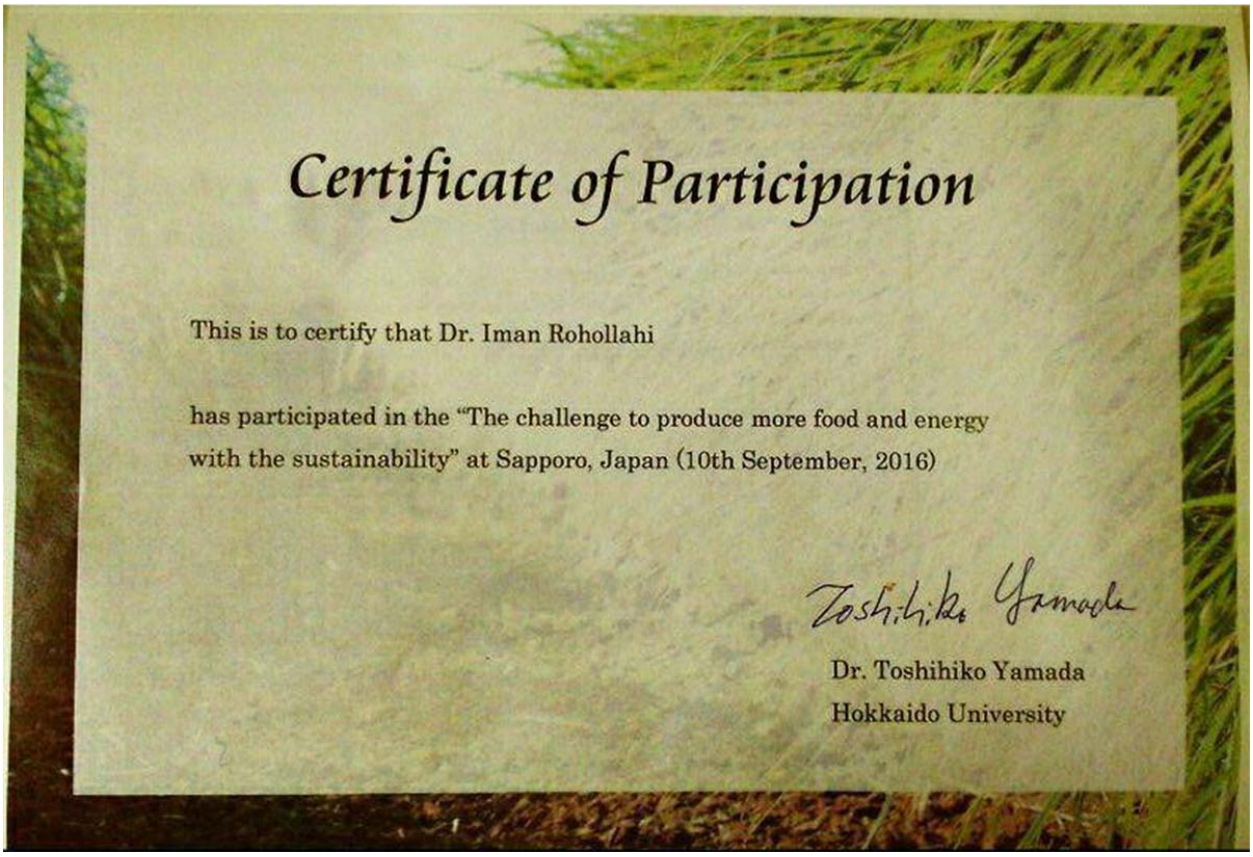
The challenge to produce more food and energy with sustainability



Certificate of Participation

This is to certify that Dr. Iman Rohollahi

has participated in the "The challenge to produce more food and energy with the sustainability" at Sapporo, Japan (10th September, 2016)

Toshihiko Yamada

Dr. Toshihiko Yamada
Hokkaido University

Isolation and expression analysis of the RbohD gene (gene encoding respiratory burst oxidase) in *Festuca arundinaceae* cv. Barvado and selected accessions of Iran under drought stress

Iman Rohollahi[1], Toshihiko Yamada[2]

1. **Assistant prof. in Shahed university**
2. **Professor in Hokkaido university**

Abstract

The objectives of this study were to investigate sequencing and expression level of FrbohD gene in leaf and shoot under drought, after establishment upon selected entry in germination stage for *Festuca arundinacea*. *F. arundinaceae* is a major cool-season forage and turf grass specie which is adapted to cold, arid and semiarid environments. The populations were evaluated under 100% field soil water capacity (FC) [-0.33 matric potential (MPa)] and at 80, 60 and 40 % FC. Isfahan had the best final emergence, longer leaf and root length compared to Barvado at 40% FC. We selected three entries on the emergence and early establishment of 14 wild *F. arundinacea* populations collected from various regions in Iran, and two commercial turf cultivars. After sequencing of FrbohD, expression level of FrbohD gene response to drought in a drought-tolerance entry (Isfahan) and a drought-sensitive entry (Quchan) were compared to those of cultivar Barvado. The plants were subjected to 4, 6 and 8-day without water before samples were taken from the leaf and shoot for the purpose of gene expression investigation. Henceforth, consistent with germination results, the transcript level of FrbohD substantially increased under drought stresses in the leaf and shoot of Quchan entry compared to cultivar Barvado as reference gene. Significantly, lower expression FrbohD gene in Isfahan entry might be as a result of the higher antioxidant enzyme activity and/or low ROS accumulation in Isfahan entry compared to those in Quchan entry. Our results showed that up-regulated of FrbohD gene in leaf under drought in Quchan and Isfahan entries compared to their control. This higher level of FrbohD gene transcript in a leaf following drought stress compared to control, indicating that FrbohD gene could be involved to mediate drought stress effect.

Keywords: Emergence, Induction, Drought tolerance, Sequence, Transcript level.

Introduction

Festuca L. is one of the largest in the family Gramineae whose members are used as forage and turf grasses in a wide range of soil and climate condition, in temperate regions of the world (Saha et al.,

2005). This genus is widely adapted to a variety of ecogeographical regions (Yamada, 2011). The primary reason that *F. arundinacea* fairs well in cold, arid and semi-arid regions being due to its ability to survive under drought conditions compared to other cool-season turfgrass species (Fry and Huang, 2004). *F. arundinacea* is widely distributed as a native grass in temperate and cool climates throughout Europe, North Africa, West and central Asia, and Siberia (Gibson and Newman, 2001). According to Rechinger (1970), *F. arundinacea* is one of the perennial grasses that are endemic to cold and dry regions of central, north-eastern and north-western Iran. The most local accessions have probably adapted to strict microclimatic and edaphic conditions in isolated mountain ranges of Iran, these accessions will be ideal source stock for germplasm selection (Sharifi et al., 2009). Because of its use as pasture and turfgrass, a large number of studies have investigated effect of drought stress on *F. arundinacea*. Wilman et al. (1998) reported *F. arundinacea* to be the most drought–tolerance grass when compared with seven others grasses. In spite of the fact that *F. arundinacea* is relatively drought resistant (Pessarakli, 2008) there is considerable genetic variation for this trait among genotypes and populations (Severmutlu et al., 2011). Details of Germination process are important for the successful establishment of grass species (Larsen and Bibby, 2004). Investigation of emergence and seedling performance under conditions of low soil moisture is an efficient process to screen genotypes of cool-season grasses for drought resistance (Gazanchian et al., 2006). In some species, the allele frequencies of seeds and established seedling differ (Cabin et al., 1997), demonstrating that germination ability can have effect on the genetic structure of plant populations which can result in individual's variation. (Hamrick, 1983; Cabin et al., 1997). Significant variation was reported in response to drought stress among and within species and accessions collected from different ecological regions of Iran (Gazanchian et al., 2006). Contemporary knowledge confirms possibility of making selection for root system basis of seedling stress tolerance (Stoytcheva and Zlatev, 2013). It is possible also to evaluate characteristics of seeds and seedling, i. e. provide selection after hybridization of the plants on the basis of the seed and seedling traits for the seed quality and also for the classic selection in the plant breeding (Stoytcheva and Zlatev, 2013; Foolad and et al., 2003). Furthermore Ramolya et al. (2004) have shown that a positive relationship exist between drought tolerance in germination and subsequent growth of *Salvadora.* Although Van Zandt et al. (2004) showed that maternal effects were no longer noticeable 10 days after germination, indicating that the indirect effect of salinity was more influential on germination timing and success than on seedling growth.

Drought stress is an important factor affecting growth and development of cool-season perennial grasses (Yu and et al., 2013). Plant drought tolerance is a complex phenomenon, involving multiple metabolic pathways (Wang et al., 2003). A number of genes involved in plant drought response and tolerance have been identified in model plants and crops (Guo et al., 2009; Khowaja et al., 2009; Campo et al., 2012). The products of these genes are thought to function not only in stress tolerance but also in the regulation of gene expression and signal transduction (Bartels and Suunker 2005; Si et al. 2009).

ROS, which are produced at the cell surface, can function as signaling molecules that mediate response to various processes such as development, programmed cell death and stomatal behaviour, and are one of the earliest events detected in the plant defense response (Si, 2010). Plant reactive oxygen species (ROS) are produced by the NADPH oxidase, respiratory burst oxidase homologue (Rboh) (Takahashi et al., 2012). Plants have evolved mechanisms of ROS generation as signaling for rapid cell to cell communication in biotic and abiotic stress which is dependent on the RbohD gene (Miller et al., 2009). The equilibrium between activities of antioxidative enzyme and/or ROS production defines whether oxidative signaling or damage will occur (Moller et al., 2007). In rice, drought-tolerant varieties indicated lower H2O2 level compared to drought-sensitive varieties (Guo et al., 2006; Rabello et al., 2008). Also Zhang et al. (2012) reported that RbohH was extremely down regulated by drought in drought tolerance but up-regulated in sensitive varieties.

In Arabidopsis, 10 Rboh genes are known, and among these, RbohD and RbohF function in ROS dependent ABA signaling for stomatal closure (Kwak et al., 2003). The rice *Oryza sativa* RbohA gene was the first Rboh gene isolated from a plant (Takahashi, 2012). Consequently, Rboh genes have been isolated from several plant species including Arabidopsis, tobacco and potato (Yoshioka, 2003). Wong et al. (2007) reported constitutively expression of all Rboh genes in root, leaf, shoot and calli of rice with the exception of RbohD in root and leaf. Groom et al., (1996) investigated showed RbohA transcript in both roots and shoots of healthy rice plants. Although Rboh did not change in roots and shoots of watermelon during polyethylene glycol treatment, Rboh induced in water melon scion when it was grafted onto a *C. colocynthis* rootstock (Si, 2010).

Genetic variation and maternal effect on drought tolerance has been detected in grasses, however its molecular dissection has mainly been investigated in the model species *Arabidopsis* (Si et al., 2009). Maternal effects in germination and seedling stage based on physiological responses studies have been undertaken; however do not include any research about the possibility of connection between

germination stage and drought tolerance related gene expression after establishment in perennial grasses. To assess possibility of selection in germination and its conduction with subsequent expression of RbohD, we investigated expression pattern of RbohD under drought stresses. We also hypothesized that this candidate gene should express more significantly under drought in *F. arundinacea* after establishment**.** To test this hypothesis we isolated and identified Rboh D gene from *F. arundinacea* CV. Barvado to gain information on its expression under drought stress condition in each selected entry after establishment. To our understanding**,** this is the first report on the cloning of RbohD gene and it expression under drought stress in *F. arundinacae*.

Material and methods

Plant materials selection (Measurements for germination and seedling establishment)

Sixteen entries were evaluated in the present study. Seeds of 14 wild *F. arundinacea* populations were collected from different cold, dry and semi-arid regions of Iran by the Rangelands and Forestry Research Institute (stored in -20 C^o for one years) (Table 1). In addition, two commercial cultivars from Europe (Bravado and Barleroy) were used as controls (Table 1). Each entry was assayed at four levels of soil moisture content (40, 60, 80 and 100% FC). The effect of varying soil moisture content on final emergence, germination rate (GR), root and leaf length, days to 50% of emergence (T50) and Seedling vigor index (SVI) were evaluated in a greenhouse (Table 1).

Finally two accessions from Iran (Quchan and Isfahan) and one commercial cultivar from Europe (Bravado) selected (Table 1). The seeds of each selected entry were germinated on moisture filter paper in the growth chamber with a 14 h photoperiod at temperature ranging from about 24-28 ˚C day/night. After 20 days, newly planted seedlings were transplanted into polyethylene pots (20 cm diameter at the top, 15 cm diameter at the bottom, and 30 cm height) filled with field soil. The plant had been established for 2 months with regular irrigation in a uniform greenhouse environment condition. After establishment, some pots were deprived of water 4, 6 and 8-day upon beginning of drought stress treatment, leaf and shoot samples from both the stressed and the control of *F. arundinacea* plants were collected at noon of each day to eliminate the possible gene expression variation from occurring during the day.

Plant water status

Relative water content (RWC) was measured during water deprivation using 1 cm leaf discs. RWC was determined according to the following equation: RWC=(FW-DW)/(SW-DW)×100, where FW is

leaf fresh weight, DW is dry weight of leaves after drying at 85 °C for 3-day, and SW is the turgid weight of leaves after soaking in water for 4 hour at room temperature (Fig. 1F).

Measurements for germination and seedling establishment

Germination and seedling establishment were monitored for 20 days. Emergence was recorded at the detection of the leaf above the soil surface of the seedlings in each pot. Days required for T50 were recorded as described by Usberti and Valio (1997). The leaf length and the maximum root length for each emerged seedling were measured for each pot at the end of the experiment (20 days after planting). In this study, GR was calculated as described by Maguire (1962). SVI was calculated (Abdul-Baki and Anderson, 1973) by multiplying the percentage of emergence for each accession by the mean length (cm) of the seedling (root plus leaf).

Sequence analysis of RbohD in *Festuca*

We used small scale CTAB (Cetyltrimethylammonium bromide) method for DNA extraction of *Festuca arundinacea* cv. Barvado (Murray et all., 1980). The primers FrbohDF1-F5 and FrbohDR1-R6 (Table 1) used for the cloning of RbohD core DNA fragment. The forward and reverse primers were designed according to the RbohD sequence in rice (accession number: ak072353, Wong et al., 2007) for the cloning of FrbohD. The polymerase chain reaction (PCR) was carried out with the primer set and LA Taq DNA polymerase (TaKaRa Bio Inc., Shiga, Japan). Amplification products were confirmed by electrophoresis on 1.0% agarose gels containing ethidium bromide. The electrophoresis was conducted at 100 V for 30 min. PCR products were purified with Nucleo Spin extract Kit (Takara, Japan) prior to sequence analysis. The PCR amplification products were cloned into the PGEM-T Easy vector (Promega Crop., Tokyo, Japan). Vectors containing DNA fragments were amplified using Escherchia coli strain JM109 (Promega Crop.) After overnight culture, plasmids were isolated using High Pure Plasmid Isolation Kit (Roche Applied Science). The DNA sequencing of plasmids and PCR products were determined by primer walking with an automatic sequencer (ABI Prisma 3130 Genetic Analyzer, Applied Biosystem).

FrbohD gene expression under drought stress

RNA isolation

After collection of samples from the three droughts treatments, the frozen shoots and leaves were ground to a fine powder with mortar and pestle. Total RNA was extracted from ground leaf tissues

from each sampling time by PureLink Plant RNA Reagent (Invitrogen) and the total RNA was quantified and checked for quality.

RQ real time RT-PCR

RQ real time RT-PCR was carried out using an ABI 27200 Real-time PCR step one plus System and step one plus software version 2.1 (Applied Biosystem, Foster City, CA). The *F. arundinaceae* specific GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) (Table 2), used as reference gene, was amplified in parallel with the target gene allowing gene expression normalization and providing quantification. Detection of Real-time PCR products was done using the SYBR Green universal master mix kit (Applied Bio system) following the manufacture's recommendations. Two microliters of cDNA were used as template for PCR. The expression stability reactions were performed under the following conditions: 20 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 62 °C in 96-well reaction plate. Three biological replicates for each sample were used for real-time PCR analysis and three technical replicates were analyzed for each biological replicate. To ensure the specificity of PCR products, a dissociation curve analysis was performed for each sample (Ririe et al., 1997). In addition, each sample was run in 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Data Analysis

We used step one plus software version 2.1 application software (Applied Biosystems, Foster City, CA) to collect the fluorescence data. The cycle threshold, C_T (the cycle at which the fluorescent signal is significantly different from background), was determined for each reaction. All replicates were pooled to estimate average C_T and standard deviation of C_T for each sample. In addition, any replicate showing nonspecific products in the dissociation curve analysis was removed. At least two of three technical replicates and six of the total replicates (from two biological samples) were included in the average C_T calculations. Raw expression values were calculated in Microsoft Excel using the average C_T values.

Result

Emergence traits and seedling growth

The highest final emergence (100%) was exhibited by population from Isfahan, and it was 50% in Barvado at 40% FC (Table 1). Final emergence for the Quchan accession was 6.7% at 40% FC

(Table 1). Whereas most populations achieved 50% or greater emergence at 40% FC, the low soil water content delayed days to 50% emergence compared to emergence at FC between 0 to 14 days depending on the accession (Table 1). Isfahan population showed the maximum GR (12.22) at 40% FC, whereas the lowest GR was obtained from Quchan (0.1) (Table 1). At 40% FC, Isfahan population displayed the best germination. In contrast, the Quchan were slow to germinate and sensitive to low soil water content (Table 1). The mean of seedling vigor index decreased by 42% with decreasing soil water content (Table 1). The highest SVI measurements occurred for the Isfahan population and the lowest for Quchan with 40% soil water content (Table 1). Under 40% FC SVI values decreased by 70% in Barvado (Table 1). For all populations, the leaf and root length of seedlings decreased significantly by more than 50% and 36% respectively between 100% and 40% FC (Table 1). Under 40% FC, Isfahan had the greatest leaf (8.8 cm) and root (6.4 cm) length, in comparison with other genotypes, whereas Quchan had the least leaf (2.3 cm) and root (2.3 cm) growth (Table 1). Leaf length and root length decreased by 52% and 21% respectively in Bravado in 40% compared to 100% FC (Table 1).

Cloning, sequence and gene expression analysis of FrbohD

The DNAs FrbohD encoding respiratory burst oxidase protein was cloned from *F. arundinacea* CV. Barvado and sequenced. We isolated a partial DNA length corresponding to FrbohD, and sequenced all clones. Partial Sequence analysis included 3903 bp nucleotide long. Furthermore FrbohD gene partial sequence (NCBI, accession number) analysis showed 76.8% similarity to RbohD (GenBank/EMBL accetion number AK072353 or Phytozome database, LOC_Os05g38980) (Wong et al., 2007) in *Oryza sativa.*

Leaf relative water content (RWC) of three entries *F. arundinacea* were measured in order to define the induction of the drought condition following water drought treatment. Upon 8 days of withholding water, the leaf RWC of all entries decreased to 37, 53 and 63% in Isfahan, Bravado and Quchan respectively (Fig. 1F). Leaf RWC in control of *F.arundinacea* was 94, 92, 93 and 93 % after 0, 2, 4, 6 and 8 days of withholding water respectively (Fig. 1). At the ninth day of drought stress, the leaves of the Isfahan and Barvado entries were completely fired under drought with RWC of 37 and 53% respectively. *F. arundinacea* entries were examined 4, 6 and 8-day after water withholding. Several pairs of primers were designed based on the sequenced of FrbohD in *F. arundinacea* CV. Barvado. After testing, primers FrbohDF6 and FrbohDR7 (Table 2) were used to analyse RbohD gene expression in three selected entries. To determine RbohD gene expression level in selected

entry, the expression of the FrbohD gene was analysed in leaf and shoot during drought stress using Barvado cultivar as a reference control (Fig. 1G and H). In leafs of Quchan entry, FrbohD expression levels indicated a 21-fold increase six days after water withholding, followed by 16.5-fold increase in control (Fig. 1G). In the shoot of Quchan entry, FrbohD expression levels showed 3.8 and 4.4 folds increase in control and drought four days after water deprivation respectively (Fig. 1H).

The specific tissue distribution of Rboh transcript, falls into 2 basic classes in rice: expression throughout the plant (Rboh A, B, C, E, F and G) or just in shoot and calli (Rboh D) (Wong et al., 2007; Groom et al., 1996). To understand how FrbohD functions in leafs and shoots of selected entry under drought stresses, the expression of the FrbohD gene was analyses 4, 6 and 8-day after water deprivation (Fig. 1A-E) using control of each entry as reference gene. We observed FrbohD expression in both leafs and shoots (Fig. 1A-E) following water withholding in all entries, although it was significant just in the leaf (Fig. 1A-C and E). The induction level in Quchan entry leaves was significant 4-day (2.3 fold) and 8-day (1.33 fold) after water deprivation (Fig. 1A-E) while Isfahan entry just showed significant induction in only 6-day (4 fold) after water withholding in leaf compared to control (Fig. 1C). There were no differences in the intensity of FrbohD expression among the drought stress and control in stem, 4 days after water deprivation (Fig. 1B). Drought stress significantly inhibited FRbohD expressions of Quchan entry shoots, six days after water withholding (Fig. 1D). Also FrbohD expression was suppressed significantly in the shoot of Barvado accession under drought stress (Fig. 1D). This observation indicates that the FrbohD gene also functions significantly during drought in the leaf in order to mediate drought stress effect. Furthermore FrbohD gene was significantly up-regulated in Quchan entry, as a sensitive entry in germination, under drought.

Discussion

There were substantially genetic differences in emergence and seedling development under conditions of drought stress among the different wild populations of *F. arundinacea* collected at different locations within Iran. Among fourteen entry that we gathered from different places in Iran, and 2 commercial cultivars, several accessions were not able to reach 50% emergence at 40% FC. FrbohD gene expression under drought stress after establishment in selected entry showed that FrbohD gene was significantly up-regulated in Quchan entry, a sensitive lot in germination, compared to Bravado entries. Furthermore this entry showed significantly higher FrbohD expression under drought stress compared to its control.

In this study, we found that seedling vigour index and final emergence of the tolerant genotypes were significantly higher under lower soil moisture levels. Studies on identification indices of drought resistance indicated that final emergence, leaf length, root length and seedling vigor index were most important and better indicators of establishment in *F. arundinaceae.* It is well known that rapid and complete germination are important during the establishment of grass species (Mut and Akay, 2010; Larsen and Bibby 2004). This finding was in agreement with Gazanchian et al. (2006) who found that none of the perennial grasses tested would emerge from soils at 25% FC. The fourth day of the water withholding stage is evidently the critical day, marking a notable decrease in the RWC thereafter (fig. 1F). The leaf RWC dropped by 57, 41 and 31 % in Isfahan, Barvado and Quchan entries respectively after 8-day of water deprivation (Fig. 1F). Furthermore 20 days after germination, leaf length decreased from 13.4, 8 and 14.5 cm to 8.8, 2.3 and 7 cm under drought stress in Isfahan, Quchan and Barvado entries respectively (Table 1).

It can be concluded that Quchan entry has the least RWC reduction under drought stress because of its slow leaf growth and transpiration rate. Furthermore, consistent with germination results, FrbohD was extremely up-regulated under drought stresses in leaf and shoot of Quchan entry compared to cultivar Barvado as reference gene. Rebello et al., (2008) investigation reported lower H2O2 level in drought–tolerance varieties compared to drought–sensitive varieties in rice. Similar to our results, Zhang et al. (2012) investigation showed that RbohH was extremely down regulated by drought in drought tolerance varieties but up-regulated in sensitive varieties. This up-regulated induction might be due to higher ROS accumulation in Quchan entry compared to Isfahan entry. Rboh proteins have important role in different cellular responses to biotic and abiotic stress in rice by facilitating the generation of ROS (Wong et al., 2007). Also similar differential induction has been reported in other plant but not systematically investigated. All Rboh genes, with exception of RbohD and RbohH, are constitutively expressed in each of the 4 organs (root, leaf, shoot and calli) (Wong et al., 2007). The products of these genes are thought to function not only in stress tolerance but also in the regulation of gene expression and signal transduction (Wong et al., 2007). However, other studies found that RbohD did not express in leaf in rice (Wong et al., 2007) our results showed that it up-regulated in leaf under drought in Quchan and Isfahan entries. It is important to examine the functions of the drought inducible genes among the populations not only for further undrestanding of the molocular mechanism of stress tolerance and response of the plant, but also for representing potentially useful germplasm for a turfgrass breeding program

In summary, our work revealed significant variation among populations *F. arundinacea* from different ecological regions of Iran in response to low soil water content. Isfahan entry had the best emergence, growth and SVI under drought conditions, while Quchan showed the worst final emergence and SVI. Partial-length DNA clone, FrbohD, encoding respiratory burst oxidase has been identified in *F. arundinacea* CV. Barvado. Consistent with germination results Quchan entry showed significantly higher FrbohD induction compared to Barvado and Isfahan entries. Furthermore, FrbohD down-regulated expression in Isfahan might be due to the higher antioxidant enzyme activity and low ROS accumulation in Isfahan entry compared to those in Quchan entry. The results indicated that the FrbohD gene also induced significantly during drought in the leaf compared to its control in order to mediate drought stress effect.

Acknowledgment

References

Abdul-Baki AA, Anderson JD. Relationship between decarboxylation of glutamic acid and vigor in soybean seed. Crop Sci 1973;13:227–32.

Abernethy GA, McManus MT. Biochemical responses to an imposed water deficit in mature leaf tissue of *Festuca arundinacea*. Environ Exp Bot 1998;40:17–28.

Bartels D, Sunkar R. Drought and salt tolerance in plants. Crit Rev Plant Sci 2005;24:23-58.

Cabin RJ, Ann SE, Randall JM. Genetic effects of germination timing and environment: an experimental investigation. Evolution 1997;1427–34

Campo S, Peris-Peris C, Montesinos L, Penas G, Messenguer J, San Segundo B. Expression of the maize ZmGF14-6 gene in rice confers tolerance to drought stress while enhancing suceptibility to pathogen infection. J Exp Bot 2012;63:983-99.

Foolad MR, Subbiah P, Kramer C, Hargrave G, Lin Y. Genetic relationships among cold, salt and drought tolerance during seed germination in an interspecific cross of tomato. Euphytica 2003;130:199–206.

Fry J, Huang B. Applied Turfgrass Science and Physiology. John Wiley & Sons, Inc. Hoboken, New Jersey, 2004.

Gazanchian A, Khosh Kholgh Sima NA, Malboobi MA, Majidi Heravan E. Relationships between emergence and soil water content for perennial cool-season grasses native to Iran. Crop Sci 2006;46:544-53.

Gibson DJ, Newman JA. *Festuca arundinacea* Schreber (*F. elatior* L. ssp. *arundinacea* (Schreber) Hackel). J Eco 2001;89:304–24.

Guo P, Baum M, Grando S, Ceccarelli S, Bai G, Li R, et al. Differentially expressed genes between drought-tolerant and drought-sensitive barley genotypes in response to drought stress during the reproductive stage. J Exp Bot 2009;60:3531–44.

Guo Z, Ou W, Lu S, Zhong Q. Differential responses of antioxidative system to chilling and drought in four rice cultivars differing in sensitivity. Plant physiol bioch 2006;44:828–36.

Groom QJ, Torres MA, Fordham-Skelton AP, Hammond-Kosack KE, Robinson NJ, Jones JDG. RbohA, a rice homologue of the mammalian gp91phox respiratory burst oxidase gene. Plant J 1996;10:515-22.

Hamrick JL. The distribution of genetic variation within and among natural plant populations. Addison-Wesley, New York, 1983. p 335-48.

Khowaja FS, Norton GJ, Courtois B, Price AH. Improved resolution in the position of drought-related QTLs in a single mapping population of rice by meta-analysis. BMC Genomics. 2009;10:276.

Kwak JM, Mori IC, Pedi ZM, Leonhardt N, Torres MA, Dangl JL, et al. NADPH oxidase AtrbohD and AtrbohF genes function in ROS dependent ABA signaling in Arabidopsis. EMB J 2003;22:2623-33.

Larsen SU, Bibby BM. Use of germination curves to describe variation in germination characteristics in three turfgrass species. Crop Sci 2004;44:891–99.

Maguire JD. Speed of germination-aid in selection and evaluation for seedling emergence and vigor. Agro J 1962;2:176-77.

Mut Z, Akay H. Effect of seed size and drought stress on germination and seedling growth of *Avena sativa*. L. Bulg J Agric Sci 2010;16:459-67.

Miller G, Schlauch K, Tam R, Cortes D, Torres MA, Shulaev V, et al. The plant NADPH oxidase mediates rapid systemic signaling in response to diverse stimuli. Sci Signal 2009;2:ra45.

Moller IM, Jensen PE, Hansson A. Oxidative modifications to cellular components in plants. Annu Rev Plant Bio 2007;58:459-81.

Murray MG, Thompson WF. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 1980;8(19):4321-25.

Pessarakli M. Hand book of turfgrass management and physiology. Taylor & Francis publishing company, Florida:CRC Press, 2008. p 690.

Rabello AR, Guimarães C, Rangel P, Silva FD, Seixas D, Souza ED, et al. Identification of drought-responsive genes in roots of upland rice (*Oryza Sativa* L). BMC Genomics 2008;9:485.

Ramolya PJ, Patel HM, Pandy AN. Effect of salinization of soil on growth and macro- and micro nutrient accumulation in seedling of *Salvadora persica* (Salvadoraceae). Forest Ecol Manag 2004;202:181-93.

Rechinger KH. Flora Iranica. 1970;70:450- 52.

Ririe KM, Rasmussen RP, Wittwer CT. Product Differentiation by Analysis of DNA Melting Curves During the Polymerase Chain Reaction. Anal Biochem 1997;245:154–60.

Saha MC, Mian R, Zwonitzer JC, Chekhovskiy K, Hopkins AA. An SSR and AFLP based genetic linkage map of tall fescue (*Festuca arundinacea* Schreb). Theor Appl Genet 2005;110: 323-36.

Severmutlu S, Mutlu N, Gurbuz E, Gulsen O, Hocagil M, Karaguzel O, et al. Drought resistance of warm–season turfgrasses grown in mediterranean region of Turkey. HortTechnology 2011;21: 726-36.

Stoytcheva m, Zaltev R. Agriculture chemistry. Published by InTec. Janeza. Croatia. Chapter 5, 2013. p 90-112.

Si Y, Zhang C, Meng S, Dane F. Gene expression changes in response to drought stress in Citrullus colcynthis. Plant Cell Rep 2009; 28:997-1009.

Si Y, Dane F, Rashotto A, Kang K, Singh NK. Cloning and expression analysis of the Ccrboh gene encoding respiratory burst oxidase in Citrullus colocynthis and grafting onto *Citrullus lanatus* (watermelon). J Exp Bot 2010;61(6):1635-42.

Sharifi Tehrani M, Mardi M, Sahebi J, Catalán P, Díaz-Pérez A. Genetic diversity and structure among Iranian tall fescue populations based on genomic-SSR and EST-SSR marker analysis. Plant Syst Evol 2009; 282:57–70.

Skriver K, Mundy J. Gene expression in response to Abscisic Acid and osmotic stress. Plant Cell 1990;2:503.

Takahashi S, Kimura S, Kaya H, Lizuka A, Wong HL, Shimamoto K, et al. Reactive oxygen species production and activation mechanism of the rice NADPH oxidase OsRboh B. J Biochem 2012;152(1):1635-42.

Usberti R, Valio IFM. Osmoconditioning effects on germination of Guinea grass (*Panicum maximum*) seeds. Seed Sci Technol 1997;25:303-10.

Wang W, Vinocur B, Altman A. Plant response to to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta 2003;218:1-14.

Wilman D, Gao Y, Leitch MH. Some Differences between eight grasses within the *Lolium Festuca* complex when grown in conditions of severe water shortage. Grass and forage Sci 1998;53:57–65.

Yu X, Bai G, Liu S, Luo N, Wang Y. Richmond DS et al. Association of candidate genes with drought tolerance traits in diverse perennial ryegrass accessions. J Exp Bot 2013;64:1537–51.

Van Zandt PA, Mopper S. The effects of maternal salinity and seed environment on germination and growth in *Iris hexagona*. Evol Ecol Res 2004;6:813–32.

Wong HL, Poinontoan R, Hayashi K, Tabata R, Yaeno T, Hasegawa K et al. Regulation of rice NADPH oxidase by binding of Rac GTPase to its N-Terminal extension. Plant Cell 2007;19:4022-34.

Yamada T. *Festuca*, In: Kole C. (ed.) Wild crop relatives: Genomic and breeding resources, millets and grasses. Springer, New York, p 153-64, 2011.

Zhang HW, Pan XW, Li YC, Wan LY, Li XX, Huang RF. Comparison of differentially expressed genes involved in drought response between two elite rice varieties. Mol plant 2012;5:1403–05.