roots	<i>Qarf3.04</i>	3	3.04	3.9-4.2	0.06	1	Mano et al. 2009
	<i>Qarf7.04</i>	7	7.04	3.0	0.04	1	Mano et al. 2009
	<i>Qarf8.03</i>	8	8.03	2.6	0.04	1	Mano et al. 2009

a: Proportion of phenotypic variance explained.

b: Mapping populations, 1; Mi29 × *Z. nicaraguensis* (presented in Fig. 3), 2; B64 × *Z. nicaraguensis*, 3; *Z. nicaraguensis* × *Z. nicaraguensis*.

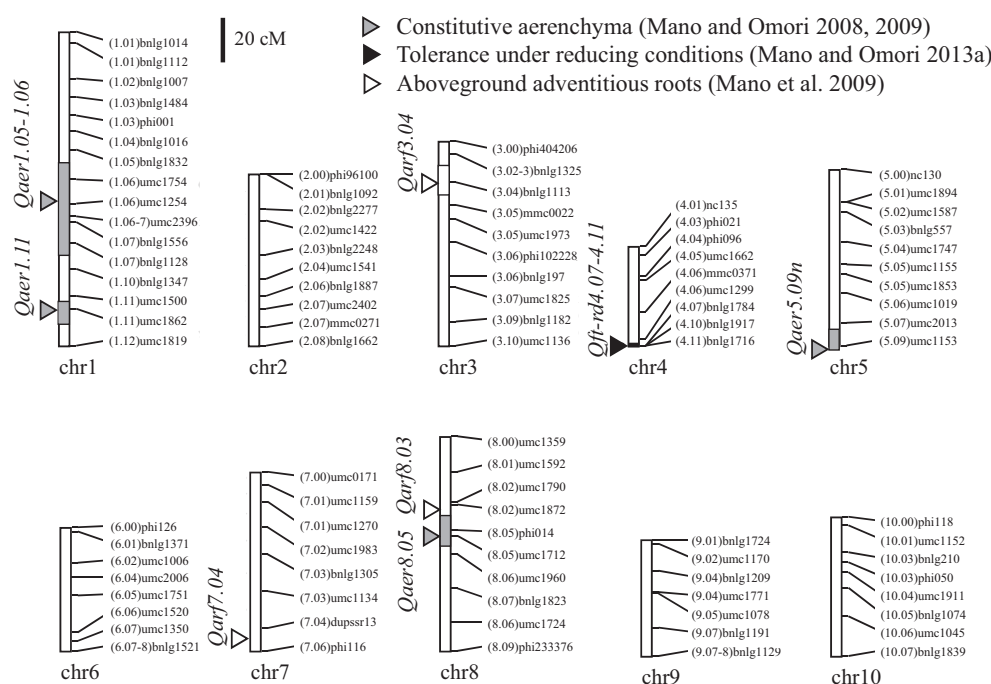


Fig. 3. Chromosome locations of QTLs for flooding-tolerance-related traits detected in mapping populations from a cross between Mi29 × *Z. nicaraguensis*. Arrowheads indicate the logarithm of odds (LOD) peak for each QTL. The shaded vertical bars on the chromosomes indicate the region(s) of *Z. nicaraguensis* introgressed into near-isogenic lines or QTL pyramids in the genetic background of maize Mi29. Currently, we developed the following three lines: 1) pyramiding line possessing *Qaer1.05*, *Qaer1.11*, *Qaer5.09n*, and *Qaer8.05* in the genetic background of Mi29, 2) near-isogenic line possessing *Qft-rd4.07-4.11* in the genetic background of Mi29, and 3) near-isogenic line possessing *Qarf3.04* in the genetic background of Mi29

investigations, it has not yet been applied to practical breeding for the development of flooding-tolerant maize cultivars. As described above, our approach has been to conduct QTL mapping analysis separately for several flooding-tolerance-related traits. We have been developing

NILs, each possessing one or more QTLs for a given flooding-tolerance trait, by using *Z. nicaraguensis* as the donor parent and elite maize inbred line Mi29 (Ikegaya et al. 1999a) as the recurrent parent. Because Mi29 is the female parent of two elite F₁ hybrid cultivars—

‘Yumesodachi’ (cv. Mi29 × cv. Na50, Ikegaya et al. 1999b) and ‘Yumechikara’ (cv. Mi29 × cv. Mi47, Ito et al. 2004)—flooding-tolerant F₁ cultivars (heterozygous for the flooding-tolerance QTL allele) can be rapidly obtained by crossing a NIL (Mi29 genetic background) as the female parent × Na50 or Mi47 as the male parent. Table 1 and Fig. 3 summarize the current mapping results using the Mi29 × *Z. nicaraguensis* population. As indicated by the map, two traits of interest in our research program are the capacity to form constitutive aerenchyma and flooding tolerance under reducing soil conditions, which are discussed in the following sections.

2. Aerenchyma

The importance of root aerenchyma or air space in cortical tissue is widely recognized as a means of adapting to flood conditions by supplying oxygen to the tips of roots through aerenchyma (summarized in Yamauchi et al. 2013). In Mi29 × *Z. nicaraguensis* mapping populations, QTLs for constitutive aerenchyma formation have been identified in four regions (Mano & Omori 2008, 2009), and pyramiding of these four QTLs into Mi29 has been completed (Mano & Omori, unpublished; Fig. 3). In our recent investigation, the capacity to form constitutive aerenchyma increased flooding tolerance at the seedling stage, as evaluated by the shoot dry-weight ratio ([shoot dry weight in flood]/[shoot dry weight in control]) of aerenchyma-forming and non-forming *Z. nicaraguensis* accessions (Mano & Omori 2013b). A

more precise comparison to assess the beneficial effects of aerenchyma formation in the same genetic background is now in progress using Mi29 and the four-QTL pyramided line described above.

3. Flooding tolerance under reducing conditions

Under flood conditions, toxic chemicals (e.g., Mn²⁺, Fe²⁺, H₂S) induced by reducing soil conditions (low redox potential) cause serious problems in addition to those posed by low-oxygen stress (Ponnamperuma 1984), and soil reduction is promoted by high-temperature conditions encountered during the maize growing season. Recently, we developed a series of introgression lines (ILs), each possessing a chromosome segment of *Z. nicaraguensis* in the genetic background of Mi29; together, these lines cover the entire *Z. nicaraguensis* genome (Mano & Omori 2013a). By screening the ILs under artificial reducing soil conditions (by adding soluble starch in tap water as reported in Mano & Takeda 2012), we selected the extremely tolerant line IL#18 (Fig. 4). This line contains a fragment representing most of the long arm of chromosome 4 in *Z. nicaraguensis*, a region where no recombination was observed (Fig. 3). Here, we name the QTL in this region that is associated with flooding tolerance under reducing conditions as “*Qft-rd4.07-4.11*”. In greenhouse experiments, the usefulness of *Qft-rd4.07-4.11* was verified in an F₁ hybrid of IL#18 × Mi47, i.e. a version of cv. ‘Yumechikara’ heterozygous for *Qft-rd4.07-4.11* (Mano &

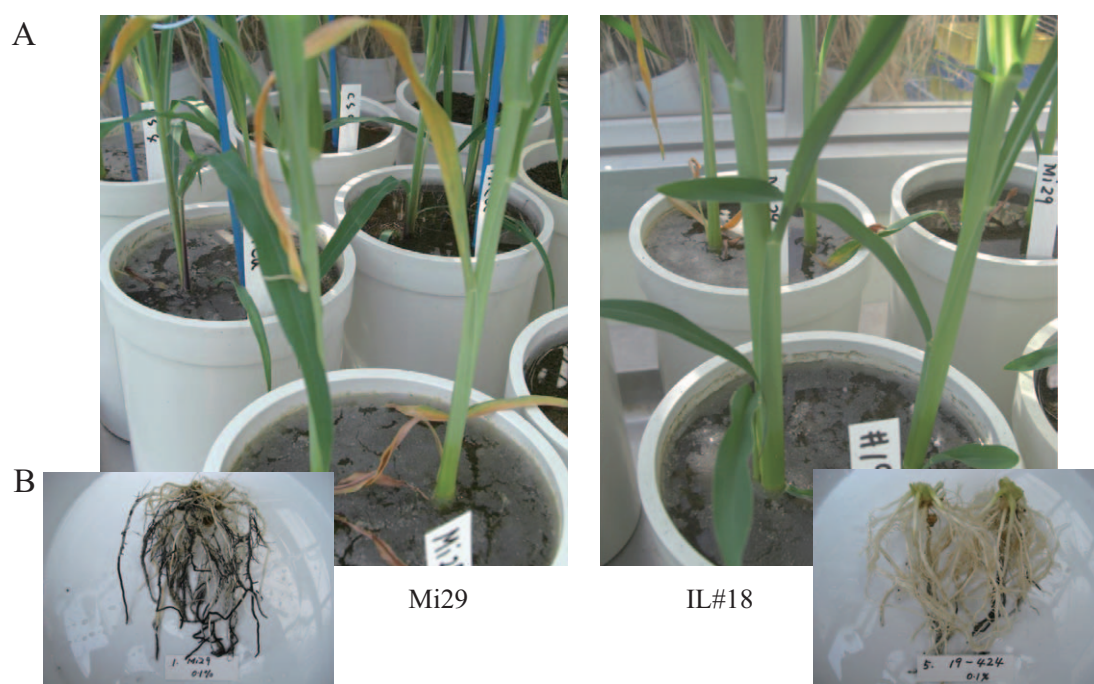


Fig. 4. Response to flooding under reducing conditions in maize Mi29 (left) and IL#18 (right)

(A) Aboveground parts of plants flooded at the five-leaf-stage with 1 g L⁻¹ soluble starch solution for two weeks.

(B) Roots of plants flooded at the three-leaf stage with 1 g L⁻¹ soluble starch solution for four weeks.

Omori 2015). As the gene action of *Qft-rd4.07-4.11* is additive or partial dominance (Mano & Omori 2013a), we introgressed the QTL into two male parents (Na50 and Mi47) used to produce F₁ hybrid cultivars, and thus completed the development of F₁ hybrids homozygous for the flooding-tolerance QTL allele (Mano & Omori, unpublished).

4. Other traits

Using the ILs described above, we have developed a NIL possessing a QTL (*Qarf3.04*) for flooding-induced aboveground adventitious roots (Fig. 3). Although flooding tolerance in the developed NIL has yet to be evaluated, we expect that the NIL will be able to obtain oxygen directly from the air via the aboveground adventitious roots, possibly increasing its flooding tolerance.

The ROL barrier is also important for flooding tolerance because it facilitates oxygen diffusion to root apices by preventing radial loss of oxygen from the roots (Colmer 2003, Watanabe et al. 2013). Most recently, our collaborators at Nagoya University have succeeded in identifying the chromosome location of a QTL for ROL barrier (Nakazono et al. 2015). NILs containing the ROL barrier QTL will be available after the removal of undesirable teosinte fragments through DNA marker-assisted backcrossing and selfing; and pyramiding QTLs for constitutive aerenchyma and ROL barrier is now in progress. In addition, QTL information regarding flooding-tolerance-related traits in *Zea* species other than *Z. nicaraguensis* is being accumulated (e.g., Mano et al. 2008, Omori & Mano 2007).

Field experiments

1. Field experiments by other researchers

Intensive studies have been done on the flooding and/or waterlogging tolerance of maize, mainly in Monsoon Asia where there is much rainfall in summer. The level of flooding tolerance can change during plant development: early growth stages are generally more sensitive to flooding than later stages (e.g. Ren et al. 2014). Other authors have reported that maize is comparatively more susceptible to excess moisture stress before the tasseling stage (e.g. Palvadi & Lal 1976, Zaidi et al. 2003), and that excessive soil moisture can prolong the anthesis–silking interval, resulting in poor grain yield (Rathore et al. 1997, Zaidi et al. 2003, Zaidi et al. 2002).

Zaidi et al. (2007) conducted field experiments in which 25 maize inbred lines were tested under excessive moisture conditions (average ponding depth of 10 cm for 12 days at V7-V8 growth stage) and found that genotypes tolerant to these conditions tended to have higher numbers of nodes with brace roots, higher ability to produce root

porosity, and lower ethanol concentrations in leaves and roots. They noted that tolerant genotypes could be classified into three groups: those having predominantly morphological adaptations, those having predominantly metabolic adaptations, and those having both types of adaptations to the same extent. Zaidi et al. (2010) also reported half-diallel (7 × 7 combinations) and line × tester (8 × 3 combinations) genetic analyses showing that both general combining ability (GCA) and specific combining ability (SCA) are significant, and have proposed reciprocal recurrent selection as an effective strategy for the improvement of flooding tolerance.

To our knowledge, no research group other than our own has yet to conduct field trials using flooding-tolerant maize lines developed via DNA marker-assisted selection or other approaches. Because we have identified some highly tolerant lines in greenhouse experiments (Fig. 4), we conducted field trials with these materials as described in the next section.

2. Field experiments at the NARO Institute of Livestock and Grassland Science

During the past few years, we have conducted experiments to detect and/or verify the tolerance of parental (inbred) and F₁ materials from our molecular breeding program under field conditions. In 2011, one of the fields at NARO was equipped with a farm-oriented enhanced aquatic system (FOEAS) that has since proven valuable in studies on flooding tolerance in soybean and other field crops (e.g. Shimada et al. 2012). This FOEAS field has allowed us to conduct large-scale experiments and accelerate our research. At the moment we are focusing on flooding tolerance at about the six-leaf stage because maize usually reaches this stage at the end of June, the most flooding-prone period of the year, and because it is the stage when constitutive aerenchyma formation promotes flooding tolerance (Mano et al. 2007). In these field experiments, the flood conditions produced by irrigating from the irrigation channel (water depth of about 5 to 10 cm) were maintained for about two weeks beginning on June 21 (Fig. 5) in 2013 and 2014, and the growth (e.g. plant height, number of leaves, silking date) and yield of each genotype were compared to those of the same genotype, sown on exactly the same date, in a non-irrigated field at our station. We have accumulated data demonstrating the high flooding tolerance of genotypes containing genomic regions from teosinte, and are planning to release the first flooding-tolerant F₁ maize hybrid within a few years.

Molecular analysis for studying flooding stress

1. Screening for genes expressed under flooding stress

Gene expression analyses of maize roots under



Fig. 5. Field test for flooding tolerance in maize F₁ hybrids. Six-leaf-stage plants were flooded for two weeks and then drained until harvest. This photo was taken on the first day of flooding

flooding stress have revealed many genes related to this response. Several studies conducted within the last decade were large-scale analyses providing an overview of gene expression patterns. For instance, flooded maize roots were analyzed by using suppression subtractive hybridization (SSH) and cDNA microarrays. The spectrum of genes induced in flooded maize roots in the first 0.5 h was found to be distinct from that induced after 2-4 h of flooding (Zhang et al. 2006). In the early stages of flooding, expression of transcription factor genes (including *r2*, which encodes a Myb protein that possibly binds to the GT-motif in the promoter of anaerobic response genes) markedly increased and reached a peak after 1 h of flooding, indicating that these gene products play a crucial role in regulating late-stage response genes related to the pathways for glycolysis, respiration, lipid metabolism, signal transduction, and protein metabolism (Zhang et al. 2006). Thirunavukkarasu et al. (2013) demonstrated the activation of these pathways with microarray data showing a large number of up-regulated genes in roots flooded for seven days, including genes associated with the synthesis of ethylene and auxin, and with the subsequent formation of aerenchyma and adventitious roots.

Tissue-specific expression analysis focused on aerenchyma formation in maize cortical cells has also been conducted with laser microdissection (Rajhi et al. 2011). In that study, upregulation or downregulation of many genes related to such molecular functions as reactive oxygen species (ROS) generation or scavenging, Ca²⁺ signaling, and cell wall modification was observed. Further, the induction or suppression of these genes was associated with ethylene perception inhibitor 1-methylcyclopropene (1-MCP), indicating that this experiment allowed the specific identification of ethylene-responsive genes that are possibly

related to lysigenous aerenchyma formation. Co-expression of some of the identified transcripts might indicate their involvement in the above-mentioned metabolic pathways. Interestingly, some of these transcripts could be targets of microRNAs (miRNAs), which could be critical post-transcriptional modulators for stress tolerance and root development in response to flooding (Liu et al. 2012, Zhai et al. 2013).

2. Candidate gene approaches

Although the above-mentioned approaches based on transcriptional analyses provide an overview of the molecular networks responsible for flooding tolerance, specific candidate genes must also be identified. To this end, some expressed sequence tag (EST) clones of possible candidate genes identified by microarray analysis and/or SSH have been aligned on the maize genome, and confirmed to be located near molecular markers tightly linked to known QTLs associated with flooding tolerance (Osman et al. 2013, Thirunavukkarasu et al. 2013, Zou et al. 2010). Similarly, miRNAs have been mapped near QTLs responsible for flooding tolerance. Interestingly, the role of these miRNAs in triggering the formation of adventitious roots, lateral roots, and crown roots, and in adapting to hypoxia via regulating transcription of the target genes may explain the function of these QTLs (Osman et al. 2013). Another candidate gene approach focused on constitutive aerenchyma formation has also been applied using *Z. nicaraguensis* (Abiko et al. 2012b). In that study, the authors compared cDNA libraries from Mi29, *Z. nicaraguensis*, and line BC₄F₁ #62, which has the teosinte-derived locus *Qaer1.05-1.06* on chromosome 1. Six genes located at or near *Qaer1.05-1.06* were identified as candidates.

3. Isolation of *Qft-rd4.07-4.11*

In addition to the efforts mentioned above, we and our collaborator, Dr. Hidekazu Takahashi, an associate professor at Akita Prefectural University, have just started a project to clone *Qft-rd4.07-4.11*, the QTL for flooding tolerance under reducing conditions, with next-generation sequencing. We used IL#18, which has the *Qft-rd4.07-4.11* region of *Z. nicaraguensis* in the genomic background of Mi29 (Fig. 3). We have obtained a large amount of sequence data from Mi29, *Z. nicaraguensis*, and IL#18, which are now in the process of being aligned with the reference genome sequence of maize inbred line B73 (Schnable et al. 2009), thereby constructing a physical map of each line. Teosinte-derived regions in the IL#18 genome (identified by comparing it with the physical maps of Mi29 and B73) will be expected to contain candidate gene(s). To enhance the precision of gene identification, we are also conducting RNA-seq analyses. Alignment of the transcript

sequences detected by RNA-seq with the physical maps might facilitate identification of the candidate gene(s). This approach should make it possible to use this QTL in other upland crops.

Conclusion

Although flooding tolerance is a complex trait, we have outlined a breeding strategy for increasing flooding tolerance in maize (Mano & Omori 2007, Mano & Oyanagi 2009). Maize materials useful for practical breeding of flooding tolerance in Japan have been developed, and we are preparing to release a flooding-tolerant F₁ variety within a few years. We expect that the genetic information and materials obtained in our study will contribute to the practical breeding of maize and other upland crops in flooding-affected regions worldwide.

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