Original research article

Screening of candidate genes associated with constitutive aerenchyma formation in adventitious roots of the teosinte *Zea nicaraguensis*

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Abstract: *Zea nicaraguensis* (teosinte), a wild relative of maize (*Zea mays* ssp. *mays*), constitutively forms aerenchyma, which contributes to plant waterlogging tolerance, in the root cortex in drained soil, whereas maize (inbred line Mi29) does not. One with highest logarithm of odds (LOD) among quantitative trait loci (QTLs) that control constitutive aerenchyma formation in *Z. nicaraguensis* is *Qaer1.05-6* on chromosome 1. Here, we attempted to identify genes in *Qaer1.05-6* by comparing cDNA libraries from Mi29, *Z. nicaraguensis* and a hybrid (BC_4F_1 #62) carrying *Qaer1.05-6*. We first confirmed that constitutive aerenchyma formation was apparently observed in the order *Z. nicaraguensis* > BC_4F_1 #62 > Mi29. Contigs were assembled from cDNAs pooled from the three lines. We identified 1,868 contigs in the region on chromosome 1 that contained *Qaer1.05-6*. These contigs were screened for contigs that were predominantly composed of cDNAs from BC_4F_1 #62 and *Z. nicaraguensis* (no more than 10% of cDNAs from Mi29). Twenty-one such contigs were found and the genes they encoded were identified. In a real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis, expression of six of these genes in BC_4F_1 #62 was at least double that in Mi29, making them candidates for genes associated with constitutive aerenchyma formation.

Keywords: maize, next generation sequencing, quantitative trait loci, teosinte

Abbreviations: QTLs, quantitative trait loci; RT-PCR, reverse transcription polymerase chain reaction; UTR, untranslated region

Introduction

Soil waterlogging is an environmental stress that reduces the growth and yield of crops such as maize (Mano and Omori 2007, Zaidi et al. 2007), wheat (Oyanagi 2008, Setter et al. 2009), and soybean (Linkemer et al. 1998, Shimamura et al. 2003, VanToai et al. 2001). Waterlogged soils are usually anaerobic and chemically reduced because of the slow diffusion of oxygen in water and rapid consumption of oxygen by soil microorganisms. Features that contribute to waterlogging tolerance in plants include the aerenchyma, a barrier to radial oxygen loss, adventitious roots at the soil surface, and resistance to toxic substances such as Fe^{2+} and H_2S (reviewed by Colmer and Voesenek 2009).

Aerenchyma formation plays a central role in providing gas space in roots, stems and leaves to transport gases (*e.g.*, oxygen and CO_2) between aerial tissues and roots (Armstrong et al. 2000). Two types of aerenchyma have been identified based on how the gas space is formed (Evans 2003, Jackson and Armstrong 1999). Schizogenous aerenchyma develops air-filled space through cell separation, without cell death. By contrast, lysigenous aerenchyma develops air-filled space by programmed cell death specifically in the cortex in roots. Lysigenous aerenchyma can be distinguished from necrotic tissues, which are characterized by uncontrolled cell death without any tissue specificity. In general,

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lysigenous aerenchyma is further classified into two types, inducible and constitutive aerenchyma (Visser and Bögemann 2006). Inducible aerenchyma is formed by stresses such as hypoxia, waterlogging, mechanical impedance, and deprivation of nutrients such as nitrogen and phosphate (Drew et al. 1979, 1989, He et al. 1992, 1996). Flooding, experimental hypoxia or mechanical impedance induces the synthesis of ethylene, which promotes the formation of inducible aerenchyma (He et al. 1996, Rajhi et al. 2011, Sarquis et al. 1991, Visser and Voesenek 2004). Exogenously applied ethylene has been shown to promote inducible aerenchyma formation (Drew et al. 1979, He et al. 1992). Recently, a microarray analysis showed that the formation of inducible aerenchyma in maize roots following waterlogging treatment was associated with the expression of genes related to Ca^{2+} signaling, reactive oxygen species, cell-wall loosening, and cell wall degradation (Rajhi et al. 2011). Constitutive aerenchyma is known to develop during root growth in wetland species under anaerobic and aerobic conditions (Arikado et al. 1990, Jackson and Armstrong 1999, Justin and Armstrong 1987, Mano et al. 2007, McDonald et al. 2002, Visser et al. 2000). However, the molecular mechanism of constitutive aerenchyma formation remains unclear.

A plant that may elucidate the mechanisms of constitutive aerenchyma formation in roots is *Zea nicaraguensis*, which was discovered in the coastal plain in Nicaragua, where it is remarkable for its ability to grow in as much as 0.4 m of standing or slowly moving water (Iltis and Benz 2000). Under drained soil conditions, *Z. nicaraguensis* constitutively forms aerenchyma in the roots, whereas maize (*Zea mays* ssp. *mays*; inbred line Mi29) does not (Mano et al. 2006). Since hybridization between *Z. nicaraguensis* and maize enables to detect quantitative trait loci (QTLs), a series of studies have been conducted for identifying the QTL genes associated with the aerenchyma formation (Mano et al. 2007, Mano and Omori 2008, 2009). In *Z. nicaraguensis*, at least four QTLs associated with constitutive aerenchyma formation have been detected — two regions on chromosome 1, one region on chromosome 5, and one region on chromosome 8 — by mapping populations developed from cross between maize inbred Mi29 and *Z. nicaraguensis* (Mano and Omori 2008, 2009). *Qaer1.05-6*, the QTL most strongly associated with constitutive aerenchyma formation, *i.e.*, the one with the highest LOD score, was detected on chromosome 1 of maize by QTL mapping using an advanced backcross progeny, BC_4F_1 (Mano and Omori 2009). This line carries the *Z. nicaraguensis* chromosome segment in this QTL region in the genetic background of Mi29, and thus should be useful for screening candidate genes associated with constitutive aerenchyma formation in adventitious roots of *Z. nicaraguensis*.

The aim of the present study was to identify candidate constitutive aerenchyma-related genes in *Qaer1.05-6* in *Z. nicaraguensis*. To this end, we prepared cDNA libraries from *Z. nicaraguensis*, maize inbred line Mi29, and a BC_4F_1 line (BC_4F_1 #62) and assembled contigs from the pooled cDNAs. We then screened these contigs for contigs that mapped to *Qaer1.05-6* and that were predominantly composed of cDNAs from BC_4F_1 #62 and *Z. nicaraguensis*. We discuss the possible contributions of the candidate genes to constitutive aerenchyma formation.

Materials and methods

Plant materials

The maize inbred line Mi29, *Z. nicaraguensis* (a wild relative of maize) and the line BC_4F_1 #62 were used. Seeds of Mi29 were provided by NARO Kyushu Okinawa Agricultural Research Center, Japan. *Z. nicaraguensis* (accession number: CIMMYT 13451) was provided by the International Maize and Wheat Improvement Center (CIMMYT), Mexico. The line BC_4F_1 #62 was provided by the NARO Institute of Livestock and Grassland Science, Japan. In BC_4F_1 #62, the *Z. nicaraguensis* chromosome segment had been heterozygously introduced into a region broadly between DNA markers bnlg1016 and bnlg1556 on chromosome 1 in Mi29. Thus, BC_4F_1 #62 was used to screen gene candidates for *Qaer1.05-6* associated with constitutive aerenchyma formation in *Z. nicaraguensis*.

Growth condition

Plants were grown in a greenhouse controlled at 30°C by day and 25°C at night, under natural light and a 14- to 14.5-h day length. Each seedling was grown in a pot (110 mm diameter, 300 mm height) in granular soil (Kureha Chemical Industry, Tokyo, Japan; 0.4 g N, 1.0 g P and 0.6 g K per 1.0 kg soil); the conditions were as described by Mano and Omori (2009). Each one plant of Mi29, BC_4F_1 #62, and *Z. nicaraguensis* seedlings were grown until the six-leaf stage. Three nodal roots that had developed from the initial node were then cut off at the root base and gently washed with water to remove the soil surrounding the root. One of these isolated nodal roots was used for assessment of aerenchyma formation. The rest of the roots were immediately frozen in liquid nitrogen and then stored at -80°C until use for extraction of total RNA.

Evaluation of aerenchyma formation

To assess the development of root aerenchyma, transverse sections (80 to 100 μm in thickness) of one nodal root (270 to 370 mm long) of each line were cut with a microtome (MTH-1, NK System Co. Ltd., Osaka, Japan) at a distance of 135 to 185 mm behind the root apex. The sections were mounted in water and viewed with bright field illumination using a microscope (Nikon Eclipse E600) fitted with a CCD camera (camera head: Nikon DS-Ri1; control unit: Nikon DS-U3) linked to a computer. The areas of the aerenchyma and the root cross-section were calculated by tracing their boundaries with Image J software (Version 1.39; National Institutes of Health, Bethesda, MD, USA). The ratio of aerenchymatous tissue to root tissue was then calculated by dividing the aerenchymatous area by the root cross-sectional area. The measurements were replicated by using three root sections prepared from one root. The significance of the differences in the ratios was analyzed by one-way ANOVA (analysis of variance).

Isolation of total RNA

Two nodal roots that had developed from the initial node of a single plant were cut and stored at -80°C. The frozen roots were ground with a Multi-Beads Shocker (Yasui-Kikai Co. Ltd., Osaka, Japan). Total RNA was then extracted with an RNeasy Plant Mini Kit in accordance with the manufacturer's instructions (Qiagen Co. Ltd., Hilden, Germany). The quality of isolated RNA was assessed by using an RNA 6000 Pico Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies Co. Ltd., Santa Clara, CA, USA). The RNA Integrity Number (RIN) of the RNA used in this study, calculated with 2100 Expert Software (Agilent, version B.02.02, eukaryote total RNA pico mode), was more than 8.0.

Construction of cDNA libraries, sequencing, contig assembly and contig identification

Total RNA was sent to TAKARA BIO Inc. (Ohtsu, Japan) for construction of cDNA libraries, 454 sequencing, contig assembly, identification of closest matches and alignment of contigs with the chromosomes in the Maize Genetics and Genomics Databases (http://www.maizesequence.org/index.html).

Briefly, second-strand cDNA was synthesized from total RNA using oligo(dT)-T7 primers. Amplified RNA was synthesized by using T7 RNA polymerase. After reverse transcription of 10 μg of amplified RNA using random hexamers, biotinylated cDNA was synthesized using a biotinylated GS FLX adaptor that incorporated oligo(dT) primer. Synthesized cDNA was sonicated to generate 3′ cDNA fragments 300 to 800 bp long. Biotinylated cDNA was bound to streptavidin magnetic beads to purify only the 3′-UTR-orientated fragment. Each specific adaptor (Mi29 5′-ACGAGTGCGT-3′; BC_4F_1 #62 5′-ACGCTCGACA-3'; *Z. nicaraguensis* 5'-AGACG CACTC-3') was ligated to the top strand of each sublibrary. After adaptor ligation, the desired single-strand DNA was eluted from the magnetic beads with an alkaline solution. Using the single-strand DNA that was constructed, sequencing was performed with a Genome Sequencer FLX System (Roche Co. Ltd., Basel, Switzerland).

Low-quality reads such as short reads (≤50 bp) were removed and poly-A/T tails and tag sequences at the 5′ ends for cDNA synthesis were trimmed. The reads for each line were pooled, and assembled into 32,025 contigs using Paracel Transcript Assembler Version 2.7 (Paracel Inc., Pasadena, CA, USA). Thus, in general, the contigs were composed of reads from all three lines. Closest matches to the contigs were found by blast searches of the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/) and MaizeGDB.

Contig selection

We selected contigs in *Qaer1.05-6*, which is located between DNA markers bnlg1016 and bnlg1556 on chromosome 1. Of the 32,025 contigs, 1,868 contigs were in this region. We then searched these contigs to select the contigs that were largely composed of reads from BC_4F_1 #62 and *Z. nicaraguensis*, *i.e.*, contigs for which no more than 10% of the reads came from Mi29.

Real-time RT-PCR

First-strand cDNA was prepared by using a SuperScript First-Strand Synthesis System for real-time RT-PCR (Invitrogen Co Ltd., Carlsbad, CA, USA). cDNA was generated by using oligo(dT) 12-18 primers. For validation, quantitative real-time RT-PCR analysis was performed using first-strand cDNA synthesized from 500 ng total RNA of Mi29 and BC_4F_1 #62. Gene-specific primers were designed within the region of the gene-specific sequence based on the Maize GDB. Information on primer sequences is presented in Table S1. The primers designed for 1064-contig3 were not available for real-time RT-PCR. The PCR product was amplified by PCR cycling, as follows: initial denaturation (94°C for 120 s); 40 cycles of denaturation (94°C for 15 s), annealing (58 to 62°C for 15 s), and extension (68°C for 30 s); then final extension (68°C for 360 s). The PCR products were amplified and detected by QuantiFast

Table 1. Aerenchyma formation in roots of Mi29, BC_4F_1 #62, and *Z. nicaraguensis* grown in drained soil

Trait	Mi29	BC_4F_1 #62	*Z. nicaraguensis*
Cross-sectional area (mm^2)	0.91±0.00	0.97±0.00*	0.95±0.01*
Area of cortex (mm^2)	0.58±0.00	0.67±0.01*	0.69±0.01*
Area of stele (mm^2)	0.20±0.00	0.19±0.00	0.19±0.01
Aerenchyma per cross-sectional area (%)[a]	N.D.[b]	4.79±0.02*	16.79±0.01*

Data are represented as mean values with standard error. The measurements were replicated by using 3 root sections shown in Fig. 1. Statistical analysis of the data was performed by one-way ANOVA ($P \leq 0.001$).
[a]Area of aerenchyma as a percentage of root cross-sectional area (%).
[b]Aerenchyma was not detected.

SYBR Green PCR (Qiagen), using a MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratories Co Ltd., Hercules, CA, USA). pCR-Blunt II TOPO vector into which fragment of *ubiquitin* (GRMZM2G118637) had been inserted was constructed by using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen) and used to make a standard curve for quantification. Independent triple samples were assayed. Each value was normalized against *ubiquitin* used as an endogenous control.

Results

Evaluation of aerenchyma formation in Mi29, BC_4F_1 #62, and Z. nicaraguensis

Using Mi29, BC_4F_1 #62, and *Z. nicaraguensis*, we compared the formation of constitutive aerenchyma by observation of sections of the middle part of the root. Root cross-sectional area was similar among Mi29, BC_4F_1 #62, and *Z. nicaraguensis* (Fig. 1 and Table 1), as were the areas of the cortex and stele (Table 1). Constitutive aerenchyma formation was observed in BC_4F_1 #62 and *Z. nicaraguensis*. The ratio of aerenchyma (air-filled space) to root (%) was 4.79±0.02% (n = 3) in BC_4F_1 #62 and 16.79±0.01% (n = 3) in *Z. nicaraguensis* (Table 1). The aerenchyma was mainly observed in the inner side of the cortical cells in BC_4F_1 #62, whereas the larger air-filled space of aerenchyma was radially formed in both the inner and outer sides of the cortical cells in *Z. nicaraguensis* (Fig. 1). By contrast, the aerenchyma was hardly observed in Mi29 (Fig. 1 and Table 1), in agreement with our recent report (Abiko et al. 2012).

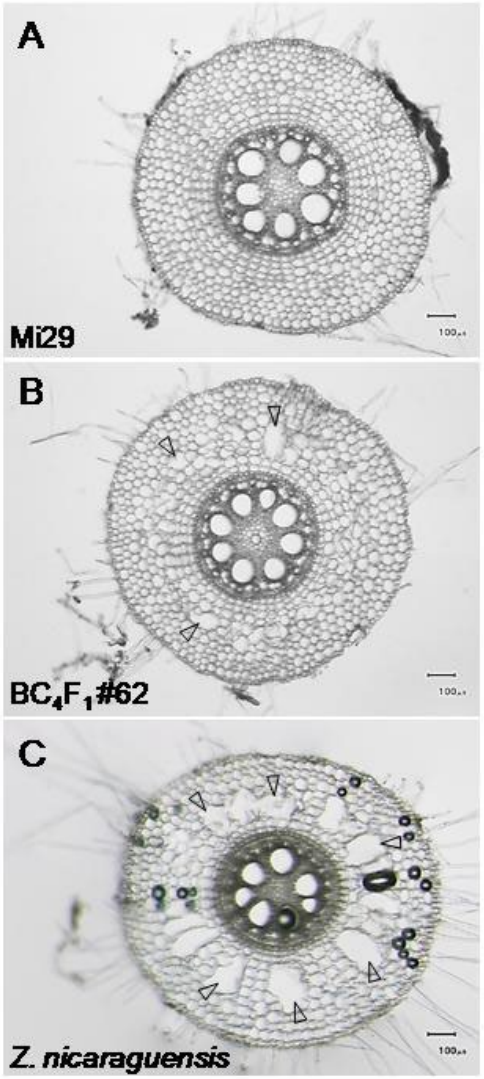

Fig. 1. Representative cross-sections of roots in Mi29 (A), BC_4F_1 #62 (B), and *Z. nicaraguensis* (C) grown under drained soil conditions. Cross-sections were taken at 135 to 185 mm behind the apex of 270 to 370 mm-long roots. White arrowheads indicate formed aerenchyma. Scale bar = 100 μm.

Contig assembly and selection

For the three lines, we obtained a total of 929,704 reads greater than 50 bp (Table 2), which were pooled and assembled into about 32,035 contigs and

Table 2. Number of reads from cDNA libraries

	Mi29	BC_4F_1 #62	*Z. nicaraguensis*
No. reads >50 bp [a]	381,554	407,310	140, 840
Mean length (bp) [b]	292	287	170

[a] The number of reads which are available for contig assembly.
[b] Mean length of reads used for contig assembly.

6,373 singlets (Table 3). The mean length of the assembled contigs was 557 bp (Table 3).

Among the 32,035 contigs, 1,868 contigs were located in the candidate region of *Qaer1.05-6*. Among these 1,868 contigs, we searched for contigs for which at least 90% of the reads were from BC_4F_1 #62 and *Z. nicaraguensis*. Twenty-one of the contigs satisfied this criterion and thus were selected as candidate contigs (Table 4).

Validation by real-time RT-PCR

The expressions of all but one of these 21 genes (1064-contig3) were successfully measured in BC_4F_1 #62 and in Mi29 by real-time RT-PCR and expressed as a BC_4F_1 #62/Mi29 ratio (Table 4). Six of these genes (shown in bold in Table 4) had ratios of at least 2.0.

Discussion

Constitutive aerenchyma was observed in BC_4F_1 #62 and *Z. nicaraguensis*, but not in Mi29 (Fig. 1 and Table 1). The ratio of aerenchyma in BC_4F_1 #62 was intermediate between those in Mi29 and *Z. nicaraguensis* (Table 1). The lower aerenchyma ratio in BC_4F_1 #62 than in *Z. nicaraguensis* may be due to the likelihood that BC_4F_1 #62 is lacking other QTLs in *Z. nicaraguensis* that also control constitutive aerenchyma formation.

Table 3. Number of contigs and singlets from pooled reads

No. contigs [a]	32,035
Mean contig length (bp) [b]	557
No. singlets [c]	6,373

[a] The number of contigs which are assembled.
[b] Mean length of contigs used for assembly.
[c] The number of contigs which are not assembled.

Six genes were identified as candidate genes located in *Qaer1.05-6* (Table 4). Two of the six genes (952-contig1 and 413-contig2) were expressed only in BC_4F_1 #62 and the other four (1630-contig2, 4258-contig1, 760-contig1, and 608-contig1) were expressed at higher levels in BC_4F_1 #62 than in Mi29. Of the first type, 952-contig1 is homologous to a gene encoding 60S ribosomal protein L31. Translation-associated genes including those encoding ribosomal proteins were also found to be upregulated in maize roots during inducible aerenchyma formation (Rajhi et al. 2011). However, the relationship between translation and aerenchyma formation is unclear. 413-contig2 is homologous to a nitrogen metabolism-related gene encoding glutamate decarboxylase. Of the second type, 608-contig1 is homologous to a gene encoding 2-oxoglutarate/malate carrier protein, which supplies 2-oxoglutarate to the glutamine synthetase (GS) and glutamate synthase (GOGAT) pathway, an important pathway for ammonium assimilation in many plants (Lancien et al. 2000). There appears to be a link between nitrogen metabolism and aerenchyma formation because nitrogen deficiency dramatically induces aerenchyma formation in maize roots (Drew et al. 1989). Thus, glutamate decarboxylase and 2-oxoglutarate/malate carrier might be involved in aerenchyma-related nitrogen metabolism. 760-contig1 is highly homologous to *SRG3* (*senescence-related gene 3*), which accumulates in senescing organs of *Arabidopsis thaliana* (Callard et al. 1996). Transcripts of *SRG3* genes accumulate at high levels in chlorotic leaves, in which cell death is occurring (Callard et al. 1996). 760-contig1 is also homologous to *glycerophosphodiester phosphodiesterase*. A mutant of *glycerophosphodiester phosphodiesterase* in *A. thaliana* has been reported as *shaven3* (*shv3*); it exhibits abnormal root hair cells (Hayashi et al. 2008). This gene is associated with changes in cellulose content and pectin modification in cell-wall components (Hayashi et al. 2008). Cell-wall modifications (*e.g.*, pectin modification) were observed before cell wall degradation during

Table 4. Candidate genes for *Qaer1.05-6* controlling constitutive aerenchyma formation

Cluster-contig ID	Number of reads			Ratio[a]	Gene ID [b]	Description
	Mi29	BC_4F_1#62	*Z. nicaraguensis*			
1064-contig3	0	132	7	N. D. [e]	GRMZM2G135177	Small subunit ribosomal protein S19
346-contig5	0	153	144	1.57±0.17	GRMZM2G304442	Pathogenesis-related protein (*Hv-1a*) precursor
1630-contig2[d]	**1**	**70**	**45**	**6.99±1.27**	**GRMZM2G084063**	**Unknown**
3060-contig1	0	19	12	0.99±0.16	GRMZM2G025190	Glutathione transferase 42
4258-contig1[d]	**1**	**15**	**8**	**2.63±0.23**	**GRMZM2G434514**	**Peptidoglycan-binding lysine-domain containing protein**
239-contig5	9	139	129	1.81±0.05	GRMZM2G037255	ZRP3 protein
3976-contig1	0	16	12	1.19±0.05	GRMZM2G40184	Peptidyl-prolyl cis-trans isomerase (cyclophilin)
174-contig1	0	52	22	1.15±0.06	AC186319.4_FG002	Nucleotide-binding, alpha-beta plait domain–containing protein
140-contig3	8	135	80	1.28±0.04	GRMZM2G132077 GRMZM2G433844	Unknown
2864-contig2	0	58	28	1.11±0.25	GRMZM2G131907	Adenine phosphoribosyl-transferase 1
6041-contig1	0	12	5	0.79±0.13	GRMZM2G142660	EXS family protein
5228-contig2	0	5	11	0.79±0.08		Similar to Prep homeobox transcription factor
3322-contig1	0	16	9	1.49±0.35		Unknown
1470-contig1	0	18	12	1.24±0.06	GRMZM2G022365	Methyl-binding domain protein MBD106
2601-contig2	0	79	17	1.14±0.01	GRMZM2G005887	Cysteine synthase
32-contig4	0	45	74	0.75±0.01	AC187262.4_FG007	Unknown
952-contig1[d]	**0**	**55**	**25**	**N. D. in Mi29[c]**	**GRMZM2G132623**	**60S ribosomal protein L31**
760-contig1[d]	**0**	**13**	**14**	**3.30±0.09**	**GRMZM2G064962**	**Glycerophosphodiester phosphodiesterase**
1143-contig1	0	18	10	1.40±0.03	GRMZM2G048129	MtN19-like protein
413-contig2[d]	**0**	**121**	**128**	**N. D. in Mi29[c]**	**GRMZM2G017110**	**Glutamate decarboxylase**
608-contig1[d]	**0**	**17**	**30**	**5.94±0.45**	**GRMZM2G436593**	**Mitochondrial 2-oxoglutarate/ malate carrier protein**

[a]Ratio of expression of each candidate gene in BC_4F_1 #62 to that in Mi29. Data are mean values with standard errors.
[b] Gene IDs in Maize sequence Database (http://www.maizesequence.org/).
[c] Gene expression was not detected in Mi29.
[d] Genes validated by real-time RT-PCR analysis (bold letters).
[e] The primers designed for 1064-contig3 were not available for real-time RT-PCR.

aerenchyma formation (Gunawardena et al. 2001, Evans 2003). According to these reports, it is possible that the gene corresponding to 760-contig1 is involved in cell-wall modifications such as changes in cellulose content and pectin modification during aerenchyma formation. Another of the candidates, 4258-contig1, is homologous to the gene encoding peptidoglycan binding protein with lysine motif (LysM). LysM, which is composed of about 40 amino acids, is a domain found in more than 4,000 proteins. For example, proteins such as lysozyme, peptidoglycan hydrolase, peptidase, chitinase, and esterase retain the LysM domain (Buist et al. 2008). In plants, the gene encoding LysM-receptor like kinase is a candidate Nod factor receptor gene (Arrighi et al. 2006). However, the function of the gene corresponding to 4258-contig1 is not still reported. The expression change of 6.99±1.27-fold in 1630-contig2 in BC_4F_1 #62 compared with Mi29 was the highest among the four candidate genes of the second type (Table 4). Unfortunately, the database survey revealed no information on the potential biological function or known domain of this gene.

Conclusions

Twenty-one gene candidates for *Qaer1.05-6* controlling constitutive aerenchyma formation in a wild relative of maize, *Z. nicaraguensis* were tentatively identified by comparing cDNA libraries of *Z. nicaraguensis*, BC_4F_1 #62 and Mi29. A real-time RT-PCR analysis of these genes identified six genes that were more strongly expressed in BC_4F_1 #62 than in Mi29. Further analyses are required to determine which of the six candidate genes are associated with *Qaer1.05-6*.

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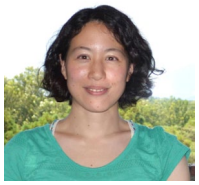
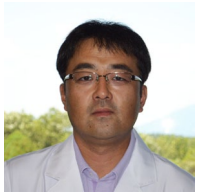
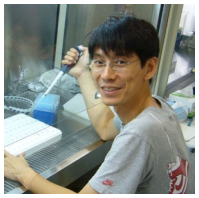


Dr. Tomomi Abiko is a plant physiologist in Japan International Research Center for Agricultural Sciences, Tsukuba, Japan. She is interested in adaptation to waterlogged and reduced soil in plant roots.



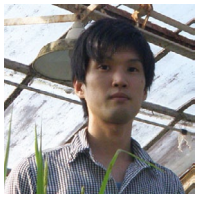
Dr. Mitsuhiro Obara has studied nitrogen utilization of rice by physiological and genetic approaches. He is interested in relationships between nitrogen utilization and root system development toward sustainable crop production in developing regions.



Dr. Fumitaka Abe's interest is the gene functions involving in the yield performance of crops. His excellent technique for producing fertile transgenic plants of wheat is our powerful tool.

Dr. Kentaro Kawaguchi has an interest in the morphological and physiological responses of crops to various environmental stresses, e.g. low- temperature, waterlogging, etc.

Dr. Atsushi Oyanagi is an agronomist in NARO Institute of Crop Science, Tsukuba, Japan. He is interested in root growth angle and vertical distribution of roots in wheat. Excess-moisture injury in the wet field is his research subject.



Dr. Takaki Yamauchi is interested in molecular mechanisms of the root aerenchyma formation in cereal crops and how the aerenchyma affects tolerance to waterlogging.

Dr. Mikio Nakazono has been trying to understand how plants cope with environmental stresses such as waterlogging and submergence. In particular, he is interested in strategies of oxygen acquisition in roots under waterlogged conditions.

Supporting Information:

Appendix S1. List of primer sequences used in real-time RT-PCR analysis

Cluster-contig ID	Forward primer sequence[a]	Reverse primer sequence[a]
346-contig5	5'-CAAGGTGTGCGGCCACTACA-3'	5'-GCTGCCAAGTAGAGGCAGAGAG-3'
1630-contig2	5'-GGTGTCCACTTCTAGTGAAAC-3'	5'-GGAGTTGTGCCAGTTCACAT-3'
4528-contig1	5'-GCAAACCTGCATGTGTGTCG-3'	5'-GCTCGAGACTACGAGCAGAA-3'
3060-contig1	5'-ATCGGAACGCGGCTTTGTGGT-3'	5'-TGGTTGGATAGCTGAGCTCG-3'
239-contig5	5'-GAGTGGTGTGGTTACACTTC-3'	5'-GTGCGTGTATATGTCTGGCCT-3'
3976-contig1	5'-GCTGTTACAAGATGCGTTTGAG-3'	5'-GGTTAATGAAGTTGCTCGCT-3'
174-contig1	5'-TGCTCAAGAGATCATCTGCA-3'	5'-CTAAGGGCCGAACGCAGTTTCT-3'
140-contig3	5'-TGCTTCGCTCAGTAGCCTCGC-3'	5'-AGGATCGAGATGCGTGTCTG-3'
2864-contig2	5'-GACTTCTCAGAGAGTTTGGC-3'	5'-ATCCTGATTGGAGCTTGTTG-3'
6041-contig1	5'-GAAATTTGTTGGCGGCTTCGG-3'	5'-CAGGAGGTATTCGTTGAGAC-3'
5228-contig2	5'-GGAAGCATTGGGTTGTAGTAGC-3'	5'-AGCTTGTGATTTGGCCACCA-3'
3322-contig1	5'-GTCCATGCAACCAAGAAAGA-3'	5'-GTTCAGTCAGGCAGTGGATG-3'
1470-contig1	5'-GCTGATGCTGTTGCTGCTCC-3'	5'-TCAACGTGAAGTGCATCTGA-3'
2601-contig2	5'-TAGAGCACACAGTTCTGGGC-3'	5'-GTTGTCGCTGGAGAACACGT-3'
32-contig4	5'-GATCTAGCTCGCTGCGTCA-3'	5'-TGTCGGTGCTGATGTGGCCT-3'
952-contig1	5'-CAAGGTCGTTGACGAAACCG-3'	5'-TCATCTCTGCCACATCCTT-3'
760-contig1	5'-GACTGACTGAATGATAGGTC-3'	5'-GTTGGTACAGTACAGGTACAG-3'
1143-contig1	5'-TGGTTAGGGTTCGGTTTGGG-3'	5'-CCTCCGTTTGACTTCTTCAC-3'
413-contig2	5'-ACGCTTGGATGCGTTGGTCCAG-3'	5'-AACATTAAACTAGAGTTACG-3'
608-contig1	5'-TCAAGACCGTCAGGTCGGAG-3'	5'-AGTTCGTCACCGCGTTCGCT-3'

[a]Primer sequences for 20 out of 21 contigs are shown.