Flooding tolerance in interspecific introgression lines containing chromosome segments from teosinte (Zea nicaraguensis) in maize (Zea mays subsp. mays)

Y. Mano* and F. Omori

Forage Crop Research Division, NARO Institute of Livestock and Grassland Science, 768 Senbonmatsu, Nasushiobara, Tochigi 329-2793, Japan

* For correspondence. E-mail: mano@affrc.go.jp

Received: 25 April 2013 Returned for revision: 28 May 2013 Accepted: 31 May 2013 Published electronically: 21 July 2013

INTRODUCTION

Sets of near-isogenic lines, which contain chromosome segments from a donor parent on the genetic background of a recurrent parent (Szalma et al., 2007), have been widely used for the detection of quantitative trait loci (QTLs) and detailed mapping related to biologically or agronomically important traits. Such sets have been called introgression libraries (ILs) in maize (Salvi et al., 2011; Welcker et al., 2011) and rye (Falke et al., 2008, 2009); introgression lines (ILs) in rice (Tian et al., 2006), barley (von Korff et al., 2004) and tomato (Eshed and Zamir, 1995; Canady et al., 2005); chromosome segment substitution lines (CSSLs) in rice (Kubo et al., 2002; Wan et al., 2004; Ebitani et al., 2005); and recombinant chromosome substitution lines (RCSLs) in barley (Matus et al., 2003; Hori et al., 2005; Sato et al., 2011). In some of these reports, wild relatives of crop species were used as donor parents because such relatives are often useful sources of biotic and abiotic stress tolerance. In rice (Oryza sativa), O. rufipogon was used as the donor parent to map loci controlling drought tolerance (Zhang et al., 2006) and resistance to rice blast (Hirabayashi et al., 2010), and O. glaberrima was used as the donor to map a QTL for rice stripe necrosis virus resistance (Gutiérrez et al., 2010). In barley, RCSLs in which segments of Hordeum spontaneum were introgressed into cultivated barley (H. vulgare) were used to identify several agronomic traits (Hori et al., 2005) and resistance to powdery mildew and leaf rust (Schmalenbach et al., 2008). Until now, however, there have been no reports of introgression libraries generated from interspecific crosses involving maize (Zea mays subsp. mays).

For the development of ILs (here defined as introgression lines), selection of a suitable donor parent is important. In our research programme, we wanted to identify potential sources of flooding tolerance to improve maize. Nicaraguan teosinte (Z. nicaraguensis), a wild relative of maize, was discovered in far northwestern Nicaragua in an area that is frequently flooded for many weeks at a time (Bird, 2000; Ilitis and Benz, 2000). Zea nicaraguensis is considered to be useful as a donor parent for IL development because it exhibits several traits related to flooding tolerance (Mano and Omori, 2007) as well as agriculturally useful morphological characteristics. Zea nicaraguensis is an outcrossing species and several of its characteristics, including morphological traits (e.g. plant height, tiller number and leaf width) and the capacity to form root aerenchyma, were found to segregate within accessions of this teosinte (Mano et al., 2012). In addition, wide genetic variation within
populations was reported in another study of *Z. nicaraguensis* in which seven simple sequence repeat (SSR) markers (Loáisiga *et al.*, 2011). Therefore, it was necessary for us to select a specific population or genotype of donor plants of *Z. nicaraguensis* to use for IL development.

In a previous study, we generated a stable aerenchyma-forming *S*₂ generation of *Z. nicaraguensis* (*nica*-S₂) plants suitable for use as donor parents (Mano and Omori, 2008). Segregating populations (*F₂*, BC₁₁F₁ and BC₂F₁) derived from a single *F₁* plant of a cross between Mi29 (a maize inbred line) and *Z. nicaraguensis* (*nica*-S₂) showed wide variation for the capacity to form root aerenchyma and the ability to form surface roots (Mano and Omori, 2007). Therefore, ILs originating from this *F₁* plant should be useful for identifying QTLs controlling traits related to flooding tolerance. By using *F₂* or backcross populations, we have previously identified several QTLs controlling traits related to low-oxygen stress in *Z. nicaraguensis*, such as the capacity to form constitutive root aerenchyma and the ability to form underground adventitious roots during flooding (Mano and Omori, 2007). By evaluating these traits in ILs, it will be possible to verify QTL positions, detect new QTLs and develop near-isogenic lines with only a few backcrosses.

In addition to the stress caused by excessive water or low oxygen levels, soil flooding under reducing or low redox potential (*Eh*) conditions also poses serious problems (Yamasaki, 1952; Pezeshki, 2001; Mano *et al.*, 2006), such as the production of phytotoxins (e.g. Fe²⁺ and H₂S; Ponnamperuma, 1984). Physiological analyses of flooding tolerance under reducing soil conditions have been reported for several wetland species. For example, root-induced iron oxidation (Begg *et al.*, 1994) and barriers to radial oxygen loss (Colmer, 2003) were found to be related to tolerance under reducing conditions. In a genetic analysis, a maize QTL for tolerance to reducing conditions was identified on chromosome 1 (bin 1.03–1.04) by using a mapping population constructed from F₁649 × H84; however, the effect of the QTL was small (Mano *et al.*, 2006). Because *Z. nicaraguensis* has shown high tolerance to reducing soil conditions (Mano and Omori, 2007), it may be possible to find additional QTLs with larger effects by using ILs.

The objective of this study was to develop a series of ILs, each containing a chromosome segment from *Z. nicaraguensis* (*nica*-S₂) on the genetic background of elite maize inbred Mi29. We then used the ILs to search for QTLs associated with flooding tolerance under reducing soil conditions. In addition, we examined one of the most important traits related to flooding tolerance: the capacity to form root aerenchyma. We tested the relationship between the capacity to form constitutive aerenchyma (formed without any external stimulus) or inducible aerenchyma (induced by external stimuli such as hypoxia, drought and mechanical impedance) and flooding tolerance under reducing soil conditions.

**MATERIALS AND METHODS**

*Development of ILs*

The teosinte *Zea nicaraguensis* H. H. Ilits & B. F. Benz (CIMMYT 13451) was obtained from the International Maize and Wheat Improvement Center (CIMMYT), Mexico. The elite maize inbred line Mi29, developed at the National Agriculture and Food Research Organization (NARO) Agricultural Research Center for Kyushu Okinawa Region (Ikegaya *et al.*, 1999), was obtained from the Forage Crop Division, NARO Institute of Livestock and Grassland Science. Our earlier studies revealed that *Z. nicaraguensis* possesses several traits related to flooding tolerance, whereas Mi29 lacks these traits (Mano and Omori, 2007, 2008). Prior to crossing to maize, *Z. nicaraguensis* was self-pollinated twice and a stable aerenchyma-forming *S*₂ plant was selected (*nica*-S₂; Mano and Omori, 2008).

Figure 1 presents the scheme used for the development of the ILs. A single *F₁* plant derived from a cross between Mi29 and *Z. nicaraguensis* (*nica*-S₂) was backcrossed in the greenhouse to Mi29 (as the female), and 36 BC₁F₁ plants were obtained. These 36 BC₁F₁ plants were backcrossed in the greenhouse using Mi29 as the pollen parent to produce BC₂F₁ seeds. Fifteen BC₂F₁ seeds from each of 36 BC₁F₁ spikes (a total of 540 seeds) were sown in the field at the NARO Institute of Livestock and Grassland Science, and 262 randomly selected BC₂F₁ plants were backcrossed a third time using Mi29 as the pollen parent. For marker-assisted selection, we tested a total of 350 BC₃F₁ individuals (representing one or two BC₂F₁ seeds from each of the 262 BC₂F₁ spikes). At the BC₃F₁ generation, 98 maize SSR markers were used for genotyping across the whole genome. A total of 45 lines were selected and self-pollinated three times to produce BC₄F₄ lines. During the

**FIG. 1. Scheme used for the development of a library of introgression lines, each containing a chromosome segment from *Z. nicaraguensis* on the genetic background of maize inbred line Mi29. MAS, marker-assisted selection.**
Floodling tolerance of the ILs under reducing soil conditions
(Experiment 1)

Experiment 1 was conducted in a greenhouse under uncontrolled conditions except that the minimum temperature was kept above 15 °C by heating. Detailed experimental conditions are presented in Table 1. Four ILs (IL#17, IL#20, IL#21 and IL#40) were not tested here because the seed supply was limited, so a total of 41 ILs and the parents (four plants per line in a single pot) were grown in drained soil in pots 16 cm in diameter and 19 cm deep, with three replications per line. The pots contained granular soil (Kureha Chemical Industry, Tokyo, Japan; pH 6.5; 0.4 g N, 1.0 g P₂O₅, 0.6 g K₂O kg⁻¹ soil; soil density 0.85 kg L⁻¹; granule diameter 1.5–3.0 mm). At 16 d after sowing (corresponding to the three-leaf stage), seedlings were flooded with 1 g L⁻¹ soluble starch solution (Wako, Osaka, Japan) to 1 cm above the soil surface for 16 d. Treatment with soluble starch has been used to promote reducing conditions in substrates in experiments on rice (Ishihara et al., 2008) and barley (Mano and Takeda, 2012). Redox potentials (Eₚ) at 10 cm depth and pH at 2 cm depth were measured with platinum-tipped electrodes and a millivolt meter (Model PRN-41, with a 4400–0.65C electrode for Eₚ and a 6550 electrode for pH; Fujiwara Scientific Company, Tokyo, Japan).

The chlorophyll contents of leaves were measured just before flooding and after 16 d of treatment at the middle of the second and third leaves by using a SPAD (Soil Plant Analysis Development) meter (SPAD-502Plus, Konica Minolta, Tokyo, Japan). After 11 and 16 d of treatment (27 and 32 d after sowing), the degree of leaf injury per plant was scored visually on a scale reflecting the degree of chlorosis, with, for example, 0 = no chlorosis, 0.1 = chlorosis in 10% of the first leaf, 0.5 = chlorosis in 50% of the first leaf, 1.0 = chlorosis in 100% of the first leaf, and 1.5 = chlorosis in 100% of the first leaf and 50% of the second leaf. Increments of 0.1 were used, and the treatment mean was calculated. Because Yamasaki (1952) reported that leaf chlorosis under reducing conditions started at the lowest leaf, and this situation was also confirmed in our previous investigation (Mano et al., 2006), this scoring method was judged to be appropriate for the present study. The shoots of each plant were harvested after 16 d of treatment, dried at 70 °C for 5 d and weighed. The same experimental procedures were applied in Experiments 2 and 3 (described below) unless otherwise stated.
Verification of chromosome location for flooding tolerance QTL (Experiment 2)

In the IL survey (Experiment 1), IL#18 showed high tolerance to flooding under reducing soil conditions (see Results). This line contained a portion of the long arm of chromosome 4 from *Z. nicaraguensis* on the genetic background of Mi29. To verify the chromosome location of this QTL, a total of 180 plants showing the expected *F*₂ segregation ratio for the long arm of chromosome 4 were obtained from the mother plant of IL#18 (*BC*₂*₉* generation; Fig. 1) which was heterozygous for this chromosome arm. A total of 180 segregating *F*₂ plants and the parents were grown in drained soil in pots 11 cm in diameter and 14 cm deep containing granular soil. At 20–22 d after sowing (corresponding to the 2-5-leaf stage), seedlings were flooded with 1 g L⁻¹ soluble starch solution for 11 d. The chlorophyll contents of leaves were measured just before the flooding treatment and after 11 d of treatment at the middle of the first, second and third leaves. Each day from days 6–11 of treatment, the degree of leaf injury was visually scored as described for Experiment 1. Comparisons of flooding tolerance (as evaluated by leaf injury and SPAD values) were made among groups of plants homozygous for Mi29 alleles (designated A), heterozygous plants (H) and plants homozygous for *Z. nicaraguensis* (B) alleles for three SSR markers on chromosome 4: bnlg1784 (bin 4-07), bnlg1917 (bin 4-10) and umc1716 (bin 4-11). The effects of the QTL on chromosome 4 were estimated using a simple linear regression model in Windows QTL Cartographer 2.5 (Wang et al., 2006).

Flooding tolerance evaluated by shoot and root biomass (Experiment 3)

To compare flooding tolerance under reducing soil conditions on the basis of shoot and root biomass, two contrasting ILs [IL#18 (tolerant) and IL#24 (sensitive)] and their parents were tested. The seedlings were grown under non-flooded conditions in free-draining pots 16 cm in diameter and 30 cm deep, at two plants per pot. Four plants (grown in two pots) of each genotype–treatment combination (flooded or non-flooded) were evaluated with three replications. At 20 d after sowing (corresponding to the 2.5–3.0 leaf stage), seedlings were flooded with 1 g L⁻¹ soluble starch solution for 15 d. The chlorophyll contents of leaves were measured after 15 d of treatment at the middle of the first to fourth leaves in non-flooded and flooded plants. The shoots and roots of each plant were harvested after 15 d of treatment, dried at 70 °C for 5 d and weighed.

Evaluation of constitutive aerenchyma formation in the ILs (Experiment 4)

To examine the relationship between the capacity to form constitutive aerenchyma and flooding tolerance under reducing soil conditions, experiments were conducted from 30 May to 23 July 2012 in a greenhouse maintained at a temperature of 28 °C during the day and 25 °C during the night with natural light for 14-5 h each day. All but one of the ILs that were tested for flooding tolerance in Experiment 1 were used here; IL#35 was not tested because the nodal root elongation of this line was inhibited (about 50 % that of maize Mi29) under normal (non-flooded) conditions. The remaining 40 ILs and the parents (four plants per line) were grown in drained soil in pots 11 cm in diameter and 30 cm deep at one plant per pot, with two replications per line. The pots contained the same type of granular soil used in Experiment 1. At ~4 weeks after sowing, one or two adventitious (shoot-borne crown) roots (30–35 cm long) emerging at the second node from the bottom of seedlings at the six-leaf stage were collected for sectioning and aerenchyma measurement.

Aerenchyma measurement was conducted as described previously (Mano et al., 2006). Briefly, cross-sections were made 10 and 15 cm from the tip of each root and up to four positions per plant were observed (i.e. up to two roots with two cross-sections per root). To evaluate a large number of samples in a short time, we used a simple measurement method, visually scoring the degree of aerenchyma formation in the root cortex on a scale of 0–3: 0 (no aerenchyma), 0.5 (partial formation), 1 (radial formation), 2 (radial formation extended to the outer cortex) and 3 (well-formed aerenchyma). Examples of photographs illustrating these degrees of constitutive aerenchyma formation were presented by Mano and Omori (2013). The average score of the four positions was used as a measure of the capacity to form constitutive aerenchyma in each plant.

Evaluation of inducible aerenchyma formation in the selected ILs (Experiment 5)

A total of seven ILs with varying levels of flooding tolerance (evaluated by leaf injury under reducing conditions in Experiment 1) and their parents were used to assess the capacity to form inducible aerenchyma. The contrasting ILs consisted of three tolerant lines (IL#18, IL#30 and IL#39, selected based on leaf injury at 11 and 16 d), one moderately tolerant line (IL#29, based on leaf injury at 11 and 16 d) and two sensitive lines (IL#22 and IL#24, selected based on leaf injury at 16 d or SPAD values for the second leaf). In addition, a line constitutively forming aerenchyma, IL#4 (selected in Experiment 4; see Results), was also tested. Root samples of these lines and the parents were collected from the same plants as those used in Experiment 1. Root aerenchyma area was measured in cross-sections from the basal (5 mm from the shoot–root junction) and middle parts of the longest adventitious root of each plant (root lengths are given in the caption of Fig. 10). Four plants of each genotype were evaluated with three replications.

The areas of root cortex and aerenchyma were measured by using ImageJ software (version 1.39; National Institutes of Health, Bethesda, MD, USA). The capacity to form inducible aerenchyma was evaluated by calculating the percentage of root aerenchyma in the root cortex.

Marker order in an inverted region of chromosome 4

During the development of the ILs in this study, we detected recombination suppression on the long arm of chromosome 4 (see Results). In addition, the precise position of the QTL on chromosome 4 in IL#18 could not be identified because of a lack of recombination in this region. To determine the detailed marker order in the recombination-suppression region, we constructed an *F*₂ mapping population from a cross between *Z. nicaraguensis*, which contains an inversion (relative to
maize) in chromosome 4 (Mano et al., 2012), and Z. luxurians (PI 441933), which was expected to have a similar inversion in chromosome 4 (Mano et al., 2008). To detect polymorphism between Z. nicaraguensis and Z. luxurians, we screened 92 SSR markers in bins 4.05–4.11 (obtained from MaizeGDB). For the linkage analysis, a total of 120 Z. nicaraguensis × Z. luxurians F₂ plants were genotyped.

To construct the F₂ linkage map of chromosome 4 for the cross between Z. nicaraguensis and Z. luxurians, we used an F₂ model in the MAPMAKER/EXP 3.0 program (Lander et al., 1987). The linkage map was constructed by using the ‘3-point’ and ‘order’ commands at log likelihood ratio (LOD) > 3.0 and then at LOD > 2.0. Markers that could not be placed on the map at LOD > 2.0 were designated ‘accessory markers’ and placed to the right of the framework markers. The calculations in MAPMAKER are based on Haldane’s mapping function.

**Statistical analyses**

Significant differences for leaf injury scores, SPAD values and shoot dry weights (Experiment 1) and the capacity to form constitutive or inducible aerenchyma (Experiments 4 and 5) between Mi29 and each IL were tested using Dunnett’s multiple comparison test. Simple Pearson correlation coefficients (r) were calculated between traits related to flooding tolerance using the adjusted means (Experiment 1). For Experiments 2 and 3, data were tested by ANOVA followed by Tukey’s test. The non-parametric Kruskal–Wallis test was used when data failed normality or equal variance tests. When two means were compared, significant differences were calculated from the paired t-test. All analyses were conducted using Microsoft Excel 2010 software for Windows.

**RESULTS**

**Development of ILs**

By using 98 SSR markers, we developed a series of 45 ILs covering nearly the entire maize genome (Fig. 2). Each IL contained one to seven chromosome segments from Z. nicaraguensis, with an average of 2.6 segments per line. The length of substituted Z. nicaraguensis segment(s) as a proportion of total genome length ranged from 0.2% to 15.2% (average 6.8%) based on the map distance in the Mi29 × Z. nicaraguensis BC₂F₁ map (Mano and Omori, 2008). No homozygotes were recovered for a segment from Z. nicaraguensis on the short arm of chromosome 3 because of segregation distortion (Fig. 2).

Some ILs produced only a small number of seeds because of small female spike size (IL#20, IL#21 and IL#40) or partial male sterility. Five ILs segregated for male-sterile or partially male-sterile plants in the BC₂F₃ and BC₃F₄ progenies grown under field conditions: IL#8, IL#11, IL#17, IL#18 and IL#25. These lines all contained a segment of Z. nicaraguensis (either homozygous or heterozygous) from bin 4.06 to bin 4.11, suggesting the presence of one or more male-sterility factors in this region. However, the map position of the gene(s) controlling male sterility could not be identified because of the presence of an exceptional line, IL#9; this line was fertile even though it contained a Z. nicaraguensis segment from bin 4.07 to bin 4.11. In addition, the male sterility in the five other lines containing segments in this region was unstable and depended upon the growing environment (data not shown).

During the development of the ILs, there was no recombination on the long arm of chromosome 4 from bnlg1784 (bin 4.07) to umc1716 (bin 4.11) in 141 and 48 individuals in the BC₂F₁ and BC₃F₂ generations, respectively. The length of the interval without recombination was estimated to be ~60–70 cM (Mano et al., 2005).

**Flooding tolerance under reducing soil conditions in the ILs**

(Experiment 1)

Figure 3 shows variation among the ILs in traits related to flooding tolerance (i.e. the degree of leaf injury, chlorophyll content of leaves (SPAD values) and shoot dry weight) after flooding with soluble starch solution. In this study, surface roots developing in response to flooding were not observed in the ILs and their parents. The leaf injury scores at 11 and 16 d varied greatly between the parents, Mi29 and Z. nicaraguensis (nica-S₂). The leaf injury scores of 11 ILs were significantly (P < 0.05) lower than those of Mi29 (indicating greater tolerance) at both 11 and 16 d after the start of treatment. In particular, five lines showed tolerance at both time points: IL#13, #18, #30, #36 and #39 (P < 0.0001). Among the sensitive ILs, IL#24 had a significantly higher leaf injury score than Mi29 at 16 d.

To obtain SPAD values, we measured the second and third leaves after 16 d of treatment. We found SPAD values significantly higher than those of Mi29 (indicating greater tolerance) in IL#6 and IL#18 for the second leaf and in IL#18 and IL#30 for the third leaf, whereas significantly lower values (less tolerance) were found in IL#22, #24, #34 and #35 for the second leaf and in a total of nine ILs for the third leaf (Fig. 3C, D). For subsequent experiments, we selected IL#18 as the most tolerant genotype (Fig. 4B) and IL#24 as the most sensitive genotype, based on the degree of leaf injury.

Flooding-tolerant line IL#18 possessed only one segment (in homozygous form) from Z. nicaraguensis, on the long arm of chromosome 4 (Fig. 2), suggesting the presence of a QTL in this region. Four other lines (IL#8, IL#9, IL#11 and IL#25) possessed the same segment of Z. nicaraguensis in heterozygous form, and three out of four of these ILs showed significantly greater flooding tolerance than Mi29 at one or both time points (Fig. 3A, B). These results support the presence of a flooding-tolerance QTL on the long arm of chromosome 4.

In addition to the QTL on chromosome 4, the data also suggested the presence of a QTL on chromosome 2. For example, IL#6 showed significantly better flooding tolerance than Mi29, but its only segment from Z. nicaraguensis was a portion of chromosome 2 (Figs 2 and 3). Three out of five other lines (IL#13, IL#30 and IL#41) that had all or part of the same segment also showed significantly better flooding tolerance than Mi29. By combining flooding tolerance data and marker information on chromosome 2, a QTL for flooding tolerance under reducing soil conditions can be mapped between bnlg1092 and bnlg2277 (17.4 cM, based on the map of Mi29 × Z. nicaraguensis; Mano and Omori, 2008), but further linkage analyses are necessary to verify this QTL.

After 16 d of treatment, Mi29 had significantly higher shoot dry weight (0.606 ± 0.047 g, mean ± s.d.) than Z. nicaraguensis (0.220 ± 0.045 g), and there were wide differences among the
**FIG. 2.**  Graphical genotypes of the introgression lines (ILs), showing introgressed chromosome segments from teosinte *Z. nicaraguensis* letter B indicate chromosomal regions homozygous for *Mi29* alleles, white bars containing the letter A indicate regions homozygous for *Mi29* alleles, and light grey bars containing the letter H indicate heterozygous regions. C and ‘open’ indicate a dominant marker (equivalent to B or H) and missing data, respectively, and are shaded based on our best estimate of the genotype. SDR and Suppression indicate the approximate positions of segregation distortion regions and recombination suppression regions, respectively.
FIG. 3. Traits related to flooding tolerance in 41 ILs, the recurrent parent (Mi29) and the donor parent [Z. nicaraguensis (nica)] in Experiment 1. Seedlings at the three-leaf-stage were flooded with 1 g L\(^{-1}\) soluble starch solution to 1 cm above the soil surface for 16 d. (A) Leaf injury score after 11 d of treatment, (B) leaf injury score after 16 d of treatment, (C) SPAD meter values in the middle of the second leaf after 16 d of treatment, (D) SPAD values in the middle of the third leaf after 16 d of treatment, (E) shoot dry weight after 16 d of treatment. Leaf injury scores are explained in the Methods section. Bars indicate mean ± s.d. (\(n = 12\)). The horizontal dotted lines indicate the mean value of Mi29 for each trait. * and ** indicate significant differences between Mi29 and the ILs at the 5 % and 1 % levels, respectively, by Dunnett’s test.
41 ILs (Fig. 3E). Because the seedlings were severely damaged by the flooding treatment with soluble starch solution, plant growth was inhibited even in the tolerant lines; therefore, shoot dry weight after flooding was largely dependent on plant growth prior to flooding. This association was confirmed by the high correlation coefficient between plant height just before treatment and shoot dry weight 16 d after treatment \( (r = 0.833, P < 0.01; \text{data not shown}) \).

Correlation coefficients among six traits (the five traits in Fig. 3 plus SPAD of the second leaf at day 0) are presented in Table 2. Highly significant correlations were observed between flooding tolerance evaluated by the degree of leaf injury 11 and 16 d after treatment and SPAD values measured in the second leaves after 16 d of treatment \( (r = -0.776 \text{ and } -0.862, P < 0.01) \). Significant correlations were observed between SPAD values just before treatment in the second leaf and those after flooding for 16 d \( (0.462 \text{ for the second leaf and } 0.323 \text{ for the third leaf}) \) (Table 2). However, these values were moderate, and the SPAD value just before treatment in the sensitive parent Mi29 \( (50.4 \pm 4.9, \text{mean } \pm \text{s.d.}) \) was higher than the mean SPAD value of all 41 ILs \( (45.4 \pm 2.0) \). For this reason, we considered the effect of the original chlorophyll content of leaves on flooding tolerance evaluated by SPAD values to be small.

### Table 2. Correlation coefficients among six traits in 41 ILs (Experiment 1). The three-leaf-stage seedlings were flooded with 1 g L\(^{-1}\) soluble starch solution to 1 cm above the soil surface for 16 d

<table>
<thead>
<tr>
<th>Trait</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>LI (11 d)</td>
<td>0.849**</td>
</tr>
<tr>
<td>SPAD second (0 d)</td>
<td>-0.337*</td>
</tr>
<tr>
<td>SPAD second (16 d)</td>
<td>-0.776**</td>
</tr>
<tr>
<td>SPAD third (16 d)</td>
<td>-0.373*</td>
</tr>
<tr>
<td>SDW (16 d)</td>
<td>-0.327*</td>
</tr>
</tbody>
</table>

*LI (11 d), LI (16 d), leaf injury under reducing soil conditions 11 and 16 d after the start of treatment; SPAD second (0 d), SPAD second (16 d), SPAD third (16 d) are SPAD values of second or third leaves just before flooding \( (0 \text{ d}) \) and after 16 d of flooding \( (16 \text{ d}) \); SDW (16 d), shoot dry weight under reducing soil conditions 16 d after the start of treatment.

**Significant at the 5 % level; **significant at the 1 % level.

**Verification of chromosome location for flooding tolerance QTL (Experiment 2)**

In Experiment 1, we selected a highly tolerant line, IL#18, containing a \( Z. \ nicaraguensis \) segment in the long arm of chromosome 4. To verify that this chromosome location is associated with flooding tolerance, we compared flooding tolerance among plants homozygous for the Mi29 alleles (designated A), heterozygous plants \( (H) \) and plants homozygous for the \( Z. \ nicaraguensis \) alleles \( (B) \) for three SSR markers that were heterozygous in the mother plant of IL#18 \( (BC_3F_4 \text{ generation}) \). No recombination was observed between these three SSR markers, which was in good agreement with our previous investigation \( (\text{Mano et al., 2012}) \). The segregation ratio of these markers \( (A:H:B = 14:86:80) \) was skewed from the expected ratio of 1:2:1, with a higher than expected frequency of \( Z. \ nicaraguensis \) \( (B) \) and a lower than expected frequency of Mi29 \( (A) \) \( (\chi^2 = 48.8, P < 0.01) \).

In the parents, leaf chlorosis began after 6 d of flooding in Mi29 and reached a score of 2.1 (chlorosis in 100 % of the first and second leaves and 10 % in the third leaves) after 11 d of flooding, whereas no or very little chlorosis was found in \( Z. \ nicaraguensis \) at any time (Fig. 5). In the progeny, leaf chlorosis began 6 or 7 d after treatment in the three genotypes and gradually increased. At the end of the treatment, flooding tolerance was highest (leaf injury score was lowest) in the \( Z. \ nicaraguensis \) \( (B) \) genotype, intermediate in the heterozygous \( (H) \) genotype and lowest in the Mi29 \( (A) \) genotype (Fig. 5). In the simple linear regression model, the effects of the QTL on chromosome 4 ranged from LOD = 11.9 and \( r^2 = 0.29 \) (6 d of treatment) to LOD = 25.5 and \( r^2 = 0.49 \) (8 d of treatment), indicating a major QTL in this region.

For SPAD values of the first, second and third leaves, no significant difference was observed among the three genotypes \( (A, H \text{ and } B) \) just before flooding treatment \( (\text{data not shown}) \). At the end of the treatment \( (11 \text{ d of flooding}) \), plants homozygous for the \( Z. \ nicaraguensis \) alleles \( (B) \) showed significantly higher SPAD values than those homozygous for Mi29 alleles \( (A) \) in
under reducing soil conditions, was located on the long arm of QTL controlling flooding tolerance, as evaluated by leaf injury. These results confirmed the finding in Experiment 1 that a flooded/control ratio (FL/C) of both control and flooded conditions (Table 3). To evaluate flooding tolerance, we calculated the flooded/control ratio (FL/C) of treatment.

Leaf injury scores are recorded each day from 6 to 11 d of flooding. Leaf injury scores were explained in the Methods section.

Flooding tolerance evaluated by shoot and root biomass (Experiment 3)

To compare flooding tolerance on the basis of shoot and root biomass, we tested two contrasting ILs [IL#18 (tolerant) and IL#24 (sensitive)] and the parents. After 8 d of flooding treatment, leaf chlorosis was observed in the sensitive lines Mi29 and IL#24. At the end of the treatment (15 d of flooding), the degree of leaf injury in Z. nicaraguensis (0.16 ± 0.16, mean ± s.d.) and IL#18 (0.01 ± 0.01) was lower than in Mi29 (1.04 ± 0.35) and IL#24 (1.34 ± 0.14), in good agreement with the results of Experiment 1. SPAD values were significantly decreased by the flooding treatment in the first, second and third leaves of all genotypes, with three exceptions out of 12 comparisons (Fig. 7). The decreases in SPAD value after treatment were small in Z. nicaraguensis and IL#18. For the fourth leaf, differences among the accessions were small, regardless of flooding treatment.

The shoot and root dry weights of Z. nicaraguensis were significantly lower than those of IL#18, Mi29 and IL#24 under both control and flooded conditions (Table 3). To evaluate flooding tolerance, we calculated the flooded/control ratio (FL/C) of shoot and root dry weight. The FL/C ratios for both shoot and root dry weight were significantly higher in Z. nicaraguensis than in Mi29 and IL#24. The ranking in order from highest to lowest FL/C value was Z. nicaraguensis, IL#18, Mi29, IL#24 for both shoot and root dry weight (Table 3). The shoot/root dry weight ratio (S/R), an important parameter for evaluating flooding tolerance, increased in response to flooding in all lines (data not shown), and no consistent difference was found in S/R between tolerant and sensitive lines.

Evaluation of constitutive aerenchyma in the ILs (Experiment 4)

The capacity to form constitutive aerenchyma was dramatically lower in Mi29 (0.11 ± 0.10, mean ± s.d.) than in Z. nicaraguensis (1.95 ± 0.27), and ranged from 0.00 (IL#29) to 0.79 (IL#8) in the 40 ILs tested (Fig. 8). The correlation coefficient between replications was 0.576 (n = 40, P < 0.01), indicating moderate repeatability of the experiment. Of the 40 ILs, four (IL#4, IL#8, IL#36 and IL#41) had constitutive aerenchyma scores significantly higher than that of Mi29. These four ILs each possessed three to seven chromosome segments of Z. nicaraguensis, so the exact chromosome locations of the QTLs controlling aerenchyma formation could not be determined at this point. The constitutive aerenchyma formation in line IL#4 (Fig. 8) might be due to the action of a previously reported QTL allele from Z. nicaraguensis on chromosome 1, Qaer1.06–1.07 (Mano et al., 2007; Mano and Omori, 2008), because IL#4 possessed a Z. nicaraguensis fragment (bin 1.03–1.10) that completely overlapped with the location of Qaer1.06–1.07. Qaer1.06–1.07 is noteworthy because its effects have been consistent throughout several experiments (Mano and Omori, 2008; Mano et al., 2012). Thus, we included
IL#4 in Experiment 5, in which we tested for the formation of inducible aerenchyma.

Correlation coefficients between the capacity to form constitutive aerenchyma and flooding tolerance in the 40 ILs were –0.289 (after 11 d of treatment) and –0.292 (after 16 d of treatment) for leaf injury and 0.264 (second leaf) and 0.080 (third leaf) for SPAD value 16 d after treatment, but neither correlation was statistically significant, suggesting the absence of a relationship between aerenchyma and flood tolerance. Figure 9 illustrates the relationship between constitutive aerenchyma score and flooding tolerance evaluated by leaf injury evaluated after 16 d of treatment. The most flooding-tolerant line, IL#18, had the same level of tolerance as Z. nicaraguensis, whereas the degree of constitutive aerenchyma in IL#18 was low, suggesting that IL#18 possesses flooding-tolerance-related traits other than the capacity to form constitutive aerenchyma.

Evaluation of inducible aerenchyma formation in the selected ILs (Experiment 5)

As seen for constitutive aerenchyma formation, the capacity to form inducible aerenchyma after 16 d of flooding under reducing soil conditions was clearly and significantly lower in Mi29 (mean ± s.d., 4.9 ± 5.5 % for basal parts of adventitious roots and 2.3 ± 1.7 % for middle parts of roots) than in Z. nicaraguensis (23.1 ± 8.0 % for basal and 9.6 ± 4.2 % for middle) (Fig. 10). Constitutive-aerenchyma-forming line IL#4 (containing a Z. nicaraguensis segment overlapping with Qaer1.06–1.07) also had a significantly higher inducible aerenchyma percentage than Mi29. In the basal parts of adventitious roots, one tolerant line (IL#18) and two moderately tolerant lines (IL#4 and IL#29) had a significantly higher inducible aerenchyma percentage than Mi29. In the middle parts of...
adventitious roots, the aerenchyma percentage was lower than in the basal parts for all nine accessions tested. The ranking of lines was similar for the basal and middle parts with one exception: line IL#29 had a high level of inducible aerenchyma formation in the basal parts of roots, but was not significantly different from Mi29 for inducible aerenchyma formation in the middle parts of roots (Fig. 10).

**Marker order on the inverted segment of chromosome 4L**

To determine the order of markers on the segment of chromosome 4 where we had mapped a QTL for flooding tolerance, we first screened for polymorphism between *Z. nicaraguensis* and *Z. luxurians* using 92 SSR markers on chromosome 4. Of the 92 markers, 26 showed only weak amplification or none at all. Of the remaining 66 markers, 30 showed clear polymorphism and the other 36 showed only a monomorphic fragment, giving a frequency of polymorphism of 45.5% among the amplified markers. Using the 30 polymorphic markers, we constructed a

![Constitutive aerenchyma score](image)

**FIG. 8.** Capacity to form constitutive aerenchyma of 40 ILs, the recurrent parent (Mi29) and the donor parent [*Z. nicaraguensis* (*nica*)] in Experiment 4. At ~4 weeks after sowing, aerenchyma formation was measured in adventitious roots emerging at the second node from the bottom of six-leaf-stage seedlings grown under well-drained conditions. Aerenchyma formation was scored visually on a scale from 0 (no aerenchyma) to 3 (well-formed aerenchyma). Bars indicate mean ± s.d. (*n* = 8).

The horizontal dotted line indicates the mean value of Mi29. **Significant difference between Mi29 and the ILs at the 1 % level by Dunnett’s test.

![Leaf injury (16 d)](image)

**FIG. 9.** Relationship between the capacity to form constitutive aerenchyma (Experiment 4) and flooding tolerance evaluated by leaf injury after 16 d of treatment (Experiment 1; higher injury scores indicate greater sensitivity) in 40 ILs. *Z. nicaraguensis* and Mi29 are indicated. **Significant correlation at the 1 % level.

![Inducible aerenchyma](image)

**FIG. 10.** Capacity to form inducible aerenchyma in seven selected ILs and their parents tested in Experiment 5. Aerenchyma area (as a percentage of root cortex area) was measured in 12 seedlings per line (*n* = 12) used in Experiment 1, in which seedlings at the three-leaf stage were flooded with 1 g L⁻¹ soluble starch solution to 1 cm above the soil surface for 16 d. (A) Inducible aerenchyma (%) in the cortex of basal parts of adventitious roots; (B) inducible aerenchyma (%) in the middle parts of roots. Aerenchyma (%) data were arcsine square-root transformed for statistical analyses. Bars indicate mean ± s.d. The horizontal dotted lines indicate the mean values of Mi29. *, **Significant differences between Mi29 and the ILs at the 5 % and 1 % levels, respectively, by Dunnett’s test.

Average root lengths (mean ± s.d.) evaluated for aerenchyma formation were 12.7 ± 1.6 cm for Mi29, 13.8 ± 1.4 cm for *Z. nicaraguensis*, 15.0 ± 1.0 cm for IL#18, 14.7 ± 1.7 cm for IL#30, 14.9 ± 1.7 cm for IL#39, 14.9 ± 1.7 cm for IL#4, 12.2 ± 1.6 cm for IL#29, 12.3 ± 1.3 cm for IL#22 and 10.5 ± 2.6 cm for IL#24.

*Z. nicaraguensis* × *Z. luxurians* linkage map for chromosome 4 from bin 4-05 to bin 4-11, covering 87.3 cM (Fig. 11E). A large inversion relative to maize was observed in the long arm of chromosome 4 between markers bnlg1189 (bin 4-07) and bnlg2244 (bin 4-08) in the *Z. nicaraguensis* × *Z. luxurians*
map (Fig. 11E; inverted region shown in grey). In addition to this large inversion, the map locations of the SSR markers in bins 4·07–4·08 were somewhat different from those on the MaizeGDB map (http://www.maizegdb.org/ssr.php): the order between bnlg1189 (bin 4·07) and umc1808 (bin 4·08) was inverted in the Z. nicaraguensis × Z. luxurians map relative to maize (Fig. 11). Therefore, based on the MaizeGDB map and sequence information, the breakpoint appears to be in the 14.2 cM region between umc1808 (bin 4·08) and bnlg2244 (bin 4·08, at 467·1 cM, between 184 746 297 and 184 746 926 bp). Some inversions have two breakpoints, but the other end of this inversion appears to be the end of maize chromosome 4L; thus, only one breakpoint was identified here.

**DISCUSSION**

**Development of ILs**

Many researchers have pointed out the importance of teosinte germplasm for the improvement of waterlogging tolerance in cultivated maize (Colmer, 2003; Bailey-Serres et al., 2012; Hufford et al., 2012; Yamauchi et al., 2013). In this study we developed an introgression library of lines containing chromosome segments of the teosinte Z. nicaraguensis on the genetic background of maize (Fig. 2).

This IL collection is particularly useful because of the difficulty of generating recombinant inbred lines (RILs) by self-pollination of a large number of F2 or F3 plants from maize × teosinte crosses, although many RILs from maize × maize crosses have been used for mapping studies (e.g. Burr and Burr, 1991). F1, F2 or F3 plants derived from maize × teosinte crosses are generally tall, with many small female spikes, and it is difficult to perform self-pollination by bagging the female spikes. For this reason, linkage map construction and QTL analyses using maize × teosinte crosses have generally been conducted in F2 mapping populations or in the progeny of backcrosses of individual plants (Doebly and Stec, 1991; Westerbergh and Doebly, 2002; Briggs et al., 2007). This situation is quite different from that in self-pollinated species: RILs have been generated using wild species, or wild relatives of crop species in rice (Cai and Morishima, 2002; Uga et al., 2003), barley (Backes et al., 2003; Yun et al., 2005) and wheat (Xie et al., 2012). Here, the female spikes in the BC3 F1 generation were generally similar to those in maize, and it was easy to conduct self-pollination. Therefore, we were able to develop ILs (Fig. 2). Some traits can be mapped directly in the ILs, and near-isogenic lines for a QTL controlling a desirable trait can be obtained by performing a few additional backcrosses with ILs containing segments of interest.
QTL controlling flooding tolerance under reducing soil conditions

A QTL for tolerance to flooding under reducing conditions in maize introbed F1649 was located on chromosome 1 in bin 1-03 (Mano et al., 2006). In the present study, ILs containing teosinte segments in bin 1-03 (IL#2 and IL#37) did not have improved flooding tolerance based on the criterion of leaf injury (Fig. 3), suggesting that Z. nicaraguensis nica-S2 did not contain the QTL allele found in maize F1649. The effect of the QTL found on chromosome 4 in this study was large (LOD = 11.9–25.5, r² = 0.29–0.49) compared with that found on maize chromosome 1 (LOD = 4.4, r² = 0.14; Mano et al., 2006) even though QTL analyses in the present study were conducted using F₂ individuals without replications. In addition to the QTL located on chromosome 4, we found a candidate region on chromosome 2 associated with increased flooding tolerance. The observation strongly supports earlier studies that indicate the importance of wild relatives as germplasm for stress tolerance (Garthwaite et al., 2003; Colmer et al., 2006). In addition, successful introgressions of both higher root porosity and a barrier to radial oxygen loss (Malik et al., 2011) and of salt tolerance (Islam et al., 2007) from Hordeum marinum to wheat were reported.

Other QTLs related to flooding tolerance in maize have been mapped. Qiu et al. (2007) found many QTLs controlling waterlogging tolerance at the seedling stage, and a major QTL for the ratio of total dry weight between flooding and control treatments was mapped to the long arm of chromosome 9. Among the ILs evaluated in the present study, IL#41, which exhibited a high level of flooding tolerance as evaluated by leaf injury, contained a piece of the long arm of chromosome 9 from Z. nicaraguensis; however, IL#41 also contained other segments from Z. nicaraguensis (chromosomes 2 and 4), making the relationship between the source of tolerance in IL#41 and that described by Qiu et al. (2007) unclear.

Relationship between capacity to form aerenchyma and flooding tolerance

The importance of root aerenchyma to adaptation to flooding conditions is widely recognized (e.g. Armstrong, 1979), as is its importance for adaptation to other environmental stress conditions (Postma and Lynch, 2010; Zhu et al., 2010). In Zea species, the capacity to form constitutive root aerenchyma can increase flooding tolerance (evaluated by examining the ratio of shoot dry weight between flooded and control treatments among Z. nicaraguensis accessions; Mano and Omori, 2013). Also, although a correlation between flooding tolerance and the degree of constitutive aerenchyma formation was not proved statistically, there appeared to be a positive trend (Fig. 9). In the series of ILs, we identified IL#4, IL#8, IL#36 and IL#41 as exhibiting a high degree of constitutive aerenchyma formation in adventitious roots (Fig. 8). Of these, IL#8, IL#36 and IL#41 showed significantly higher flooding tolerance (less leaf injury under reducing soil conditions) than Mi29, and IL#4 showed tolerance slightly greater than or equivalent to that of Mi29 (Fig. 3). On the other hand, IL#18 does not form constitutive aerenchyma (Fig. 8) yet it showed the highest tolerance to flooding (evaluated by leaf injury) among the ILs. Further investigation revealed that IL#18 possessed a high degree of inducible aerenchyma formation (Fig. 10), which suggests that this trait might contribute to enhanced flooding tolerance in IL#18. However, factors other than the formation of constitutive or inducible aerenchyma formation also appear to be involved in flooding tolerance under reducing soil conditions because the flooding-tolerant lines IL#30 and IL#39 had the same level of constitutive (Fig. 8) and inducible (Fig. 10) aerenchyma formation as Mi29. Other studies have also suggested that factors other than aerenchyma formation (e.g. radial oxygen loss) play a role in flooding tolerance (Armstrong, 1979; Garthwaite et al., 2003).

Application of a Z. nicaraguensis × Z. luxurians map

IL#18 produced relatively few seeds because of small female spike size and partial male sterility. Therefore, an increase in seed productivity of IL#18 is necessary if it is to be used as practical breeding material; this might be achieved by using backcrossing to eliminate undesirable chromosome fragments from teosinte that decrease spike size. In a previous study, we observed recombination suppression on the long arm of chromosome 4 in a linkage map of the cross between maize and Z. nicaraguensis that was used to generate the ILs (Mano and Omori, 2008). In a recent investigation, we found that recombination suppression on chromosome 4 in Z. nicaraguensis was caused by an inversion relative to maize (Mano et al., 2012). The inversion limited our ability to dissect the QTL by linkage analysis; within the recombination-suppression region on the long arm of chromosome 4, detailed map positions of QTLs (genes) controlling useful traits cannot be determined in populations derived from maize × Z. nicaraguensis. This observation illustrates a major problem when using a wild species as a donor: mapping is difficult in chromosomal regions having different structures in the two parental species. Because the genetic similarity of Z. nicaraguensis and Z. luxurians was previously established by using SSR markers (Fukunaga et al., 2005), random amplified polymorphic DNA (RAPD) markers and internal transcribed spacer sequences (Wang et al., 2011), we crossed Z. nicaraguensis and Z. luxurians and constructed a linkage map of the long arm of chromosome 4 (Fig. 11). This map will be useful for identifying QTLs for desirable traits located on the long arm of chromosome 4, e.g. by searching for phenotypic differences between Z. nicaraguensis and Z. luxurians.

Conclusions

We developed an IL by using an interspecific cross in Zea. Because we used a cross between domesticated maize and a wild relative (teosinte), ILs should be useful both for identifying QTLs controlling traits related to flooding tolerance and for genetic analyses of domestication-related traits (e.g. spike size, spike number and tiller number) and reproductive barriers, all of which segregated in the IL library. In addition, we selected a flooding-tolerant genotype, IL#18, which will be valuable not only for maize breeding but also for physiological analyses of flooding tolerance. Furthermore, we successfully constructed a linkage map of a region of suppressed recombination on chromosome 4 by using a population derived from Z. nicaraguensis × Z. luxurians. Because Z. nicaraguensis and Z. luxurians showed clear differences in flooding tolerance as evaluated by leaf injury under reducing soil conditions (Y. Mano, unpubl.)
ACKNOWLEDGEMENTS

We thank the International Maize and Wheat Improvement Center (CIMMYT) in Mexico for providing seed of *Z. nicaraguensis*, the North Central Regional Plant Introduction Station in Ames, IA, for supplying seed of *Z. luxurians* and the NARO Agricultural Research Center for Kyushu Okinawa Region for providing seed of maize inbred Mi29. We also thank Dr. A. Oyanagi (NICS) for critically reviewing the manuscript, and Dr. R. Mck. Bird (North Carolina State University), Dr. B. Kindiger (USDA/ARS) and Dr C. H. Loa´ isiga (Universidad Nacional Agraria) for supporting the work. This work was supported from the National Agriculture and Food Research Organization (NARO), Japan, and by grants from the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN) and the Ministry of Agriculture, Forestry and Fisheries of Japan (Genomics for Agricultural Innovation, FBW1101 and Development of mitigation and adaptation techniques to global warming in the sectors of agriculture, forestry, and fisheries, 4203).

LITERATURE CITED


