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Research Article

Screening of Multiple Abiotic Stress-Induced Genes in Italian Ryegrass leaves

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ABSTRACT

Cold, salt and heat are the most critical factors that restrict full genetic potential, growth and development of crops globally. However, clarification of genes expression and regulation is a fundamental approach to understanding the adaptive response of plants under unfavorable environments. In this study, we applied an annealing control primer (ACP) based on the GeneFishing approach to identify differentially expressed genes (DEGs) in Italian ryegrass (cv. Kowinearly) leaves under cold, salt and heat stresses. Two-week-old seedlings were exposed to cold (4°C), salt (NaCl 200 mM) and heat (42°C) treatments for six hours. A total 8 differentially expressed genes were isolated from ryegrass leaves. These genes were sequenced then identified and validated using the National Center for Biotechnology Information (NCBI) database. We identified several promising genes encoding light harvesting chlorophyll a/b binding protein, alpha-glactosidase b, chromosome 3B, elongation factor 1-alpha, FLbaf106f03, *Lolium multiflorum* plastid, complete genome, translation initiation factor SUI1, and glyceraldehyde-3-phosphate dehydrogenase. These genes were potentially involved in photosynthesis, plant development, protein synthesis and abiotic stress tolerance in plants. However, this study provides new insight regarding molecular information about several genes in response to multiple abiotic stresses. Additionally, these genes may be useful for enhancement of abiotic stress tolerance in fodder crops as well a crop improvement under unfavorable environmental conditions.

(**Key words** : Abiotic stress, Forage, Gene, Italian Ryegrass)

Ⅰ. INTRODUCTION

Abiotic stresses mainly cold, salt, heat, drought and heavy metals are the major limitations of crop productivity which restrict full genetic potential, growth and productivity of crops globally (Tuteja et al., 2011). Cold stress induces several philological constrains through i) generating of reaction oxygen species (ROS), ii) reducing the photochemical efficiency (PSII), and that inhibits photosynthesis in plants (Paredes and Quiles, 2015). Salt stress negatively affects to cells by disturbing the ionic and osmotic equilibrium, whereas, heat stress leads to overproduction of free radicles, induce oxidative stress and subsequent non-reversible damage plant growth (Chalanika et al., 2017). In near future, it is expected that the initiation of heat weave with higher stress intensity will more severe due to global warming (Wang et al., 2016). Abiotic stresses induce a series of physiological, biochemical, molecular and morphological changes in crops including forages (Staniak et al., 2015; Rahman et al., 2015, 2016a). In addition, crop performance and yield are the consequence of genotypic expression that is modulated by crop interaction with environment. Thus, screening and functional characterization of genetic resources under abiotic stress would be useful for better understanding the adaptive response of plants to multiple abiotic stresses.

Italian ryegrass (*Lolium multiflorum*) is the winter forage crop mostly cultivated in Southern Brazil and other South American countries (Pavinato et al., 2014). Recently, it has been extensively cultivated in Korea. Ryegrass is economically most important forage species, considered as excellent source

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of high productivity with high nutritive value (Gerdes et al. 2005). Despite of its many superior properties of ryegrass, some species are known to be sensitive to abiotic stresses, thus limiting its cultivation in certain area (Hulke et al. 2008). Plants have evolved some strategies to avoid and/or tolerate several abiotic stresses, and molecular breeding approach using suitable candidate genes provides excellent support for forage crop improvement against multiple abiotic stresses (Alam et al., 2010; Singer et al., 2017).

Numerous molecular candidates have been extensively disclosed in several forages legume and grass species including perennial ryegrass (Hulke et al. 2008), alfalfa (Rahman et al., 2015, 2016 a,b), tall fescue (Lou et al., 2017), and Siberian wild rye (Xie et al., 2017), teff grass (Lee et al., 2018) under abiotic stresses. Advanced molecular tools give the new opportunities for understanding of global gene expression in plants. Regulation of gene expression, and functional responses towards abiotic stress tolerance have been demonstrated in forage and turf grass (Kopecký and Studer, 2014), *Arabidopsis* (Azevedo et al., 2016), and alfalfa (Li et al., 2017). Despite of great progresses in diverse plants, still needs to be elusive in Italian ryegrass (cv. Kowinearly).

Sequence-based several molecular approaches present a better alternative to identify and analyze gene expression. For instance, the serial analysis of gene expression (SAGE) was considered as suitable approach for gene expression profiling (Velculescu et al., 1995), followed by massively parallel signature sequencing (MPSS) (Brenner et al., 2000), suppression subtractive hybridization (SSH) (Sahu and Shaw, 2009), and next-generation sequencing (NGS) technologies (Jain, 2012) which were used to target range of transcript and low abundant genes in plants. In recent years, a GeneFishing approach is being successfully used to identify the differentially expressed genes (DEGs) in plants (Rahman et al., 2016b; Lee et al., 2018). Hence, the benefit of this suitable approach is that it increases the annealing specificity to the template and able to amplify only the genuine gene products. In this study, we applied annealing control primer (ACP) based gene fishing technique to identify cold, salt and heat-induced DEGs in Italian ryegrass (cv. Kowinearly), subsequently would be target genes to develop abiotic stress tolerant forage crops.

Ⅱ. MATERIALS AND METHODS

1. Plant materials, growth conditions and application of abiotic stresses

Italian ryegrass (*Lolium multiflorum* cv. Kowinearly) seeds were collected from the Division of Grassland and Forages, National Institute of Animal Science (NIAS). Italian ryegrass seeds were sterilized using 70% ethanol for 2 minutes and washed three times by Mlli-q, followed by the seeds were treated by 30% sodium hypochlorite (NaOCl) solution for 30 min. Finally, surface sterilized seeds were moved to germination medium containing 3% sucrose and half MS (Murashige & Skoog). The culture room environment was maintained in the room temperature as 25 ±1°C with of 400 μmol m^{-2} s^{-1} light and 14 h light/8 h dark period. Two-week old ryegrass seedlings were exposed to cold (4 °C), salt (200 mM NaCl) or heat (42 °C) treatments for 6 h, respectively. The control plants were maintained at 25 ±1°C temperature and/or irrigated by only water. After 6 h of all stress treatments the plants leaves were cut from shoot and leaf transition zone, washed properly using Milli-q water for three times, quickly frozen in liquid N_2 for further molecular analyses.

2. Isolation of total RNA and cDNA synthesis

Total RNA was extracted from abiotic stresses treated and non-treated (control) ryegrass leaves using TRIzolreagent (Qiagen, CA, USA). These RNA samples were used for the first stand cDNAs synthesis by reverse transcriptase. The reserve transcription reaction was carried out as described earlier by Lee et al. (2009). After completing of first-strand cDNAs synthesis, samples were diluted at by adding 80 μl of one fold dilution of Milli-q water, and prepared for the GeneFishing™ approach.

3. GeneFishing by annealing control primer (ACP)-based approach

DEGs using control primer (ACP)-based approach was carried out. For this molecular technique, Gene Fishing-kit (Seegene, Inc., Republic of Korea) was used. Subsequently, synthesis of

the second strand cDNA was performed according to traditional thermal cycler's protocol and experimental methods of Lee et al. (2011). Finally, the amplified products were collected and separated by using 1% agarose gel containing RedSafe™ (5μL/100 mL solution; ABC Scientific, USA).

4. Cloning and identification of DEGs

Abiotic stress-induced DEGs were isolated from the gel by using the GENCLEAN II Kit (Q-BIO gene, Carlsbad, CA, USA), and subsequently cloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA) based the manufacturer's protocol. Followed by the sequencing of cloned plasmids were performed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Finally, the sequences of specific genes were validated and confirmed by comparing to corresponding homologs in NCBI database (https://blast.ncbi.nlm.nih.gov/Blast).

Ⅲ. RESULTS AND DISCUSSION

We successfully screened and identified abiotic stress-induced several DEGs including *light harvesting chlorophyll a/b binding protein* (named as DEG 1), *alpha-glactosidase b* (DEG 2), *chromosome 3B* (DEG 3), *elongation factor 1-alpha* (*EF1a*; DEG 4), *FLbaf106f03* (DEG 5), *Lolium multiflorum* plastid, *complete genome* (DEG 6), *translation initiation factor SUI1* (DEG7), nd *glyceraldehyde-3-phosphate dehydrogenase* (DEG 8) in Italian ryegrass leaves (Figs. 1-3; Table 1). In the following sections, we have clarified the potential role of these identified candidate genes by evaluating previous genomics, abiotic stress responses, and biological importance in plant system.

A light complex is comprised of several subunit proteins which are supercomplex of photosystem, the functional unit of photosynthesis. We have identified cold stress-induced a gene encoding *light harvesting chlorophyll a/b binding protein* (LHCB; DEG 1). The *LHCB* gene is known as key apoprotein of the light-harvesting complex of photosystem II (PSII), this proteins is known to be associated in guard cell signaling. The up-regulation of *LSCB* in response to cold stress suggests that it is possibly inv *LHCB* protein gene containing important stress responsive element, showed similarities among dark, heat, salinity and drought response at developmental stage in barley (Qin et al., 2017). In our study, we identified cold-induced *LHCB*, therefore we hypothesized that this *LHCB* gene has pivotal role in photosynthesis and developmental stages in Italian ryegrass leaves.

Salt-induced DEG2 expression is enhanced by salt in Italian ryegrass leaves and identified as *alpha-glactosidase b* (*α-Gal b*; Table 1, Fig. 1). The *α-Gals* are member of the glycoside hydrolase family, which have pivotal role in leaf elongation during plant development (Chrost et al., 2007). Moreover, it was documented that *α-Gal* showed tolerance in plants against freezing (Pennycooke et al., 2003). In this study, α-*Gal* gene was found to be highly induced response to salinity, which indicates α-*Gal* might be also involved in salt tolerance in Italian ryegrass. Therefore, this gene would be used as suitable biomarker in abiotic stress response as well as development of abiotic stress tolerant forage crops. DEG3 is identified as encoded *chromosome 3B*, Choulet et al. (2014), produced a reference sequence of 1 gigabase (gb) *chromosome 3B* in *Triticum aestivum* and distributed its structural and functional features. Moreover, they clarified the relationship, gene expression function and chromosome location under15 conditions, also revealed that the several distal regions were enriched wherein some are responded to abiotic stimulus. However, the gene response in Italian ryegrass to abiotic stress such as NaCl in our study, was supported by this observation.

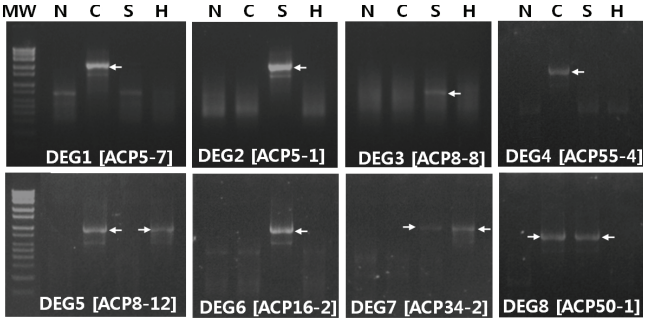

Fig. 1. Cold, salt, and heat-induced genes in Italian ryegrass leaves. Agarose gel image shows the differentially expressed genes (DEGs) induced by cold (4 °C), salt (NaCl; 200 mM), and heat (42 °C). Arrows on gel image indicate the stress specific DEGs. MW, molecular weight size marker; N, non-treatment; C, cold; S; salt (NaCl); H, heat; ACP, annealing control primer

Cold-induced DEG4 identified as *elongation factor 1 alpha* (*EF-1 alpha*) in ryegrass leaves. *LeEF-1 alpha* led to enhance

protein synthesis in tomato plants (Pokalsky et al., 1989). In addition, they also found that the increased expression of *LeEF-1 alpha* mRNA was correlated with higher levels of protein synthesis in developing tissues of tomato. Therefore, up-regulation of *EF-1 alpha* in our study would be recommend as a candidate for enhancement of protein synthesis in forage leaves, as our main deal with fodder improvement specially for livestock. In this study, we identified some genes in ryegrass leaves, and those specific function still unknown and uncharacterized. For example, the combined cold-heat induced *FLbaf106f03* (DEG 5), and a salt-induced *Lolium multiflorum plastid, complete genome* (DEG6). However, further exploration concerning abiotic stress-induced functional regulation of these genes is needed to develop our understanding.

Salt and heat-induced DEG7 was identified as translation initiation factor S*hortened Uppermost Internode1* (*SUI1*). The member of *SUI* genes family has significant importance in the development of plant aerial part (Yin et al., 2013). In our study, we hypothesized that the up-regulation of *SUI1*gene might be involved in the response of several abiotic stress regulations, as it induced significantly by salt and heat in ryegrass leaves. Moreover, Yin et al. (2013) found that overexpression of *SUI1* and *SUI2* led to enhance growth of internodes at the stages of vegetative development in rice. However, these above observations provide the role of *SUI1* in abiotic stress response along with plants development.

We also identified the *glyceraldehyde-3-phosphatedehydrogenase* (*GAPDH*, DEG8) enzyme in ryegrass leaves; this is a ubiquitous enzyme that plays important role in the glycolysis process. *GAPDH* is known to be participated in metabolic processes as well as abiotic stress tolerance in plants. Zeng et al. (2016) observed that the expression of several *GAPDH* genes, which were modulated by abiotic stimuli and associated with enhanced abiotic stress tolerance in wheat. However, the up-regulation of *GAPDH* in rye grass suggests that it would be potential candidate for conferring stress tolerance in forage crops.

Table 1. Cold-induced DEGs in Italian ryegrass leaves.

DEG No.	Cold induced genes	Name of (blastx search)	Total score	E value	Identity	Accession
01	Cold	Light harvesting chlorophyll a/b binding protein [*Tritivum aestivum*]	510	4.00E-141	87%	HM362991.1
04	Cold	Elongation factor 1-alpha (EF1a) [*Deschampsia antarctica*]	1628	0.0	92%	HM208299.1

Table 2. Salt‐induced DEGs in Italian ryegrass leaves.

DEG No.	Salt induced	Name of (blastx search)	Total score	E value	Identity	Accession
02	Salt	Alpha-glactosidase b (partial) [*Aegilops tauschii* subsp. *tauschii*]	990	00	95%	XM_020300011.1
03	Salt	Chromosome 3B [*Tritivum aestivum* cv. Arina]	111	3.00E-21	96%	HE996635.1
06	Salt	*Lolium multiflorum* plastid,complete genome [*Lolium multiflorum*]	2498	0.0	99%	JX871942.1

Table 3. Cold, heat, and salt-induced DEGs in Italian ryegrass leaves.

DEG No.	Cold-heat and salt	Name of (blastx search)	Total score	E value	Identity	Accession
05	Cold, Heat	FLbaf106f03, [*Hordeum vulgare* subsp. *vulgare*]	368	2.00E-98	87%	AK251177.1
07	Salt, Heat	Translation initiation factor SUI1 [*Brachypodium distachyon*]	512	1.00E-141	86%	XM_003562949.1
08	Cold, Salt	Glyceraldehyde-3-phosphate dehydrogenase [*Tritivum aestivum* cv. *wyuna*]	1474	0.0	93%	FN429985.1

Ⅳ. CONCLUSION

In this study, several DEGs including *light harvesting chlorophyll a/b binding protein*, *alpha-glactosidase b*, *chromosome 3B*, *elongation factor 1-alpha*, *FLbaf106f03*, *Lolium multiflorum plastid, complete genome*, *translation initiation factor SUI1*, and *glyceraldehyde-3-phosphate dehydrogenase* were identified using ACP-based GeneFishing approach. These genes were significantly up-regulated by various abiotic stresses (cold, salt and heat) in Italian ryegrass leaves. These identified genes were involved in photosynthesis, plant development, protein synthesis and abiotic stress tolerance. The identification of these genes in ryegrass leaves would be effective as promising candidates for the fodder crops improvement under multiple abiotic stresses.

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