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Chapter 4

Ameliorating Drought-Induced Stress in Turfgrass through Genetic Manipulation

Mostafa K. Sarmast

Additional information is available at the end of the chapter

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Abstract

To delineate the major processes associated with short water scarcity in four tall fescue species, we examined their enzymatic and nonenzymatic antioxidant activity and *FaSGR* expression levels. Moreover, we examined the possibility of *Agrobacterium*-mediated transformation of *Arabidopsis P5CS1* gene in tall fescue. According to the results, proline has been introduced as an important compatible osmolyte, so as to protect enzymes and cellular structures under water scarcity. In addition to that, superoxide dismutase (SOD) along with proline can be used as a core physiological indicator for the assessment of adaptability to environmental conditions. Results indicated that most of the superoxide that was produced as a result of drought stress was converted to H_2O_2 by SOD and subsequently detoxified by ascorbate peroxidase (APX) into H_2O. Notably, the *FaSGR* transcript increased drastically over the course of the drought stress in Pixie and Mini-mustang, in contrast to jaguar and h–d, supporting the notion of Stay GReen (*SGR*)-mediated chlorophyll degradation in the less drought-tolerant cultivars. Different modulations of ROSs quenching system in tall fescue genotypes suggest that even one stress signal causes different signaling responses in different cultivars. The heterologous transformation of *P5CS1* in *Festuca arundinacea* background, confirmed by PCR and transient *GUS* assay, most probably can improve tall fescue tolerance to drought stress.

Keywords: drought stress, enzymes, proline, *SGR*, tall fescue

1. Introduction

1.1. Drought stress

A world in which average temperatures reach 4°C above preindustrial levels would likely see unprecedented heat waves, severe drought, major flooding, and up to 1 m of sea-level rise (http://

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www.worldbank.org/). The aforementioned change in environment clearly is because of the careless human activities. In nature, plants as sessile organisms must respond to different stimuli throughout their life cycle; otherwise, fluctuations of these abiotic stresses outside their normal ranges can cause serious consequences on plant growth and reproduction. Many physiological and biochemical processes will be changed in plants under stress by affecting RNA stability, protein, and ion transport [1].

Stress is a specific environmental condition herein plant growth and development disrupted and its full genetic potential defeated [1]. Up to 70% of food crops yield is negatively influenced by biotic stresses, such as drought, cold, high salinity, and heat, which obviously threaten food security worldwide. Many organic compounds such as proline, glycine betaine, and polyamines, a variety of sugars (mainly fructose and sucrose), sugar alcohols, complex sugars (such as trehalose and fructans), and organic acids (oxalate, malate) can be aggregated inside the cell, while plants have faced with salt, drought, and cold to prevent cellular dehydration and protect cellular proteins. These osmoprotectants presumably accumulate several folds more than unstressed condition, without disturbing the intracellular biochemistry. Drought stress is by far the most important environmental factor contributing to yield losses in crops [2]. Plants experienced drought stress either when the water supply to roots becomes difficult or when the transpiration rate becomes very high. These two conditions often coincide under arid and semiarid climates. Imbalance between light capture and its utilization caused by drought stress inhibits photosynthetic activity [3]. The changes in the photochemistry of chloroplasts due to fluctuation of electrons in the leaves of drought-stressed plants result in the dissipation of excess light energy in the PSII core and antenna, thus generating reactive oxygen species (ROS), which are potentially dangerous under drought stress conditions [4]. Due to multigenic and quantitative properties of aforementioned stresses, advanced plant breeding purposes have been challengeable. High throughput sequencing and functional genomics tools provide new window to underlie signal perception and transduction of the molecular regulatory networks. The mechanism by which plants perceive and transmit the signals down to active adaptive response inside the cell is of great importance to mimic this strategy for manipulating organelle to convey tolerance properties under stress conditions. To do this, merging physiological, biochemical, and gene regulatory network knowledge is inevitable [5]. The objectives of the present work were as follows:

1. Elucidating cellular enzymatic and nonenzymatic antioxidative defense strategies in the drought stress response in leaves of four genotypically distinct *F. arundinacea* Schreb. genotypes during midsummer
2. Studying the regulatory role of *NYE*/Stay GReen (*SGR*) transcript during drought stress
3. Overexpressing of the *P5CS1* gene in tall fescue background aimed at improving drought tolerance of this species

1.2. Turfgrasses importance

The *Poaceae* family comprise over 9000 monocot species in which environmental grasses distribution is pretty diverse. Perennial grass has a specific role in providing food for livestock,

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bioenergy, fiber products, soil and water improvement and conservation, habitats for wildlife population, and recreation and beautification [6]. Over 50 grass species are cultivated as turfgrass, which have a major role in our daily life by providing a ground cover in parks, home lawns, sports fields, and golf courses [7]. Turfgrasses are also used along roadsides, edges of waterways, or in preserved land, where they may act as barriers to reduce pollution, water runoff, and wind erosion of the underlying soil [7]. The turfgrass industry potentially is a multibillion-dollar-a-year business. Turfgrass provide attractive vegetative ground cover, which is consistently mowed.

Turf species are divided into two groups based on origin and geographical distribution: cool-season grasses and warm-season grasses. Cool-season turf species are typically adapted to the cool–humid and cool–arid zones, and warm-season turf species are best adapted to the warm–arid and warm–humid regions. Most of the cool-season grasses are propagated by seeds, while a majority of the warm-season grasses are vegetatively propagated. Maintaining the quality of turf under adverse environmental conditions is of prime importance and actually a big challenge in turf growth and production. This challenge could be addressed through molecular breeding to create stress-tolerant germplasm. Traditional breeding techniques resulted in the production of many genotypes with an improved stress tolerance; however, traditional breeding progress is limited mostly due to the lack of superior stress-tolerant grass germplasm and because of poor understanding of the different aspects of physiological and molecular mechanisms for stress tolerance in perennial grass species. Finding and overexpressing of some drought-related candidate genes, either transcription factors, key enzymes, or channel proteins, recognized by RNA seq and other high-throughput techniques resulted in improving plant adaptation to drought stress [8]. The molecular mechanism and genetic information underlying drought stress have not yet been well addressed in turfgrass species.

1.3. Origin, taxonomy, and cytological features of *Festuca arundinacea* Schreb.

An increasing interest in tall fescue (*Festuca arundinacea* Schreb.) in western Europe and elsewhere is mainly because of its better drought resistance and yield potential in contrast to that of perennial ryegrass (*Lolium perenne* L.) [9]. Tall fescue is native to Europe; it also grows on the Baltic coasts throughout the Caucasus, in western Siberia, and extends into China [10].

The genus *Festuca* encompasses over 80 species, including two agriculturally important forage crops *Festuca arundinacea*, which is one of the most widely used cool-season species, and *F. pratensis* Huds [11]. Tall fescue is a wind-pollinated species with a high degree of self-incompatibility. Its genome size is approximately 6×10^3 Mbp (mega base pair) and contains three genomes (P, G1, and G2). The P ($2x$) genome is from the diploid species, meadow fescue (*F. pratensis*), while the G1 and G2 ($4x$) genomes are from the tetraploid species, *F. arundinacea* var. "glaucescens" [12, 13]. In comparison to other cool-season perennial grasses, tall fescue exhibits a high degree of stability when confronting drought stress. In contrast to having low rate of water use, tall fescue's deep and expansive root system allows this species to avoid drought conditions by continually having access to water [14]; even under continuous dry periods, the cells remain turgid. Transpiration is another strategy for tall fescue in order to cool itself via vast leaves [15]. This relative drought tolerance makes it an ideal option for

cultivation in urban landscapes throughout transitional climates. Tall fescue is a coarse-textured vigorous perennial bunchgrass. This species reproduces through tillering and seed. Its desirable agronomic characteristics encompass high yields of herbage, excellent persistence, adaptation to a wide range of soil conditions, compatibility with various management practices, long grazing season, and low incidence of pest problems [16, 17]. Given the continuous drought stress in many areas of the world, the necessity of highly tolerant turf species for landscaping, xeriscaping, and other usage is inevitable. Selection and/or production of high-tolerant transgenic turfgrass would be one of the key ways that leads to ameliorate their drought tolerance threshold in the realm of landscaping.

2. Drought stress and experimental design

Two replicate experiments with potted *F. arundinacea* were conducted. The treatment for each genotype had four replicates (four pots). The control plants were maintained in an optimal soil water condition (FC) during the whole experimental period. The experiment was carried out in loamy soil that was collected at a height of 20 cm from the top of the department's research field in plastic pots (45 cm in height). Potted plants grown under greenhouse conditions were subjected to drought by withholding irrigation for 8 days, whereupon leaves withered and started to discolor in most genotypes. Mini-mustang, Pixie, Jaguar, and h–d were selected in this experiment to capture the range of genetic and phenotypic (aesthetic value) diversity observed in the 11 cultivars. Fully expanded youngest mature leaves of the aforementioned genotypes were collected at days 2, 4, 6, and 8 after the start of the drought treatment for biochemical experiments. Well watered pots (irrigated daily) served as a control for collecting leaf tissue. Leaf samples were collected at midday and were immediately frozen in liquid nitrogen and stored at −80°C until analysis.

2.1. Biochemical experiment

2.1.1. Enzyme assay

The methods used to determine the activities of catalases (CAT) and ascorbate peroxidase (APX) were those reported by Ref. [18]. Superoxide dismutase (SOD) activity was estimated by measuring the decrease in absorbance of superoxide nitro blue tetrazolium complex by the enzyme [19]. The activity of peroxidase (POX) was determined using the method of Ref. [20], with minor changes. Proline content was quantified using the protocol described by Ref. [21].

2.2. qRT-PCR analysis

Total Ribonucleic acid (RNA) extraction, cDNA synthesis, and qRT-PCR analysis were performed as described previously [22].

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2.3. Tall fescue tissue culture and callus regeneration

Tall fescue (*F. arundinacea*) seeds were submerged into 25% sulfuric acid for 30 min and then prewashed in tap water overnight. These seeds were moved into the airflow cabinet hood; then they were soaked into 25–50% Clorox (containing 5.25% sodium hypochlorite) solution containing 0.02% household detergent for 30 min for surface sterilization and then rinsed six times with sterilized distilled water. Finally, the dehusked seeds were cut longitudinally and placed onto MS [23] basal medium supplemented with 0–16 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D), in which explants gained the ability of callus induction at dark in 2 weeks. These calli were subcultured consequently for more than a year, every 4 weeks, on MS media containing 5–8 mg/l 2,4-D. We tried to keep the embryogenic calli in subcultures which were identifiable easily by the yellowish color and firm texture compared to the nonembryogenic calli. In all these experiments, the calli were kept at dark. BAP (0.1, 0.5 mg/l) and kinetin (0.1 and 0.2 mg/l), W/O 2,4-D, have been considered for plant regeneration of tall fescue calli. The pH of all media was adjusted to 5.8 by 0.1 N HCl before autoclaving for 15 min at 121°C and 1.5 kg/cm^2 pressure. Cultures were kept at 25 ± 2°C temperature under cool white fluorescent light (30 μmol/m^2/s), with 16/8 h day/night photoperiods.

2.4. *Agrobacterium*-mediated transformation

pGV3101 strain of *Agrobacterium tumefaciens* harboring pBI121 plasmid as a binary vector contains the *gus* gene under the control of the cauliflower mosaic virus' (CaMV) 35S promoter and the selectable marker neomycin phosphotransferase II (nptII) under the control of the CaMV promoter. Aforesaid bacteria were grown and selected in rotator (200 rpm) in LB liquid media (tryptone 10 g/l, NaCl 5 g/l, yeast extract 5 g/l) supplemented with 50 μg/ml kanamycin for 24 h at 28°C. The cells were harvested by centrifugation (8000 rpm for 10 min at 4°C) and further resuspended in 10–15 ml of MS medium. Acetosyringone (AS) was added to the medium up to 100 μM. For transformation, 4 × 4 mm of calli grown at dark were considered for inoculation with *A. tumefaciens* (OD_{600} = 0.5–1) under 400 mg Hg pressure for 10–15 min; then, the callus pieces and *Agrobacterium* were incubated together for 20 min with gentle shaking. Excess bacteria were removed after the incubation. The infected callus pieces were transferred onto filter papers for a few minutes and then placed onto cocultivation MS medium supplemented with 100 μM acetosyringone (AS) and 5 mg/l 2,4-D for almost 3 days (in dark) at 25°C. Sometimes, we used to use an empty petri dish containing a Whatman filter paper, moisturized with sterile water, for cocultivation. After cocultivation, the explants were washed with sterile distilled water once; for the second time, the explants were incubated with either 500 mg/l cefotaxime or 400 mg/l Timentin for about 30 min, along with gentle shaking to prevent *Agrobacterium* overgrowth later on (in regeneration MS media); then they were blotted onto sterilized filter papers and placed on MS medium supplemented with 5–8 mg/l 2,4-D, 150–200 mg/l G418 for the selection of transformed explants and 200 mg/l Timentin to prevent *Agrobacterium* overgrowth for 2 weeks. For plant regeneration, explants were transferred to regeneration media supplemented either with BA (6-Benzylaminopurine) or kinetin in light. Cultures were kept at 25 ± 3 °C under cool white fluorescent light (30 μm/m^2/s), for 16 h each day.

2.4.1. PCR confirmation and gus histochemical staining

The presence of *uidA* gene in embryogenic calli was confirmed by PCR amplification of tall fescue-calli genomic DNA with gene-specific primers: *gus*-F (GCTGTGCCAGGCAGTTTTAAC) and *gus*-R (ATATCGTCCACCCAGGTGTTC). The predicted size of the amplified DNA fragments of *uidA* was 425 bp. DNA amplifications were performed in a total volume of 20 μl containing 1 μl of 10 μM forward pimer, 1 μl of 10 μM reverse primer, 2 μl of 10× Ex Taq Buffer, 0.5 μl of dNTP mixture (2.5 mM each), 0.1 μl of TakaRa Ex Taq enzyme (5 unit/μl) (Takara, Shuzo, Kyoto, Japan), by Thermocycler (Bio-Rad, United States). PCR was carried out for screening of regenerated transformed plantlets with an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C, 30 min; 58°C, 30 min; 72°C, 30 s; and a final extension, 75°C for 10 min. The PCR products were separated in 1% agarose gel containing 0.5 μg/ml ethidium bromide. The size of the amplification products was estimated using a 100 bp DNA ladder (GeneRuler DNA Ladder Mix # SM 0331, Fermentas). GUS histochemical staining was performed as described previously [24].

3. Tall fescue biochemical response to short drought stress

In response to water stress, plants exert adaptive modifications in their morphological, physiological, and biochemical properties. Photosystem II (PSII) is more vulnerable than PSI during drought stress in which inhibition of CO_2 assimilation, coupled with the changes in photosystem activities and photosynthetic electron transport capacity, results in accelerated production of reactive oxygen species (ROS). The damaged targets by ROS attack are recovered by repair or by replacement via de novo biosynthesis. However, under severe drought stress, cell death would be inevitable. The fate of stressed cells is determined by the duration of stress as well as the protective capacity of the plant. Reactive oxygen species play a crucial role in causing cellular damage under drought stress [25]. Reactive oxygen species not only play a signaling role in coordinating nuclear gene expression in order to protect cells during biotic and abiotic stress responses, but can also cause lipid peroxidation and consequently membrane injury, protein degradation, and enzyme inactivation [26, 27]. All plants have ROS detoxification mechanisms, which are enzymatic, with superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POD), glutathione reductase (GR), and monodehydro ascorbate reductase (MDAR), and nonenzymatic detoxification mechanisms through flavanones, anthocyanins, carotenoids, and ascorbic acid (AA). The activity of antioxidant enzymes under drought stress will be enormously flexible among several plant species and even between two cultivars of one species. It has been demonstrated that enzymatic breakdown of ROS is one of the major processes the plant uses to scavenge this signaling molecule [28]. Superoxide dismutase, the first enzyme in the detoxifying process, converts superoxide anion radicals ($O^{2?}$) to hydrogen peroxide (H_2O_2), and APX reduces H_2O_2 to water using ascorbic acid as a specific electron donor [29–31].

It has been stated that ROS not only cause damage to membrane, but also disturb the correct functions of DNA and proteins. Enzyme-mediated disruption of ROS effectively quenches ROS in plant cells [28].

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To underlie the key processes concomitant with drought tolerance in tall fescue, four genotypes (Jaguar, h–d, Pixie, and Mini-mustang) were assessed for enzymatic and nonenzymatic antioxidant activity as well as *SGR* expression during water shortage. These cultivars were selected from a larger set of 11 tall fescue cultivars based on the genetic relationships delineated via intersimple sequence repeat (ISSR) analysis [22]. These cultivars were thus selected for preliminary experiments in which drought stress was induced by ceasing irrigation in plastic pots for up to 8 days. Two antioxidant enzymes' activity (markers of drought stress) increased in all four cultivars due to drought stress—APX and SOD. Increased SOD activity of Mini-mustang, Pixie, jaguar, and h–d (6 days only) is in agreement with [32], who observed similar patterns when heat stress was applied on two annual and two perennial cool-season turfgrasses. A strong correlation between SOD activity and drought-induced oxidative stress tolerance has been well established [33]. At day 8, SOD activity slightly declined in h–d. Such decline in SOD activity in severe drought stress has been previously reported [34]. Chlorophyll stability was associated with leaf APX activity in the Jaguar and h–d leaves. APX is known as a chlorophyll protector in leaves [35, 36]. The connection between SOD and APX activity in most genotypes suggests that most of the superoxide that was produced as a result of drought stress was converted to H_2O_2 and subsequently detoxified by APX into H_2O [37]. In transgenic tall fescue, high levels of APX and SOD were considered as defense mechanisms during stress conditions [38]. Regardless of Jaguar, all cultivars showed a nonsignificant decline in CAT activity at 8 days. Such decline in CAT activity has been reported by several authors in different stress conditions [33, 34, 39, 40]. Pixie and h–d genotypes experienced a nonsignificant decline in catalase activity, which does not seem to be in accordance with chlorophyll changes which is in line with the reports of [41, 42]. Identification of some chloroplast proteins that potentially can be involved in the regulation of drought stress in *F. arundinacea* has been reported by [43] during short water scarcity in tall fescue. In another report, the higher photosynthetic capacity of an intergeneric hybrid of *L. multiflorum*/*F. arundinacea* was likely due to higher efficiency of Calvin cycle during the drought stress [44]. The kinetic variation observed among the antioxidant activity most likely depends on species and cultivars, time and type of stress, tissue or organ types, as well as severity of stress [28, 45].

4. Osmoregulatory effects of proline

Proline is a very specific amino acid essential for primary metabolism, which can accumulate in eubacteria, protozoa, marine invertebrates, and plants under stress condition. First proline accumulation report has been addressed in welting perennial ryegrass [46]. Soon after that, numerous reports have shown proline accumulation under drought [47], high salinity [48], high light and ultraviolet (UV) irradiation [49], heavy metal [50], oxidative stress [32], and biotic stress [51], in which proline metabolism by far has mainly been studied in response to osmotic stress [52]. Proline is synthesized in cytosol mainly from glutamate, which can convert to proline in two steps. First, glutamate is reduced to glutamate-semialdehyde (GSA) and then spontaneously converted to pyrroline-5-carboxylate (P_5C) by the pyrroline-5-carboxylate synthetase (P_5CS) and P_5C reductase (P_5CR), respectively [53, 54]. Proline is eventually

catabolized to P_5C in mitochondria by proline dehydrogenase (PDH) or proline oxidase, and then P_5C dehydrogenase (P_5CDH) converts P_5C to glutamate [55]. Kumar et al. [56] evaluated T_1 transgenic plants of *indica* rice overexpressing a *P_5CS* gene. Their research indicated a better growth performance, biomass production, higher proline accumulation, and lower rate of lipid peroxidation in transgenic plants compared to nontransgenic plants under 150 mM NaCl stress. Furthermore, the ameliorating effects of proline on heavy metal stress have been reported on *Chlamydomonas reinhardtii*, which was able to express *P_5CS* gene 80% higher than wild type [57].

Proline can be encoded by *P_5CS_1* and *P_5CS_2* in cytosol. While *P_5CS_1* translational fusion of GFP normally localizes in cytosol, but right after osmotic stress, the *P_5CS_1* signal was detectable in chloroplast but not *P_5CS_2*. Székely et al. [58] and Strizhov et al. [59] reported the augmentation of proline under osmotic stress in chloroplast, which was induced by *P_5CS*. This report sheds light on different subcellular compartments of proline biosynthesis, which is pretty different based on the environmental conditions. A proline uniporter, which facilitates proline transport into the mitochondrial matrix, and a proline/glutamate antiporter, which appears to have an important role in the Pro/Glu shuttle between the mitochondrial matrix and the cytosol, have been identified in *Triticum durum* Desf. mitochondria [60]. In *Arabidopsis*, *P_5CS_2* is a house-keeping gene, whereas *Arabidopsis P_5CS_1* is induced by osmotic and salt stresses [48]. Proline has been shown to function as a molecular chaperone able to protect protein integrity and enhance the activities of different enzymes. Proline acts as a singlet oxygen quencher and has a ROS scavenging activity. Hien et al. [61] reported that proline accumulation in *Oryza sativa* was related to induction of proline biosynthesis by osmotic stress and not proteolysis. In *Opuntia* spp. exposed to heat, salinity, and water-deficit stress, an increase in the proline content was reported, but this accumulation was not necessarily correlated with P5CS enzyme activity, whereas the transcript level of *P5CS* was correlated with proline accumulation in the report of Silva-Ortega et al. [62]. We evaluated the proline content of the tall fescue leaf during the course of drought stress, because predominately proline accumulation is a physiological plant response to biotic and abiotic stress. The research results indicated that proline content in all tested genotypes increased significantly, especially at the highest deficit irrigation treatment. For example, Pixie showed over 50-fold increase in proline content than control plants, while this was about twofold increase in h–d genotype. We concluded that the substantial increase in proline content in Pixie and a lesser extent in Mini-mustang likely are because of their dependency on proline, so as to maintain cell homeostasis during water stress. However, other drought-tolerance mechanisms may be responsible for slight changes of proline in h–d and Jaguar. Our research results collectively suggest that proline is an important compatible osmolyte which serves as a protectant for enzymes and cellular structures of tall fescue under severe drought stress.

5. Overexpression of *Arabidopsis P5CS* in *F. arundinacea* calli

Particle bombardment–genetic transformation of *F. rubra* L. was one of the preliminary reports by Ref. [63]. However, 12 years later, the first successful *F. arundinacea Agrobacterium*-mediated

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transformation was published [64]. Since the success of *Agrobacterium*-mediated transformation of rice in the early 1990s, transformations of some other monocotyledonous species occurred routinely. *Agrobacterium*-mediated transformation has several advantages over the biolistic method, not the least of which is the stable integration and expression of the target gene in offspring, mostly due to lower copy number, and fewer rearrangements in genome [47, 65]. Bettany et al. [64] were able to regenerate only one transgenic *F. arundinacea* expressing *uidA* gene after establishment in soil, even though they failed to produce seeds. The *Agrobacterium* LBA4404 was the strain that had been used in their research. They reported a huge variability in their transgenic events, which in most cases did not follow Mendelian segregation [64].

Soon after the last aforementioned report, Wang and Gee [66] developed a much more efficient *Agrobacterium*-mediated transformation procedure aimed at regenerating transgenic tall fescue fertile plants. They reported that the number of hygromycin-resistant calli obtained per dish was in the range of 2.0–5.8; the number of transgenic plants recovered per dish was in the range of 0.4–1.7, and a 1:1 segregation ratio of the transgenes was found in the progenies. Gao et al. [67] gained 10.5% average transformation efficiency across the four callus lines of *F. arundinacea*. In their report, *Agrobacterium*-mediated transformation appears to be the preferred method for producing transgenic tall fescue plants. Zhao et al. [68] examined whether salt tolerance can be improved stably by overexpressing vacuolar $Na^{+/}H^{+}$ (*AtNHX1*) antiporters in tall fescue. Their research leads to identifying a single-copy inheritance of $AtNHX_1$—performing better in the presence of 200 mM NaCl than control plants—in most of the T1 and T2 lines of tall fescue after *Agrobacterium*-mediated transformation, with a near 1:1 segregation ratio which subsequently has been approved for release by the Chinese Department of Agriculture [68]. Dong and Qu [69] reported that *Agrobacterium*-mediated transformation of tall fescue yields 34% *hyg* B-resistant calli and had 8% overall transformation efficiency. Hu et al. [53] had improved the cold resistance of tall fescue through *Agrobacterium* transformation of *ipt* gene. Transgenic plants have had higher chlorophyll content and stayed greener, besides having a higher tillering ability as in contrast to that of control plant, which is of great economic importance to improve the tolerance of this plant to environmental stress. To the best of our knowledge, the *Agrobacterium*-mediated transformation of *F. arundinacea* by *P5CS* gene has not yet been reported.

The finding that abiotic stress is involved in chloroplast–stroma protein degradation under drought stress in numerous plant species, together with the demonstration of enhanced osmotic stress tolerance in plants by proline overproduction, spurred us on applying P_5CS gene encoding proline for improving drought stress in *F. arundinacea*. The preliminary work on callus production resulted in a high amount of callus during a year and so, with different types of calli. Soft, yellowish calli were chosen as a primary sample for agrotransformation. Whereas the callus regeneration rate was astonishing, plant regeneration was far behind callus regeneration. An increase in regeneration efficiency was observed in media supplemented with 8 mg/l 2,4-D, which was almost six times greater than 2 mg/l 2,4-D treatment by dehusked, longitudinally sliced seeds. Apart from 2,4-D concentration, dehusked *Festuca* mature seeds showed a further callus induction efficiently as opposed to intact seeds. The callus regeneration

frequency from small *Festuca* callus explants was also enhanced in dark as compared with light situation. Acetosyringone in cocultivation media greatly improved *Agrobacterium* growth, while acetosyringone-free MS media delayed *Agrobacterium* growth and subsequently, most probably, could decrease *Agrobacterium*-mediated transformation efficiencies. The heterologous transformation of *P5CS* in *F. arundinacea* background was confirmed by PCR and transient *GUS* assay, which most probably can improve tall fescue tolerance more to drought stress.

6. NYE/SGR protein role during drought-mediated chlorophyll degradation

During senescence, plant-recycled valuable nutrient components from the leaves and leaf chlorophyll (Chl) are usually converted to a colorless product through six known Chl catabolism enzymes (CCEs) and a metal-chelating substance (MCS) [70]. First, Chl *b* reductase (CBR) reduces 7-formyl group of Chl *b* to a hydroxymethyl group. There are two CBR isoforms which are encoded by *NON-YELLOW COLORING 1* (*NYC1*) and *NYC-LIKE* (*NOL*) genes. Another key regulator of Chl degradation is hydroxymethyl Chl reductase (HCAR), which reduces Chl *b* to Chl *a* [71, 72]. HCAR has recently been identified in *Arabidopsis*. Then, the Mg^{+} atom of Chl *a* is removed by MCS, which is then called pheophytin *a* (Phein *a*). Pheophytinase (PPH) produces Pheophorbide *a* (Pheide *a*) by catalyzing Phein *a*. Subsequently, the chlorin macrocycle of Pheide *a* is oxygenolytically opened by Pheide *a* oxygenase (PAO) [73], and red Chl catabolite (RCC) which is the product of this reaction is reduced to a nonphototoxic primary fluorescent Chl catabolite (*p*FCC) by RCC reductase (RCCR). Besides CCEs and MCS, STAY-GREEN1 (SGR1) or nonyellowing mutant can cause a stay-green phenotype in many plant species, such as *Arabidopsis* and rice [74]. The *NYE1* (nonyellowing) gene in *Arabidopsis* has been identified by positional cloning. NYE1 is now widely referred as SGR (Stay GReen) [75]. The *nye1-1* mutant could retain 50% chlorophyll at the end of 6-day dark incubation, whereas the wild type (*Columbia-0*) has resulted in the degradation of chlorophyll to less than 10% [75]. In addition to that, qPCR result has outlined *AtNYE1* as an extremely induced gene by senescence signals [75].

In *Festuca arundinacea*, this gene is called NONYELLOWING PROTEIN1 [nonyellowing gene (NYE)], which has 278 amino acids [76]. *FaNYE1* or *SGR1* has a high similarity to *Arabidopsis NYE1*, either by sequences or by function. *FaNYE* has been identified recently by RACE-PCR [76]. Overexpression of *AtNYE1* results in pale-yellow leaves to even albino seedlings [74]. Degreening phenotype in tall fescue occur during severe stress conditions and harsh seasons. BLAST analysis revealed that *FaNYE* has 89% sequence similarity to *Triticum urartu* Tumanian ex Gandilyan and 83% sequence similarity to *Hordeum vulgare* (NCBI). The negative correlation of *FaNYE* transcript with chlorophyll content has been previously addressed in *F. arundinacea* affected by dark treatment and natural senescence, which in 9 days' dark incubation augmented *FaNYE* transcript level just about 52-fold. Furthermore, overexpressing *FaNYE* ORF in *Col-0* background leads to accelerated senescence [76]. Leaf chlorophyll concentration of the mutant diminished very slowly in rice *sgr* mutant, but steeply decreased in wild type

during the grain filling. However, no difference in photosynthetic activity was observed between the stay green mutant and the yellowing wild-type leaves [77].

We hypothesized that the SGR-mediated chlorophyll degradation in fescue is genetically dependent on *FaSGR*. To confirm this, we evaluated the *FaSGR* transcripts in our selected genotypes during drought stress by real-time PCR. The substantial increase in *FaSGR* transcript in Pixie and Mini-mustang cultivars suggests their SGR-mediated chlorophyll degradation. The *FaNYE1/SGR* role in chlorophyll degradation during dark treatment and natural senescence has been published [76]. During stress conditions, chloroplast metabolism modulates leaf senescence. Hence, prevention of chlorophyll degradation can be an interesting subject in order to generate new varieties of plants with higher performance. In a preliminary transient experiment on tall fescue leaves, we targeted tall fescue *SGR* via CRISPR-Cas9 technology under heat and salinity stress, and then realized that the leaves that were exposed to CRISPR-Cas9 construct vividly maintained more chlorophyll than control lines.

7. Conclusion

The kinetic results of this research noticeably suggest that proline, SOD, and APX probably can be key components to protect tall fescue cells from severe drought stress. Furthermore, the aforementioned components can be key targets for genetic manipulation of tall fescue, so as to maintain its cell homeostasis against drought stress. In addition, proline and SOD can be considered as the main physiological indicators for the assessment of adaptability to environment conditions. Also, targeting SGR in leaves of tall fescue can open a new window for future research on leaf senescence.

Author details

Mostafa K. Sarmast

Address all correspondence to: mkhsarmast@gau.ac.ir

Department of Horticultural Science, Faculty of Plant Production, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Golestan, Iran

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