



***RD29A-IPT* expression enhances drought tolerance in transgenic perennial ryegrass**

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Abstract

Genetic improvement and the identification of drought-tolerant cultivars are crucial in perennial ryegrass (*Lolium perenne* L.) turfgrass to enhance germplasm reserves for molecular breeding and the development of sustainable landscapes in arid and semi-arid green spaces. Cytokinins (CKs) are plant hormones that regulate various physiological processes, including cell division, shoot growth, and leaf senescence, and also are known to regulate plant responses to drought stress. This study aimed to enhance drought tolerance in perennial ryegrass cultivars by utilizing the drought-inducible *RD29A* promoter to drive the expression of the *IPT* gene, which boosts cytokinin levels. The research also compared the performance of these transgenic plants with wild-type (WT) plants and local perennial ryegrass accessions under varying irrigation conditions. Results showed that certain transgenic plants and local accessions displayed higher drought tolerance based on turf quality, physiological, and biochemical characteristics. The expression of the *IPT* gene was confirmed in transgenic plants exposed to drought stress. Transgenic lines including GM24, GM12, GC8, GC6, NC12, NC14, NS14, and GC3 exhibited increased drought tolerance, maintaining higher levels of cytokinins in the leaves, improving water content, photosynthetic rate, and antioxidant activity while reducing damage indicators. Catalase and superoxide dismutase activities were more influential than peroxidase in drought adaptation and recovery. The efficiency of the *RD29A* promoter and the use of the UBQ10 intron in the *IPT* gene construct affected gene expression. Moreover, the significant genotypic variation among local accessions indicates opportunities for improving drought tolerance through targeted breeding in sensitive and moderately tolerant genotypes. Further research is recommended to investigate hormonal balance and osmoregulation in transgenic and wild-type plants under multiple stresses. Additionally, identifying candidate genes involved in drought tolerance, particularly in local ryegrass accessions, should be a focus for future studies.

Keywords Accession · Isopentenyl adenosine · *IPT* expression · *Lolium* · Photochemical efficiency · *RD29A* promoter

Introduction

Perennial ryegrass (*Lolium perenne* L.) is one of the most important turfgrass species for sports fields, golf course fairways, and urban landscapes in areas with a temperate climate (Miao et al. 2022). The good characteristics of this turfgrass are its rapid establishment and tolerance to shade. However, the poor ability to survive in dry regions has limited its growth and distribution. Native plants have adapted to their growing regions and can be economically valuable as genetic reserves. Other economic aspects of these species are animal feed as forage and landscape design as turf, prevention of soil and wind erosions. Turfgrasses employ multiple strategies to cope with drought, including changes in growth parameters, photosynthetic pigments, antioxidant

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enzyme activation, and osmolyte accumulation (Miao et al. 2022; Pirnajmedin et al. 2024).

Cytokinins (CKs) are synthesized predominantly in the regions of active root development, and they play a significant role in the plant's response to abiotic stressors as well as the modulation of growth. CKs generally decrease under drought conditions, leading to reduced shoot growth and enhanced root growth, which aids water uptake. CKs regulate protective responses in plants under abiotic stresses from roots to shoots (Wu et al. 2021). Cytokinins have reportedly regulated antioxidant activity that results from enzymatic processes in leaf samples (i.e. SOD, POD, and CAT), triggering defense mechanisms in the leaves against abiotic stress (Xu et al. 2016). Furthermore, Zhang and Ervin (2004) indicated that CK exerts antioxidant potential to safeguard leaves from stress-driven oxidations. Drought tolerance in plants reportedly increased via applications of exogenous CK that enhanced concentrations of indigenous CK (Zhang and Ervin 2004; Masoabi et al. 2023) or by genetically modifying genes to achieve overexpression for isopentenyl transferase (*IPT*) from *Agrobacterium tumefaciens* (Merewitz et al. 2011b; Beznec et al. 2021).

Cytokinin homeostasis and signaling components have emerged as engineered targets for the development of stress-tolerant crops. The rate-limiting step of isoprenoid CK biosynthesis is catalyzed by *isopentenyl transferase* (*IPTs*) (Hai et al. 2020; Nguyen et al. 2021). Studies conducted in plants on the effects of *IPT*-driven modifications suggest that, at a physiological level, *IPT* expression enhances stay-green characteristics, delays senescence, reduces stress-induced oxidative damage, and protects the photosynthetic machinery. These enhancements frequently lead to increased or stabilized crop yields, particularly when exposed to environmental stress (Nguyen et al. 2021).

Expression of the *IPT* gene under the control of stress-inducible promoters such as senescence-associated receptor protein kinase (SARK) promoter delayed cellular senescence and, led to drought tolerance in tobacco (Rivero et al. 2009), wheat (Beznec et al. 2021; Ijaz et al. 2025), rice (Peleg et al. 2011), and maize (Muruo et al. 2023) and under a senescence-specific promoter (SAG12) in Creeping bentgrass (Xu et al. 2009; Merewitz et al. 2010, 2011a).

Few studies have directly compared the drought tolerance of local turfgrasses and transgenic plants at physiological and biochemical levels, with most research examining these groups separately. Understanding how perennial ryegrass responds to drought stress is essential for developing more resilient cultivars through molecular breeding. Research at the molecular and genetic levels has provided valuable insights into the mechanisms that enable perennial ryegrass to adapt to adverse conditions (Miao et al. 2022).

Few studies have directly compared local turfgrasses and transgenic plants in terms of drought tolerance at

physiological and biochemical levels and more researchers have examined these groups separately. Understanding the mechanisms of perennial ryegrass response to drought stress is crucial for obtaining superior stress- drought tolerant cultivars through molecular breeding. Studies of perennial ryegrass at the molecular and genetic levels have revealed a lot of useful information to understand the mechanisms of perennial ryegrass adaptation to an adverse environment (Miao et al. 2022). However, the research on drought stress-related genes of perennial ryegrass is very limited, and also molecular mechanisms in response to the drought environment are mostly unknown. Therefore, the identification and characterization of genes associated with drought resistance are critical in clarifying the mechanisms of perennial ryegrass adaptation to drought stress. Understanding CK regulating *IPT* transgenic perennial ryegrass and their local accession responses to drought stress is critically important for improving plant drought tolerance.

According to our previous works on *IPT* gene transformation to two cultivars of perennial ryegrass (Esmaeili et al. 2019), this present research aimed to analyze *IPT* gene expression under inducible RD29A promoter in drought stress conditions. The second aim of this research was to gather and identify local accessions of perennial ryegrass in Fars province, Persia, and assess their drought tolerance in comparison with transgenic and WT plants. Understanding the mechanisms of drought tolerance of turfgrasses can provide better usage of these cultivars in the landscape of arid and semi-arid cities.

Materials and methods

Plant materials and drought treatment

This experiment was conducted in the Research Greenhouse of the Department of Horticultural Science, School of Agriculture, Shiraz University, Shiraz, Iran (52° 32'E and 29° 36'N, 1810 m asl). In previous research, *IPT* gene-transformed samples of Grassland and Numan cultivars of perennial ryegrass which originated from callus, seed, and meristem were obtained (Esmaeili et al. 2019). To obtain a detailed assay, *IPT* gene expression was analyzed for transgenic and wild-type plants that were exposed to drought stress due to containing an inducible RD29A promoter. Moreover, the most important factors related to the drought tolerance of these plants and their local plants (collected from Fars province) were evaluated. Plant material including two WT cultivars (GW and NW are Grassland and Numan), *Rd29A-IPT* transgenic (NC1, NC4, NC8, NC11, NC12, NC13, NC14, GC3, GC4, GC5, GC6, GC8, GC9, GC16, NS14, NS22, GS1, GM12, GM21, GM24), and local accessions (Bereshneh1, Bereshneh 2, Chamsorabkhani, Delkhan 1, Delkhan 2,

Homayjan 1, Homayjan 2, Homayjan 3, Homayjan- Bereshneh, Komehr 1, Komehr 2, Khosroshirin, Margon, Sheshpir, Pirsabzali, Shirmohamadi, and Tangeh Ardakan). In all text and tables GC and NC show Grassland and Numan cultivars generated from transgenic callus; GM: Grassland and Numan cultivars generated from transgenic meristem; GS and NS: generated from Grassland and Numan cultivars generated from transgenic seed.

Transgenic, WT, and local plants were cultured in plastic pots with an area of 0.06 m² (5.5 kg soil mixture pot⁻¹) containing a mixture of 1:2 farm soil and decomposed manure (v/v). After one month of establishment, the plants were exposed to 10 days of drought stress after withdrawing watering. For *IPT* gene expression analysis, leaf samples were collected of transgenic and WT plants on the tenth day and were immediately immersed in liquid nitrogen and quickly transferred to a – 80 °C freezer. Visual quality and physio-biochemical factors were measured in three stages (well-irrigated, drought, and after 14 days of recovery). Field capacity and permanent wilting point of tested soil were measured at 33% and 19%, respectively. The average temperature and light intensity of the greenhouse were 25 ± 2 °C and 50,000 lx. Physico-chemical features of the soil were evaluated and reported (Table 1).

Turf quality, total chlorophyll (TChl), and relative water content (RWC)

The drought treatment was applied by withholding irrigation for 10 days. Turf quality was visually rated in well-irrigated, drought, and recovery conditions. Turf quality was scaled according to leaf wilt, uniformity in appearance, density, and color, rated from 1 to 9, where 1 describes brown and poor-quality turf, 6 describes a threshold for acceptable turf, and 9 describes dense- lush turf (Turgeon 2011).

Dimethyl sulfoxide (DMSO) assisted in extracting chlorophyll based on Hiscox and Israelstam (1979) method with few modifications. The chlorophyll content was recorded according to Arnon (1940). Leaf extracts were measured for absorbance values (645 nm and 663 nm) using an Epoch Microplate Spectrophotometer (Company Biotech, USA).

Leaf hydration status was assessed by measuring the relative water content (RWC) of each leaf sample according to Barrs and Weatherley (1962).

Photochemical efficiency (Fv/Fm) and photosynthesis rate

Fv/Fm determined variable fluorescence (Fv) to maximum fluorescence (Fm) ratio using a chlorophyll fluorescence meter (OS- 30p model, USA). The Fv/Fm ratio was read with a fluorescence meter in each replication with three recording data. Leaf clips were used to adapt individual leaves to darkness for 30 min before reading the Fv/Fm ratio.

Photosynthesis rate was measured under well-irrigated, drought and recovery conditions of all treated plants using a photosynthetic meter (Lci Console model, UK). Five fully expanded leaves were used for measuring the photosynthesis rate in each replication with three recording of data. The leaves were placed in the chamber for one minute to mix the gases well with a small ventilation system inside the chamber.

Electrolyte leakage (EL) and proline content

For determining the EL, after collecting fresh leaf samples (0.1 g), they were rinsed in deionized water and immersed in 20 ml. The leaves were incubated with a shaker within 24 h at 50 rpm. The initial conductivity (C primary) was measurable, and the leaves were then macerated before the autoclave process at 140 °C for half an hour. The tissue conductivity value (Cmax) was measurable, and the relative EL became calculable as C primary (%) over Cmax (Blum and Ebercon 1981).

Proline was quantified spectrophotometrically (UV-120 -20, Japan) by the ninhydrin method according to Bates et al. (1973). Leaves (100 mg) from treated plants were incubated in 10 ml of 3% sulphosalicylic acid for 48 h. Then 2 ml of leaf extract was applied to assay proline content. Ninhydrin (1.25 g) was added to glacial acetic acid (30 ml) and then phosphoric acid (20 ml, 6 M) was added and kept for 24 h at 40 °C. Two ml of plant extract, 2 ml of acid Ninhydrin, and glacial acetic acid (2 ml) were added and the mixture was boiled at 100 °C for 1 h inside a water bath. Then, the solution was cooled and the reaction was terminated. About 4 ml of toluene was added to the contents and mixed vigorously for a few seconds and OD values for the colored component were measured (520 nm) via toluene, the blank. From OD values, proline content (μmoles g⁻¹ FW) was calculated separately.

Table 1 Physico-chemical features of soil mixture

Soil texture	Sand (%)	Silt (%)	Clay (%)	pH	Ec (dS m ⁻¹)	Total N (%)	P (mg kg ⁻¹)	K (mg kg ⁻¹)
Sandy clay loam	55.68	22.28	22.04	7.35	0.71	0.295	10.5	454.4

Leaf CK content

To determine the endogenous CK content of transgenic, WT, and some local plants (those that performed well under drought stress), one gram of leaf samples was extracted into methanol (80% v/v), and hydrophilic contaminants were removed by centrifuge (2500 g) for 15 min. The supernatant was filtered by a Millipore 45 µm pore size membrane. Twenty µl of the solution was used for HPLC tests. The separation of cytokinins was achieved by HPLC with a Unicam, model Crystal- 200 fitted with a C18 reverse-phase column (Altex Ultrasphere-ODS, 250 × 4.6 mm, 5 µm) and a model 160 UV-detector monitoring at 260 nm. The HPLC solvents were Baker HPLC-grade acetonitrile and 40 mM triethylammonium acetate buffer pH 3.35. The column was eluted at 1 ml min⁻¹ with the following gradient: initial conditions were 6% acetonitrile, 94% triethylammonium acetate buffer, 6 to 11% acetonitrile over 15 min; and 11 to 35% acetonitrile within 15 min. Total CK was calculated as the sum of the contents of isopentenyl adenine (iPA), zeatin riboside (ZR), and dihydro zeatin riboside (DHZR). (Doumas and Zaerr 1988; Zhang et al. 2013).

Antioxidant enzymes assay and malondialdehyde content (MDA)

Superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) enzymes were measurable as stated by Zhang and Kirkham (1996). Accordingly, 0.5 g ground leaf tissues were processed in homogenization via sodium phosphate buffer (50 mM, pH 7.0) that contained 0.2 mM ethylenediamine tetra-acetic acid (EDTA) and 1% (w/v) polyvinylpyrrolidone (PVP). The lipid peroxidation (MDA content) was determined according to Dhindsa and Matowe (1981). A 0.5 ml enzyme extraction was dissolved in trichloroacetic acid (20%) that contained thiobarbituric acid (0.5% w/v). The tubes were placed inside a preheated 95 °C water bath for 20 min and then processed in a centrifuge at 10,000 g for 10 min. MDA concentrations were determined by subtracting OD600 from OD532 and OD450.

Gene expression analysis

To obtain RNA through extraction-based procedures, 100 mg of young leaf samples were aimed for water-deficit treatments. They were harvested and immediately immersed in the liquid N₂. Then, the RNeasy mini kit was used for isolating RNA (Topaz gene, Karaj, Iran). Spectrophotometric analysis determined the qualitative and quantitative features of RNA (NanoDrop ND 1000 Spectrophotometer-Thermo Fisher Scientific, Wilmington, DE, USA), and visual comparison of RNA was detected electrophoretically on 1% agarose gel.

Before use, RNA samples were stored at – 80 °C, followed by reverse transcription PCR (RT-PCR). Complementary DNA (cDNA) samples were stored at – 20 °C until further use. To validate the accuracy of cDNA synthesis, one µl of synthesized cDNA with a pair of specific reference genes of perennial ryegrass, eukaryotic elongation factor- 1α (*eEF- 1α*), based on Martin et al. (2008) was used for RT-PCR reaction which had the most expression (100%) in leaf samples. RT-PCR reaction programs for *eEF- 1α* and *IPT* genes are described in Table 2.

Statistical analysis

Experiments were conducted in a completely randomized design with three replications. All statistical analyses were performed using Minitab 17 software. Data were analyzed using SAS 9.4 Software and means were compared via the least significant difference (LSD) test ($p < 0.05$). Heat map clustering, principal component analysis (PCA), and correlation analysis of multivariate data were performed across all perennial ryegrass genotypes using the R 4.1.2 software.

Results

Visual quality

Under the well-irrigated condition, WT plants and transgenic lines displayed no significant differences in visual

Table 2 Primer sequences, RT-PCR reaction programs, and amplicon sizes of the internal gene (*eEF- 1α*) and target gene (*IPT*)

Gene	Primer sequence designed for quantitative RT-PCR (5' → 3')	RT-PCR programs	Amplicon length (bp)
<i>eEF-1α</i>	(F) GGCTGATTGTGCTGTGCTTA (R) CTCACCTCAAGGGTGAAAGC	Initial denaturation: 95 °C (3'), denaturing: 94 °C (30"), annealing: 60 °C (30"), extension: 72 °C (20"), final extension: 72 °C (7')	114
<i>IPT</i>	(F) GCCTCTACCTGGACGACA (R) GCTTGTGGCGGATGATGTG	Initial denaturation: 94 °C (4'), denaturing: 94 °C (1'), annealing: 65 °C (1') extension: 72 °C (25"), final extension: 72 °C (5')	200

quality after that these plants were grown in a greenhouse for 2 months. During drought stress, WT plants exhibited stress symptoms (i.e. leaf rolling and wilting) after 3–4 days of drought stress while the *RD29A-IPT* plants started to show drought symptoms after 7–10 days. After that, all plants were clipped and re-irrigated under optimal watering conditions until new growth of leaves for 14 days.

After 10 days of drought stress, the leaves of WT plants were completely desiccated and dried during recovery while, most of the transgenic lines had an acceptable appearance during drought stress except NC1, NC13, and NS22. Despite these observations during drought treatment, these plants were able to produce new and green leaves after 14 days of recovery. Furthermore, most of the accessions exhibited drought symptoms earlier in transgenic plants. Large leaf area and low crown density of accessions can be one possible reason for its response to drought stress compared to transgenic plants with small leaf area and thicker crowns (Fig. 1).

Chlorophyll content and RWC

There were insignificant differences among the transgenic lines and WT plants regarding total Chl content under well-irrigated conditions. The lowest Chl content belonged to Homayjan1. Chl content significantly declined ~68% in accessions such as Bereshneh2 and Homayjan3 under

drought stress compared to well-irrigated conditions. On the other hand, a decline of about 19% and 35% in Chl content was found in Tange Ardakan and Komehr1 accessions, respectively.

Chl content was notably declined in wild-type plants in water scarcity compared to *IPT* transgenic plants (Table 3). The highest Chl content was recorded for GM12 with a decrease of about 16% compared to the well-irrigated condition, while the GW plant exhibited a decline of about 65% during drought stress in comparison with the well-irrigated condition. Moreover, Chl content significantly decreased by about 54% in NC13 in response to drought stress than in well-irrigated conditions. There was negligible variation in total Chl content among transgenic plants. Overall, some of the transgenic lines of ‘Numan’ such as NC8 and NC4 had higher total Chl content compared to ‘Grassland’ under well-irrigated conditions.

RWC significantly decreased under drought stress in all plants, especially wild types. It should be noted that RWC showed a dramatic difference among plants under drought stress. The greatest decline in RWC belonged to WT plants (about 91–92%) under drought stress. In ‘Grassland’, the minimum and maximum decrease in RWC of leaves was recorded in GC3 and GC6 (about 18% and 43%) compared to the well-irrigated condition, respectively. In ‘Numan’s transgenic lines generated from callus, RWC had variability among transgenic lines, e.g., NC8 and NC1 had the

Fig. 1 Visual quality of transgenic (TG) and wild-type (GW: ‘Grassland’ and NW: ‘Numan’) plants (A) and local accessions (LA) (B) of perennial ryegrass after 14 days of recovery stage

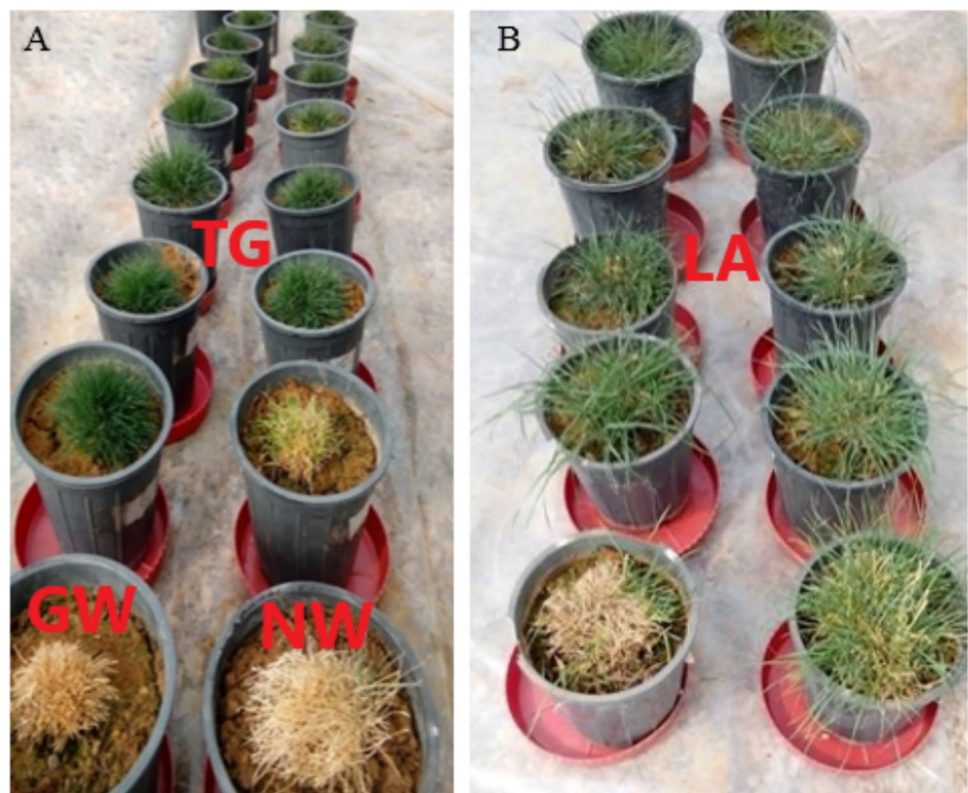


Table 3 Chlorophyll content of transgenic, and wild-type plants and local accessions under well-irrigated, drought and recovery states

Treated Plants	Chlorophyll content (mg Chl. g ⁻¹ FW)		
	Well-irrigated	Drought	Recovery
GW	4.51 ± 0.08	1.57 ± 0.01	*
NW	4.39 ± 0.02	1.75 ± 0.33	*
NC1	4.28 ± 0.06	2.64 ± 0.05	2.91 ± 0.10
NC4	4.79 ± 0.19	3.66 ± 0.23	3.90 ± 0.34
NC8	4.96 ± 0.05	3.43 ± 0.18	4.09 ± 0.25
NC11	4.57 ± 0.03	3.81 ± 0.06	4.05 ± 0.23
NC12	4.26 ± 0.039	3.33 ± 0.27	3.70 ± 0.60
NC13	4.34 ± 0.10	1.99 ± 0.28	3.03 ± 0.34
NC14	4.4 ± 0.13	3.47 ± 0.21	3.95 ± 0.20
GC3	4.61 ± 0.07	2.22 ± 0.00	3.79 ± 0.27
GC4	4.74 ± 0.05	3.43 ± 0.35	4.28 ± 0.03
GC5	4.27 ± 0.07	2.38 ± 0.19	3.50 ± 0.30
GC6	4.46 ± 0.13	3.10 ± 0.04	4.01 ± 0.17
GC8	4.27 ± 0.28	3.28 ± 0.26	4.09 ± 0.45
GC9	3.92 ± 0.08	2.94 ± 0.02	3.80 ± 0.26
GC16	4.29 ± 0.02	2.46 ± 0.29	3.94 ± 0.31
NS14	4.45 ± 0.15	2.78 ± 0.29	3.05 ± 0.16
NS22	4.26 ± 0.08	2.43 ± 0.37	3.83 ± 0.49
GS1	4.12 ± 0.25	2.47 ± 0.32	3.72 ± 0.29
GM12	4.26 ± 0.02	3.35 ± 0.12	4.22 ± 0.04
GM21	4.23 ± 0.04	3.05 ± 0.25	4.31 ± 0.07
GM24	4.25 ± 0.02	3.58 ± 0.23	4.85 ± 0.56
Bereshneh1	3.97 ± 0.06	1.93 ± 0.14	2.81 ± 0.13
Bereshneh2	3.24 ± 0.19	1.03 ± 0.03	2.37 ± 0.28
Chamsohrabkhani	4.26 ± 0.11	2.66 ± 0.32	3.54 ± 0.14
Delkhan1	4.22 ± 0.15	2.62 ± 0.30	3.99 ± 0.06
Delkhan2	4.29 ± 0.28	1.92 ± 0.11	3.27 ± 0.27
Homayjan1	3.05 ± 0.03	1.58 ± 0.19	2.25 ± 0.03
Homayjan2	3.78 ± 0.04	1.72 ± 0.35	2.85 ± 0.29
Homayjan3	3.58 ± 0.07	1.15 ± 0.015	3.02 ± 0.28
Homayjan-Bereshneh	3.32 ± 0.25	1.25 ± 0.07	2.17 ± 0.07
Komehr1	4.75 ± 0.20	3.07 ± 0.09	4.44 ± 0.15
Komehr2	4.30 ± 0.07	2.42 ± 0.13	3.64 ± 0.32
Khosroshirin	4.03 ± 0.01	1.83 ± 0.58	3.34 ± 0.06
Margon	4.20 ± 0.05	2.53 ± 0.13	4.01 ± 0.14
Pirsabzali	4.24 ± 0.06	2.38 ± 0.12	3.35 ± 0.57
Shirmohamadi	4.32 ± 0.02	2.03 ± 0.27	3.37 ± 0.18
Sheshpir	3.86 ± 0.16	1.73 ± 0.05	3.10 ± 0.10
Tangeh Ardakan	3.90 ± 0.40	3.15 ± 0.52	3.52 ± 0.25

Data are presented as means ± SE

W wild-type including GW and NW. from Grassland and 'Numan' cultivars. *Rd29A-IPT* is transgenic including NC1 to GM24. Which GC and NC: 'Grassland' and 'Numan' cultivars generated from transgenic callus; GM Grassland and Numan' cultivars generated from transgenic meristem; GS and NS generated from Grassland and 'Numan' cultivars generated from transgenic seed. Local accessions include Bereshneh1 to Tangeh Ardakan

*WT plants that did not recover. LSD value = 2.57

minimum and maximum decline of about 26% and 56% in comparison with the well-irrigated condition, respectively (Table 4). There was no significant difference in RWC among 'Grassland' transgenic lines produced from meristem tips as explants. According to the results, the GM24 transgenic line showed a decrease of about 18% in leaf RWC in conditions of water scarcity than the well-irrigated condition. In perennial ryegrass accessions, the most significant decrease was found in Bereshneh2 (77%) and a minimal decline was found in Komehr1 (34%) as compared to the well-irrigated condition.

Photochemical efficiency (Fv/Fm)

Leaf photochemical efficiency declined in all plants after 10 days of drought stress in comparison with those of the well-irrigated condition. As shown in Table 5, the Fv/Fm ratio significantly decreased (by about 84% and 62%) in NW and GW (WT plants) under drought stress in comparison to the well-irrigated condition.

The Fv/Fm ratio was significantly higher in *RD29A-IPT* transgenic lines compared to WT plants in response to drought stress (Table 5). Among transgenic plants, 'Numan' NS22 and NC1 showed a significant decline of about 48% and 29% in the Fv/Fm ratio compared to the well-irrigated condition. Some perennial ryegrass accessions such as Komehr1, Delkhan2, and Shirmohamadi showed higher Fv/Fm ratios compared to other treated plants under the well-irrigated condition. The Fv/Fm ratio in local accessions showed differences under drought stress, so Homayjan 3 and Bereshneh 2 exhibited a significant decline of about 55.7% and 47.4%, compared to the well-irrigated condition, respectively.

Photosynthetic rate (Pn)

Plant lines differed in photosynthetic rate under the well-irrigated condition (Table 6). The highest photosynthetic rate was observed in GC6 and the least was recorded in GC9. In local accessions, the highest photosynthetic rate was recorded in Komehr1 and the lowest photosynthetic rate in Homayjan1 under the well-irrigated condition. In this study, the photosynthetic rate of all plants significantly decreased under drought stress. However, the photosynthetic rate of transgenic lines such as NC4 showed a smaller decline (of about 33.8%) compared to WT plants (83%).

Leaf CK content

The total CK content in leaf samples declined in response to the shortage of water supply in WT plants, compared to *RD29A-IPT* transgenic plants. Similarly, all transgenic plants maintained a greater iPA content than the WT plants

Table 4 Leaf RWC of transgenic, and wild-type plants and local accessions in well-irrigated, drought and recovery states

Treated Plants	Leaf RWC (%)		
	Well-irrigated	Drought	Recovery
GW	83.10 ± 1.57	7.41 ± 0.86	*
NW	84.23 ± 0.17	5.90 ± 0.60	*
NC1	82.08 ± 3.01	35.38 ± 0.99	77.87 ± 2.18
NC4	85.75 ± 1.83	63.24 ± 3.26	75.11 ± 5.29
NC8	74.69 ± 2.61	54.40 ± 3.84	81.35 ± 7.37
NC11	87.00 ± 0.97	64.66 ± 1.53	82.91 ± 0.89
NC12	87.62 ± 0.72	67.7 ± 1.34	85.48 ± 2.86
NC13	89.69 ± 0.53	55.22 ± 2.74	76.79 ± 0.76
NC14	82.05 ± 1.06	51.23 ± 2.78	76.44 ± 1.12
GC3	85.10 ± 0.49	72.50 ± 2.78	81.53 ± 2.18
GC4	88.70 ± 4.66	64.17 ± 0.44	85.4 ± 0.99
GC5	87.97 ± 0.09	49.91 ± 0.71	84.94 ± 0.87
GC6	84.16 ± 0.93	59.93 ± 0.79	78.61 ± 1.06
GC8	84.29 ± 1.64	67.17 ± 2.26	78.90 ± 0.64
GC9	85.13 ± 0.74	58.90 ± 4.18	88.21 ± 1.68
GC16	89.31 ± 1.15	55.73 ± 1.65	82.90 ± 1.53
NS14	81.41 ± 1.08	54.28 ± 3.85	76.80 ± 1.63
NS22	84.30 ± 0.89	44.30 ± 2.87	78.60 ± 0.67
GS1	83.94 ± 1.14	54.63 ± 1.30	80.83 ± 1.20
GM12	87.10 ± 1.81	70.53 ± 1.55	87.67 ± 1.79
GM21	84.99 ± 0.70	66.45 ± 1.67	86.95 ± 0.34
GM24	85.70 ± 1.30	69.91 ± 0.87	88.07 ± 0.84
Bereshneh1	74.64 ± 0.80	23.27 ± 1.66	65.89 ± 5.06
Bereshneh2	73.95 ± 0.84	12.15 ± 0.45	58.85 ± 1.34
Chamsohrabkhani	75.14 ± 2.04	47.37 ± 3.06	78.19 ± 1.02
Delkhan1	76.21 ± 1.21	51.23 ± 3.94	75.30 ± 1.51
Delkhan2	76.60 ± 1.81	57.97 ± 9.58	73.83 ± 4.43
Homayjan1	71.55 ± 2.93	16.04 ± 1.85	68.28 ± 1.88
Homayjan2	65.72 ± 8.05	14.81 ± 1.42	70.83 ± 0.18
Homayjan3	76.71 ± 2.77	21.66 ± 7.66	58.64 ± 3.71
Homayjan-Bereshneh	76.99 ± 0.67	14.53 ± 1.09	50.30 ± 0.32
Komehr1	79.65 ± 0.39	52.42 ± 1.71	82.64 ± 0.56
Komehr2	81.76 ± 0.94	41.27 ± 3.32	80.78 ± 1.21
Khosroshirin	78.27 ± 1.27	31.00 ± 3.21	79.83 ± 1.26
Margon	75.76 ± 6.65	36.88 ± 0.82	83.69 ± 0.12
Pirsabzali	71.46 ± 4.53	22.05 ± 2.09	64.92 ± 7.58
Shirmohamadi	76.68 ± 2.64	36.88 ± 0.82	77.83 ± 1.02
Sheshpir	76.851 ± 0.78	37.38 ± 0.55	78.733 ± 0.74
Tanghe Ardakan	78.07 ± 1.57	40.75 ± 4.92	78.08 ± 1.74

Data are presented as means ± SE

W wild-type including GW and NW. from Grassland and 'Numan' cultivars. *Rd29A-IPT* is transgenic including NC1 to GM24. Which GC and NC: 'Grassland' and 'Numan' cultivars generated from transgenic callus; GM Grassland and Numan' cultivars generated from transgenic meristem; GS and NS generated from Grassland and 'Numan' cultivars generated from transgenic seed. Local accessions include: Bereshneh1 to Tanghe Ardakan

*WT plants that did not recover. LSD value = 7.92

Table 5 Photochemical efficiency (Fv/Fm) of transgenic, and wild-type plants and local accessions under well-irrigated, drought and recovery states

Treated Plants	Photochemical efficiency (Fv/Fm)		
	Well-irrigated	Drought	Recovery
GW	0.755 ± 0.008	0.282 ± 0.042	*
NW	0.763 ± 0.0105	0.124 ± 0.008	*
NC1	0.775 ± 0.007	0.553 ± 0.109	0.778 ± 0.011
NC4	0.764 ± 0.005	0.758 ± 0.029	0.804 ± 0.007
NC8	0.720 ± 0.004	0.684 ± 0.087	0.797 ± 0.009
NC11	0.774 ± 0.010	0.716 ± 0.057	0.794 ± 0.004
NC12	0.761 ± 0.005	0.709 ± 0.082	0.806 ± 0.002
NC13	0.779 ± 0.004	0.717 ± 0.041	0.787 ± 0.017
NC14	0.751 ± 0.008	0.732 ± 0.0106	0.790 ± 0.010
GC3	0.751 ± 0.007	0.690 ± 0.034	0.811 ± 0.004
GC4	0.757 ± 0.002	0.680 ± 0.047	0.760 ± 0.033
GC5	0.778 ± 0.006	0.692 ± 0.053	0.803 ± 0.001
GC6	0.775 ± 0.000	0.754 ± 0.027	0.790 ± 0.003
GC8	0.758 ± 0.014	0.737 ± 0.011	0.812 ± 0.000
GC9	0.737 ± 0.011	0.690 ± 0.034	0.790 ± 0.001
GC16	0.773 ± 0.004	0.769 ± 0.003	0.787 ± 0.012
NS14	0.776 ± 0.004	0.614 ± 0.013	0.745 ± 0.009
NS22	0.762 ± 0.010	0.398 ± 0.073	0.797 ± 0.006
GS1	0.753 ± 0.002	0.718 ± 0.005	0.760 ± 0.003
GM12	0.762 ± 0.006	0.735 ± 0.008	0.852 ± 0.057
GM21	0.768 ± 0.007	0.749 ± 0.005	0.792 ± 0.004
GM24	0.749 ± 0.005	0.768 ± 0.011	0.809 ± 0.006
Bereshneh1	0.763 ± 0.011	0.748 ± 0.010	0.760 ± 0.004
Bereshneh2	0.793 ± 0.015	0.417 ± 0.06	0.788 ± 0.005
Chamsohrabkhani	0.800 ± 0.012	0.738 ± 0.018	0.811 ± 0.006
Delkhan1	0.775 ± 0.001	0.757 ± 0.009	0.782 ± 0.008
Delkhan2	0.802 ± 0.002	0.612 ± 0.092	0.805 ± 0.003
Homayjan1	0.749 ± 0.003	0.640 ± 0.032	0.776 ± 0.007
Homayjan2	0.778 ± 0.008	0.646 ± 0.055	0.812 ± 0.008
Homayjan3	0.778 ± 0.006	0.345 ± 0.080	0.804 ± 0.004
Homayjan-Bereshneh	0.782 ± 0.005	0.667 ± 0.029	0.796 ± 0.007
Komeher1	0.812 ± 0.002	0.766 ± 0.006	0.801 ± 0.006
Komeher2	0.775 ± 0.001	0.669 ± 0.010	0.784 ± 0.008
Khosroshirin	0.767 ± 0.004	0.737 ± 0.010	0.772 ± 0.028
Margon	0.793 ± 0.005	0.669 ± 0.067	0.786 ± 0.001
Pirsabzali	0.790 ± 0.002	0.685 ± 0.068	0.798 ± 0.011
Shirmohamadi	0.802 ± 0.007	0.749 ± 0.015	0.804 ± 0.016
Sheshpir	0.699 ± 0.006	0.547 ± 0.174	0.800 ± 0.005
Tanghe Ardakan	0.796 ± 0.005	0.760 ± 0.018	0.811 ± 0.006

Data are presented as means ± SE

W wild-type including GW and NW are presented as 'Grassland' and 'Numan' cultivars, respectively. *Rd29A-IPT* transgenic including: from NC1 to GM24, which GC and NC: 'Grassland' and 'Numan' cultivars generated from transgenic callus; GM Grassland and 'Numan' cultivars generated from transgenic meristem; GS and NS generated from 'Grassland' and 'Numan' cultivars generated from transgenic seed. Local accessions include: from Bereshneh1 to Tanghe Ardakan

Table 5 (continued)

*WT plants that did not recover. LSD value = 3.83

on the 10 th day of the drought treatment. However, leaf iPA content in the GM21 transgenic line was higher than in other plants. Although leaf ZR levels were less than iPA and DHZR under drought stress, their maximum value was found in GM24. As shown in Table 7, total CK content in GC4 leaf samples exhibited a marked increase of about 49.7% in comparison with WT plants. Among local accessions, Komehr1 had a higher content of iPA in the leaves compared to the other accessions of perennial ryegrass. However, the highest leaf DHZR content was observed in the Margon accession which had a higher total CK content than in the other accessions (Table 7).

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Proline content

Proline content increased in all plant samples in response to drought stress in comparison with the stage before stress. Among *RD29A-IPT* transgenic plants, SN14, GM12, GM24, and NC13 lines exhibited higher leaf proline content than WT plants under drought stress. The highest proline content in response to the drought treatment was related to Homayjan 1 (Table 8).

Leaf electrolyte leakage (EL)

Leaf EL increased in all plants exposed to drought stress, except some transgenic plants. For instance, 3 lines generated from meristems had dramatically smaller EL values than the other plants. In GM12, GM21, and GM24 lines, a reduction of about 85.2%, 85.7%, and 88.7% in EL percentage compared to WT plants was found under drought stress (Table 9).

Leaf malondialdehyde (MDA)

Leaf MDA contents in all plants were lower under the well-irrigated condition than under drought stress. Under drought conditions, leaf MDA content in WT plants was considerably higher than in *RD29A-IPT* lines. For instance, a decline ranged from 93.8% to 97.2% in the leaf MDA of GC3, GC8, GM12, GM24, and GM21 (Table 10).

Table 6 Photosynthesis rate (Pn) of transgenic, and wild-type plants and local accessions in well-irrigated, drought and recovery states

Treated plants	Leaf photosynthesis rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)		
	Well-irrigated	Drought	Recovery
GW	7.723 $\pm$ 0.575	1.297 $\pm$ 0.104	*
NW	7.037 $\pm$ 0.483	1.150 $\pm$ 0.025	*
NC1	6.393 $\pm$ 0.038	3.400 $\pm$ 0.156	5.407 $\pm$ 0.218
NC4	6.587 $\pm$ 0.294	4.240 $\pm$ 0.070	5.727 $\pm$ 0.351
NC8	6.060 $\pm$ 0.382	2.460 $\pm$ 1.100	5.703 $\pm$ 0.217
NC11	6.060 $\pm$ 0.382	3.457 $\pm$ 0.264	5.703 $\pm$ 0.217
NC12	6.647 $\pm$ 0.215	3.550 $\pm$ 0.295	5.957 $\pm$ 0.681
NC13	7.243 $\pm$ 0.458	3.703 $\pm$ 0.127	6.860 $\pm$ 0.490
NC14	7.307 $\pm$ 0.489	3.930 $\pm$ 0.355	6.953 $\pm$ 0.948
GC3	7.307 $\pm$ 0.489	3.930 $\pm$ 0.355	7.287 $\pm$ 0.787
GC4	7.030 $\pm$ 0.0321	4.653 $\pm$ 0.0521	7.153 $\pm$ 0.287
GC5	9.020 $\pm$ 1.190	4.500 $\pm$ 0.300	8.370 $\pm$ 1.010
GC6	10.10 $\pm$ 0.124	4.827 $\pm$ 0.222	9.647 $\pm$ 0.084
GC8	8.727 $\pm$ 0.840	3.803 $\pm$ 0.131	8.480 $\pm$ 0.733
GC9	5.917 $\pm$ 0.366	3.373 $\pm$ 0.179	5.240 $\pm$ 0.669
GC16	9.133 $\pm$ 0.972	4.793 $\pm$ 0.079	8.950 $\pm$ 0.285
NS14	8.517 $\pm$ 0.294	2.827 $\pm$ 0.228	7.007 $\pm$ 0.456
NS22	9.153 $\pm$ 0.798	2.883 $\pm$ 0.176	8.367 $\pm$ 0.797
GS1	7.093 $\pm$ 0.679	3.127 $\pm$ 0.315	7.037 $\pm$ 0.238
GM12	8.763 $\pm$ 0.513	5.193 $\pm$ 0.262	8.520 $\pm$ 0.356
GM21	8.240 $\pm$ 0.711	3.803 $\pm$ 0.275	7.827 $\pm$ 0.533
GM24	7.803 $\pm$ 0.542	4.370 $\pm$ 0.099	8.170 $\pm$ 0.408
Bereshneh1	5.093 $\pm$ 0.201	1.793 $\pm$ 0.180	3.797 $\pm$ 0.348
Bereshneh2	4.430 $\pm$ 0.189	2.087 $\pm$ 0.193	3.457 $\pm$ 0.315
Chamsohrabkhani	5.907 $\pm$ 0.443	2.363 $\pm$ 0.253	4.130 $\pm$ 0.488
Delkhan1	7.897 $\pm$ 0.442	3.117 $\pm$ 0.17	5.613 $\pm$ 0.377
Delkhan2	6.793 $\pm$ 0.719	2.343 $\pm$ 0.154	4.467 $\pm$ 0.697
Homayjan1	4.493 $\pm$ 0.223	2.123 $\pm$ 0.325	4.963 $\pm$ 0.365
Homayjan2	7.190 $\pm$ 0.472	1.830 $\pm$ 0.371	5.527 $\pm$ 0.447
Homayjan3	4.757 $\pm$ 0.376	1.653 $\pm$ 0.102	4.313 $\pm$ 0.638
Homayjan-Bereshneh	5.893 $\pm$ 0.182	1.667 $\pm$ 0.394	3.527 $\pm$ 0.491
Komehr1	6.397 $\pm$ 0.341	3.247 $\pm$ 0.356	4.720 $\pm$ 0.365
Komehr2	6.580 $\pm$ 0.750	2.323 $\pm$ 0.222	7.200 $\pm$ 0.156
Khosroshirin	5.700 $\pm$ 0.675	1.953 $\pm$ 0.339	6.157 $\pm$ 0.173
Margon	6.493 $\pm$ 0.132	2.273 $\pm$ 0.316	5.747 $\pm$ 0.477
Pirsabzali	7.183 $\pm$ 0.182	1.190 $\pm$ 0.131	6.327 $\pm$ 0.505
Shirmohamadi	4.687 $\pm$ 0.285	1.467 $\pm$ 0.056	4.843 $\pm$ 0.206
Sheshpir	5.063 $\pm$ 0.321	1.450 $\pm$ 0.173	4.800 $\pm$ 0.571
Tanghe Ardakan	7.193 $\pm$ 0.533	1.463 $\pm$ 0.105	6.343 $\pm$ 0.544

Data are presented as means $\pm$ SE

W wild-type including GW and NW are presented as 'Grassland' and 'Numan' cultivars, respectively. *Rd29A-IPT* transgenic including: from NC1 to GM24, which GC and NC: 'Grassland' and 'Numan' cultivars generated from transgenic callus; GM Grassland and Numan cultivars generated from transgenic meristem; GS and NS generated from 'Grassland' and 'Numan' cultivars generated from transgenic seed. Local accessions include: from Bereshneh1 to Tanghe Ardakan

*WT plants that did not recover. LSD value = 2.96

Table 7 HPLC analysis for leaf ZR, DHZR, iPA, and total CK of transgenic plants, wild-type plants, and local accessions under drought stress

Treated plants	Leaf CK content (pmol g ⁻¹)			
	ZR	DHZR	iPA	Total CK
GW	2.94 ± 0.02	4.20 ± 0.01	3.63 ± 0.04	10.76 ± 0.05
NW	1.45 ± 0.02	3.18 ± 0.01	3.26 ± 0.01	7.89 ± 0.02
NC1	2.25 ± 0.03	4.13 ± 0.01	6.72 ± 0.01	13.11 ± 0.03
NC4	3.44 ± 0.07	3.72 ± 0.07	5.29 ± 0.09	12.45 ± 0.04
NC12	2.12 ± 0.19	5.75 ± 0.20	7.48 ± 0.37	15.35 ± 0.53
NC13	3.15 ± 0.04	5.65 ± 0.03	8.13 ± 0.03	16.94 ± 0.05
NC14	2.71 ± 0.09	4.50 ± 0.051	6.53 ± 0.04	13.75 ± 0.13
GC3	1.99 ± 0.29	3.92 ± 0.09	4.90 ± 0.05	10.81 ± 0.3
GC4	4.30 ± 0.03	9.77 ± 0.12	7.47 ± 0.02	21.55 ± 0.14
GC5	2.08 ± 0.04	6.24 ± 0.13	4.10 ± 0.06	12.42 ± 0.16
GC6	2.94 ± 0.03	5.62 ± 0.09	6.90 ± 0.36	15.46 ± 0.42
GC8	4.15 ± 0.03	6.17 ± 0.23	5.86 ± 0.22	16.18 ± 0.29
GC9	1.13 ± 0.04	4.80 ± 0.02	6.10 ± 0.04	12.03 ± 0.08
GC16	2.01 ± 0.03	6.80 ± 0.12	5.96 ± 0.09	14.78 ± 0.20
NS14	2.66 ± 0.18	4.58 ± 0.24	6.64 ± 0.19	13.88 ± 0.61
NS22	1.71 ± 0.18	2.30 ± 0.03	3.98 ± 0.04	7.99 ± 0.22
GM12	2.78 ± 0.14	5.95 ± 0.05	8.11 ± 0.18	16.84 ± 0.30
GM21	3.97 ± 0.07	6.15 ± 0.09	8.44 ± 0.04	18.56 ± 0.17
GM24	4.31 ± 0.45	6.72 ± 0.16	9.16 ± 0.03	20.18 ± 0.34
Cham sohrabkhani	2.10 ± 0.06	3.71 ± 0.18	4.80 ± 0.12	10.60 ± 0.24
Delkhan	1.60 ± 0.02	3.70 ± 0.23	3.39 ± 0.28	8.69 ± 0.52
Komehr1	2.05 ± 0.11	4.98 ± 0.08	6.12 ± 0.08	13.14 ± 0.27
Margon	2.63 ± 0.21	6.17 ± 0.014	5.4 ± 0.05	14.20 ± 0.30
Tangeh Ardakan	2.05 ± 0.03	3.98 ± 0.04	4.44 ± 0.08	10.47 ± 0.08
LSD value	0.396	0.3509	0.4265	0.7985

Data are presented as means ± SE

W wild-type including GW and NW are presented as 'Grassland and 'Numan' cultivars. *Rd29A-IPT* transgenic including from NC1 to GM24, which GC and NC: 'Grassland and 'Numan' cultivars generated from transgenic callus; *GM* Grassland and 'Numan' cultivars generated from transgenic meristem; *GS* and *NS* generated from 'Grassland and 'Numan' cultivars generated from transgenic seed. Local accessions include: Cham sohrabkhani to Tangeh Ardakan. ZR zeatin riboside, DHZR dihydro zeatin riboside, iPA isopentenyladenine

Leaf antioxidant enzymes (SOD, POD, and CAT)

Our results showed that POD activity in the leaves of all plants increased except in WT plants during drought stress. However, a significant increase of about 41.89% and 41.05% was found in the leaves of GM12 and GC8 in response to drought stress than in the well-irrigated condition, respectively. Among local accessions, Komehr1 significantly showed an increase of about 13% in leaf POD activity in drought stress than in the well-irrigated condition. POD activity decreased after 14 days of rewatering. In general,

Table 8 Leaf proline contents of transgenic and wild-type plants and local accessions in well-irrigated, drought and recovery states

Treated plants	Leaf proline content (μmol g ⁻¹ FW)		
	Well-irrigated	Drought	Recovery
GW	0.03 ± 0.00	9.22 ± 0.29	*
NW	0.04 ± 0.02	4.33 ± 0.21	*
NC1	3.83 ± 0.75	26.72 ± 0.50	1.84 ± 0.14
NC4	2.98 ± 0.27	17.07 ± 2.31	0.74 ± 0.16
NC8	3.67 ± 1.04	17.26 ± 0.47	0.53 ± 0.02
NC11	1.48 ± 0.16	19.83 ± 0.74	5.07 ± 1.03
NC12	2.39 ± 0.54	13.03 ± 1.42	2.69 ± 0.57
NC13	0.24 ± 0.04	29.17 ± 2.15	4.09 ± 0.79
NC14	4.01 ± 0.89	19.70 ± 0.15	1.28 ± 0.29
GC3	1.98 ± 0.42	13.93 ± 0.04	1.25 ± 0.34
GC4	4.61 ± 0.84	26.80 ± 4.03	2.56 ± 0.67
GC5	1.25 ± 0.36	16.91 ± 0.84	5.34 ± 0.40
GC6	3.43 ± 0.94	15.47 ± 0.93	2.05 ± 0.32
GC8	2.92 ± 0.34	22.03 ± 0.27	3.55 ± 0.17
GC9	1.45 ± 0.29	17.93 ± 0.32	1.91 ± 0.45
GC16	1.42 ± 0.29	20.76 ± 1.87	1.42 ± 0.43
NS14	5.58 ± 0.20	30.90 ± 1.87	2.75 ± 0.39
NS22	1.67 ± 0.34	13.05 ± 1.61	1.91 ± 0.33
GS1	7.38 ± 0.40	24.98 ± 6.46	2.85 ± 0.39
GM12	1.47 ± 0.19	28.87 ± 1.64	2.77 ± 0.39
GM21	1.87 ± 0.23	26.10 ± 1.56	2.94 ± 0.33
GM24	1.66 ± 0.22	27.78 ± 1.41	2.29 ± 0.62
Bereshneh1	1.86 ± 0.66	18.06 ± 1.15	1.76 ± 0.31
Bereshneh2	1.33 ± 0.29	17.32 ± 1.11	7.16 ± 0.71
Chamsohrabkhani	2.28 ± 0.49	27.34 ± 4.36	1.43 ± 0.42
Delkhan1	3.74 ± 0.49	28.61 ± 1.13	0.68 ± 0.42
Delkhan2	2.30 ± 0.84	22.61 ± 1.82	1.48 ± 0.18
Homayjan1	1.63 ± 0.15	11.58 ± 0.412	0.45 ± 0.08
Homayjan2	3.40 ± 0.06	31.38 ± 2.15	1.04 ± 0.22
Homayjan3	5.98 ± 0.20	29.36 ± 2.51	1.15 ± 0.46
Homayjan-Bereshneh	4.70 ± 0.19	16.31 ± 3.29	0.80 ± 0.33
Komehr1	4.45 ± 0.30	15.35 ± 2.21	0.59 ± 0.37
Komehr2	4.33 ± 0.23	17.87 ± 1.10	0.18 ± 0.03
Khosroshirin	2.73 ± 0.23	57.51 ± 3.37	0.31 ± 0.02
Margon	1.54 ± 0.11	27.86 ± 1.69	1.57 ± 0.11
Pirsabzali	4.64 ± 0.14	5.05 ± 0.23	1.81 ± 0.42
Shirmohamadi	3.30 ± 0.02	11.30 ± 0.61	1.06 ± 0.42
Sheshpir	2.87 ± 0.52	22.31 ± 0.63	0.88 ± 0.41
Tangeh Ardakan	2.72 ± 0.41	15.29 ± 1.65	1.90 ± 0.21

Data are presented as means ± SE

W wild-type including GW and NW are presented as 'Grassland and 'Numan' cultivars, respectively. *Rd29A-IPT* transgenic including: from NC1 to GM24, which GC and NC: 'Grassland and 'Numan' cultivars generated from transgenic callus; *GM* Grassland and 'Numan' cultivars generated from transgenic meristem; *GS* and *NS* generated from 'Grassland and 'Numan' cultivars generated from transgenic seed. Local accessions include: from Bereshneh1 to Tangeh Ardakan

*WT plants that did not recover. LSD value = 2.90

Table 9 Leaf electrolyte leakage (EL) of transgenic and wild-type plants and local accessions in well-irrigated, drought and recovery states

Treated plants	Leaf EL content (%)		
	Well-irrigated	Drought	Recovery
GW	10.35 ± 0.30	85.77 ± 7.37	94.50 ± 5.50
NW	8.26 ± 0.39	90.20 ± 4.96	100.00 ± 0.00
NC1	7.31 ± 1.21	60.43 ± 2.91	8.37 ± 0.44
NC4	16.43 ± 0.64	54.20 ± 2.70	18.33 ± 0.55
NC8	9.04 ± 0.72	50.27 ± 0.70	11.15 ± 3.73
NC11	10.03 ± 0.23	25.87 ± 0.38	7.99 ± 0.38
NC12	7.89 ± 1.01	16.22 ± 2.56	8.29 ± 0.73
NC13	12.73 ± 0.52	47.37 ± 2.99	7.91 ± 0.71
NC14	14.07 ± 1.02	62.10 ± 2.03	8.71 ± 1.85
GC3	5.71 ± 1.10	61.31 ± 0.99	14.53 ± 1.33
GC4	7.39 ± 0.92	14.98 ± 0.41	8.88 ± 0.36
GC5	9.35 ± 0.86	41.91 ± 1.99	4.29 ± 0.28
GC6	6.35 ± 0.65	61.60 ± 2.99	9.26 ± 0.68
GC8	7.22 ± 1.08	14.20 ± 2.02	6.66 ± 1.28
GC9	14.67 ± 1.47	20.80 ± 1.41	8.19 ± 1.41
GC16	9.44 ± 0.08	27.96 ± 3.64	9.79 ± 0.29
NS14	7.17 ± 0.95	31.03 ± 1.11	5.88 ± 0.28
NS22	5.04 ± 0.57	63.5 ± 3.67	7.31 ± 1.02
GS1	16.77 ± 0.50	46.97 ± 0.00	8.41 ± 0.78
GM12	6.70 ± 0.45	12.62 ± 0.54	6.60 ± 1.49
GM21	6.53 ± 0.48	12.19 ± 0.31	5.47 ± 0.44
GM24	5.30 ± 0.29	9.70 ± 0.07	7.80 ± 0.09
Bereshneh1	14.99 ± 0.81	81.58 ± 4.66	10.77 ± 1.60
Bereshneh2	11.50 ± 1.31	61.87 ± 1.64	4.87 ± 0.66
Chamsohrabkhani	10.74 ± 1.40	62.07 ± 1.21	7.51 ± 0.23
Delkhan1	18.15 ± 0.31	87.76 ± 5.80	6.65 ± 1.12
Delkhan2	5.42 ± 0.14	35.68 ± 3.21	8.26 ± 2.17
Homayjan1	15.17 ± 2.03	75.90 ± 0.77	7.85 ± 1.10
Homayjan2	9.78 ± 0.99	45.84 ± 4.05	7.56 ± 1.47
Homayjan3	6.27 ± 0.36	92.33 ± 5.44	9.22 ± 3.25
Homayjan-Bereshneh	11.80 ± 0.31	84.53 ± 5.20	4.69 ± 1.01
Komehr1	8.29 ± 0.45	73.59 ± 0.75	9.41 ± 0.00
Komehr2	8.79 ± 0.52	43.10 ± 1.00	21.15 ± 0.51
Khosroshirin	13.92 ± 1.87	74.61 ± 3.67	3.99 ± 0.00
Margon	12.79 ± 1.38	90.15 ± 6.20	9.39 ± 4.13
Pirsabzali	18.66 ± 3.65	58.94 ± 1.37	8.74 ± 2.58
Shirmohamadi	29.59 ± 2.39	88.13 ± 0.50	13.88 ± 0.00
Sheshpir	18.24 ± 4.97	44.06 ± 4.87	5.12 ± 1.03
Tangheh Ardakan	9.15 ± 1.26	82.74 ± 4.11	4.72 ± 0.13

Data are presented as means ± SE

W wild-type including GW and NW are presented as Grassland and 'Numan' cultivars, respectively. *Rd29A-IPT* transgenic including: from NC1 to GM24, which GC and NC: 'Grassland' and 'Numan' cultivars generated from transgenic callus; *GM* Grassland and 'Numan' cultivars generated from transgenic meristem; *GS* and *NS* generated from 'Grassland' and 'Numan' cultivars generated from transgenic seed. Local accessions include: from Bereshneh1 to Tangheh Ardakan

*WT plants that did not recover. LSD value = 7.59

Table 10 Leaf malondialdehyde (MDA) of transgenic and wild-type plants and local accessions in well-irrigated, drought and recovery states

Treated plants	Leaf MDA content (nmol ⁻¹ g ⁻¹ FW)		
	Well-irrigated	Drought	Recovery
GW	3.17 ± 0.54	22.60 ± 1.40	*
NW	2.53 ± 0.34	15.53 ± 2.13	*
NC1	2.04 ± 0.34	15.38 ± 1.25	4.88 ± 0.33
NC4	2.73 ± 0.33	14.38 ± 1.53	6.40 ± 0.39
NC8	1.92 ± 0.45	6.92 ± 0.62	5.13 ± 0.13
NC11	2.77 ± 0.71	7.20 ± 0.05	4.99 ± 0.44
NC12	1.55 ± 0.23	8.74 ± 0.84	4.96 ± 0.49
NC13	1.20 ± 0.22	6.26 ± 0.37	5.04 ± 0.46
NC14	4.76 ± 0.22	11.39 ± 0.67	5.96 ± 0.07
GC3	2.36 ± 0.08	11.33 ± 0.88	4.07 ± 0.07
GC4	4.16 ± 0.32	9.67 ± 0.40	5.07 ± 0.99
GC5	2.16 ± 0.29	13.27 ± 1.08	4.61 ± 0.25
GC6	2.20 ± 0.21	10.97 ± 1.38	5.24 ± 0.59
GC8	1.60 ± 0.25	4.82 ± 0.34	3.16 ± 0.05
GC9	1.21 ± 0.29	5.53 ± 0.07	4.89 ± 0.21
GC16	3.60 ± 0.10	12.45 ± 0.61	4.54 ± 0.14
NS14	1.74 ± 0.20	6.83 ± 0.98	4.39 ± 0.50
NS22	2.23 ± 0.19	13.01 ± 0.64	5.75 ± 0.14
GS1	4.61 ± 0.33	7.97 ± 0.14	4.94 ± 0.30
GM12	1.49 ± 0.20	2.72 ± 0.24	1.46 ± 0.25
GM21	0.82 ± 0.12	2.70 ± 0.30	1.57 ± 0.12
GM24	1.25 ± 0.18	3.80 ± 0.26	1.60 ± 0.35
Bereshneh1	4.58 ± 1.08	20.57 ± 1.44	4.53 ± 0.11
Bereshneh2	3.379 ± 0.04	20.71 ± 1.42	5.65 ± 0.18
Chamsohrabkhani	3.77 ± 0.04	11.39 ± 0.50	4.31 ± 0.26
Delkhan1	2.70 ± 0.29	11.40 ± 0.65	3.60 ± 0.15
Delkhan2	3.97 ± 0.14	9.91 ± 0.55	3.47 ± 0.76
Homayjan1	5.32 ± 0.24	19.68 ± 0.42	7.27 ± 0.73
Homayjan2	7.51 ± 1.42	14.77 ± 0.56	4.84 ± 0.65
Homayjan3	5.61 ± 0.38	19.56 ± 1.00	6.62 ± 0.38
Homayjan-Bereshneh	3.97 ± 0.30	17.41 ± 1.08	5.25 ± 0.13
Komehr1	3.22 ± 0.34	11.96 ± 0.72	4.49 ± 0.30
Komehr2	6.80 ± 0.21	13.15 ± 1.52	6.23 ± 0.24
Khosroshirin	5.10 ± 0.29	18.11 ± 1.37	6.83 ± 0.41
Margon	7.22 ± 0.07	20.47 ± 1.66	9.08 ± 0.40
Pirsabzali	4.35 ± 0.08	19.00 ± 1.01	8.91 ± 0.54
Shirmohamadi	4.74 ± 0.34	12.60 ± 0.416	5.20 ± 0.16
Sheshpir	5.71 ± 0.06	13.76 ± 1.20	4.83 ± 0.44
Tangheh Ardakan	8.7 ± 0.15	20.90 ± 1.16	8.63 ± 0.43

Data are presented as means ± SE

W wild-type including GW and NW are presented as 'Grassland' and 'Numan' cultivars, respectively. *RD29A-IPT* transgenic including: from NC1 to GM24, which GC and NC: 'Grassland' and 'Numan' cultivars generated from transgenic callus; *GM* Grassland and 'Numan' cultivars generated from transgenic meristem; *GS* and *NS* generated from 'Grassland' and 'Numan' cultivars generated from transgenic seed. Local accessions include: from Bereshneh1 to Tangheh Ardakan

*WT plants that did not recover. LSD value = 9.22

POD activity became smaller in value in local accessions than in transgenic plants (Table 11).

Under the well-irrigated condition, leaf CAT activity was different among all plants. The highest leaf CAT activity was observed in the Khosroshirin accession. Drought stress significantly decreased leaf CAT activity in WT plants, whereas transgenic plants and most local accessions had high leaf CAT activity. A notable increase of about 9- and sevenfold occurred in leaf CAT activity in GM24 and GC8 accessions. Leaf CAT activity in local accessions Komehr1 and Margon increased by about 1.8- and 1.3-fold compared to the well-irrigated condition (Table 12).

SOD activity significantly decreased by 55.44% and 69.85% in 'Grassland' and 'Numan' WT plants under drought stress in comparison with the well-irrigated condition. Transgenic plants maintained higher SOD activity than WT plants and local accessions under drought stress. Among transgenic plants, the highest SOD activities belonged to GC8, GM12, and GM24, respectively (Table 13).

Multivariate data analysis

The genetic variation of drought tolerance in WT, *Rd29A-IPT* transgenic, and local accessions of perennial ryegrass was determined by Ward's cluster analysis using VQ, and physio- biochemical characteristics. All samples were classified into four groups (Fig. 2). The first cluster (A): GM12, GM24, GC8, GC4, and GM21 with high VQ, RWC, Fv/Fm, Pn, TChl, and antioxidant enzyme activities, and low EL, MDA, and proline. These results showed Grassland cultivars originated from meristem tips (GM12, GM24, and GM21), and callus explants (GC8 and GC4) showed good drought tolerance. Among local accessions Chamsohrabkhani, Delkhan1, Delkhan2, Komehr2, and Komehr1 are grouped in the second cluster (B) as moderately drought tolerant. WT plants from 'Grassland' (GW) and 'Numan' (NW) cultivars are grouped in the three clusters (C) as moderately drought sensitive. Our results suggested that there was a great potential for improving drought tolerance in these local accessions due to greater genotypic variation of drought tolerance. NC1, NS22 and other local accessions are grouped in the four cluster (D).

A principal component analysis (PCA) was conducted to analyze the impact of parameters on plant response. The results in Fig. 3 revealed a biplot showing the relationship between parameters and genotypes. The first PC explained 59.2% of the variance in the data, and the second PC explained 12.7% of the variance, respectively. Trait correlations are depicted by the angle between vectors, with a larger angle indicating a weaker correlation. Three distinct trait groups were observed: the first (Pn, CAT, and SOD), and second groups (VQ, TChl, RWC, and Fv/Fm) strongly associated with PC1, and the third group

Table 11 Leaf POD activities of transgenic and wild-type plants and local accessions under well-irrigated, drought and recovery states

Treated plants	Leaf POD activity (Unit g ⁻¹ FW)		
	Well-irrigated	Drought	Recovery
GW	0.030 ± 0.003	0.012 ± 0.001	*
NW	0.034 ± 0.002	0.015 ± 0.002	*
NC1	0.028 ± 0.003	0.032 ± 0.001	0.023 ± 0.006
NC4	0.038 ± 0.012	0.196 ± 0.054	0.043 ± 0.004
NC8	0.022 ± 0.001	0.224 ± 0.047	0.023 ± 0.006
NC11	0.090 ± 0.035	0.453 ± 0.018	0.030 ± 0.006
NC12	0.030 ± 0.003	0.459 ± 0.370	0.013 ± 0.004
NC13	0.027 ± 0.009	0.111 ± 0.027	0.012 ± 0.000
NC14	0.087 ± 0.011	0.283 ± 0.028	0.044 ± 0.003
GC3	0.032 ± 0.007	0.247 ± 0.094	0.007 ± 0.002
GC4	0.043 ± 0.002	0.139 ± 0.017	0.042 ± 0.010
GC5	0.024 ± 0.006	0.197 ± 0.032	0.015 ± 0.002
GC6	0.026 ± 0.007	0.173 ± 0.034	0.021 ± 0.008
GC8	0.017 ± 0.004	0.715 ± 0.061	0.028 ± 0.001
GC9	0.047 ± 0.003	0.042 ± 0.007	0.026 ± 0.002
GC16	0.125 ± 0.001	0.369 ± 0.130	0.047 ± 0.002
NS14	0.072 ± 0.053	0.160 ± 0.026	0.016 ± 0.000
NS22	0.026 ± 0.001	0.020 ± 0.006	0.030 ± 0.005
GS1	0.007 ± 0.000	0.239 ± 0.020	0.010 ± 0.000
GM12	0.007 ± 0.002	0.300 ± 0.097	0.012 ± 0.001
GM21	0.035 ± 0.015	0.230 ± 0.040	0.021 ± 0.007
GM24	0.014 ± 0.005	0.330 ± 0.017	0.028 ± 0.000
Bereshneh1	0.057 ± 0.022	0.071 ± 0.026	0.020 ± 0.000
Bereshneh2	0.030 ± 0.003	0.339 ± 0.047	0.058 ± 0.014
Chamsohrabkhani	0.049 ± 0.008	0.280 ± 0.00	0.029 ± 0.008
Delkhan1	0.053 ± 0.002	0.252 ± 0.064	0.010 ± 0.000
Delkhan2	0.033 ± 0.010	0.325 ± 0.045	0.031 ± 0.000
Homayjan1	0.012 ± 0.003	0.149 ± 0.019	0.010 ± 0.002
Homayjan2	0.030 ± 0.003	0.255 ± 0.008	0.030 ± 0.014
Homayjan3	0.019 ± 0.001	0.233 ± 0.004	0.007 ± 0.001
Homayjan-Bereshneh	0.018 ± 0.003	0.339 ± 0.047	0.026 ± 0.001
Komehr1	0.042 ± 0.009	0.589 ± 0.041	0.035 ± 0.003
Komehr2	0.012 ± 0.004	0.075 ± 0.002	0.030 ± 0.006
Khosroshirin	0.052 ± 0.003	0.144 ± 0.020	0.023 ± 0.000
Margon	0.016 ± 0.001	0.214 ± 0.030	0.033 ± 0.002
Pirsabzali	0.064 ± 0.008	0.448 ± 0.047	0.085 ± 0.012
Shirmohamadi	0.017 ± 0.004	0.286 ± 0.021	0.035 ± 0.012
Sheshpir	0.017 ± 0.006	0.310 ± 0.040	0.010 ± 0.001
Tangeh Ardakan	0.089 ± 0.021	0.378 ± 0.0242	0.083 ± 0.048

Data are presented as means ± SE

W wild-type including GW and NW are presented as 'Grassland' and 'Numan' cultivars, respectively. *Rd29A-IPT* transgenic including: from NC1 to GM24, which GC and NC: 'Grassland' and 'Numan' cultivars generated from transgenic callus; GM Grassland and Numan cultivars generated from transgenic meristem; GS and NS generated from 'Grassland' and 'Numan' cultivars generated from transgenic seed. Local accessions include: from Bereshneh1 to Tangeh Ardakan

*WT plants that did not recover. LSD value = 2.41

Table 12 Leaf CAT activities of transgenic and wild-type plants and local accessions under well-irrigated, drought and recovery states

Treated plants	Leaf CAT activity (Unit g ⁻¹ FW)		
	Well-irrigated	Drought	Recovery
GW	6.79 ± 0.87	4.74 ± 1.63	*
NW	8.77 ± 0.85	2.49 ± 0.48	*
NC1	6.07 ± 0.90	26.09 ± 3.04	13.2 ± 2.25
NC4	7.32 ± 0.62	30.81 ± 0.44	18.22 ± 7.03
NC8	4.87 ± 2.21	25.18 ± 3.71	9.95 ± 2.65
NC11	6.66 ± 0.68	25.69 ± 3.34	9.36 ± 1.63
NC12	10.96 ± 1.10	23.15 ± 1.01	7.27 ± 1.85
NC13	10.46 ± 0.80	27.56 ± 2.29	8.12 ± 1.32
NC14	8.02 ± 0.89	37.21 ± 0.87	11.54 ± 0.78
GC3	8.32 ± 1.54	24.92 ± 3.06	6.91 ± 1.33
GC4	3.10 ± 1.20	33.71 ± 3.26	7.61 ± 0.78
GC5	11.12 ± 2.14	24.47 ± 1.66	7.77 ± 1.31
GC6	4.94 ± 1.51	23.45 ± 2.43	16.80 ± 2.18
GC8	5.43 ± 0.40	46.50 ± 1.60	14.32 ± 0.88
GC9	4.72 ± 1.06	20.91 ± 2.82	9.24 ± 2.86
GC16	5.89 ± 0.711	30.25 ± 3.52	8.63 ± 1.30
NS14	2.54 ± 0.57	31.68 ± 0.84	6.50 ± 1.42
NS22	2.44 ± 0.49	35.53 ± 4.45	5.43 ± 0.75
GS1	8.88 ± 1.41	25.74 ± 4.47	5.76 ± 1.32
GM12	10.66 ± 0.87	47.59 ± 4.15	10.56 ± 1.26
GM21	5.99 ± 0.48	38.53 ± 3.05	10.96 ± 0.58
GM24	4.67 ± 0.71	47.57 ± 3.65	18.17 ± 2.06
Bereshneh1	9.36 ± 1.36	13.76 ± 0.88	4.92 ± 0.31
Bereshneh2	9.80 ± 2.38	20.56 ± 1.45	5.31 ± 1.38
Chamsohrabkhani	9.61 ± 0.75	22.23 ± 4.27	9.72 ± 1.38
Delkhan1	10.10 ± 1.60	19.54 ± 2.70	4.87 ± 0.98
Delkhan2	11.54 ± 0.78	11.68 ± 2.19	10.86 ± 0.40
Homayjan1	8.32 ± 1.41	19.64 ± 1.63	9.80 ± 1.11
Homayjan2	12.03 ± 1.56	20.46 ± 3.64	6.00 ± 1.26
Homayjan3	13.05 ± 3.08	13.76 ± 1.80	7.87 ± 0.68
Homayjan-Bereshneh	8.32 ± 1.62	17.06 ± 0.35	10.30 ± 1.51
Komehr1	8.02 ± 0.64	22.84 ± 0.77	7.06 ± 2.01
Komehr2	11.47 ± 1.19	16.85 ± 2.36	12.64 ± 2.81
Khosroshirin	15.58 ± 1.45	12.94 ± 3.00	5.39 ± 1.21
Margon	11.78 ± 2.34	20.61 ± 0.94	14.31 ± 2.38
Pirsabzali	8.90 ± 0.19	13.20 ± 1.79	16.14 ± 0.77
Shirmohamadi	5.84 ± 0.97	10.37 ± 1.19	9.71 ± 0.22
Sheshpir	10.91 ± 0.40	4.32 ± 0.33	10.60 ± 0.64
Tangheh Ardakan	8.63 ± 0.36	12.13 ± 1.66	13.96 ± 2.38

Data are presented as means ± SE

W wild-type including GW and NW. from 'Grassland' and 'Numan' cultivars. *Rd29A-IPT* transgenic including: NC1 to GM24. which GC and NC: 'Grassland' and 'Numan' cultivars generated from transgenic callus; GM Grassland and Numan cultivars generated from transgenic meristem; GS and NS generated from 'Grassland' and 'Numan' cultivars generated from transgenic seed. Local accessions include: Bereshneh1 to Tangheh Ardakan.

*WT plants that did not recover. LSD value = 8.09

Table 13 Leaf SOD activities of transgenic and wild-type plants and local accessions under well-irrigated, drought and recovery states

Treated plants	Leaf SOD activity (Unit g ⁻¹ FW)		
	Well- irrigated	Drought	Recovery
GW	102.57 ± 1.79	45.70 ± 1.44	*
NW	99.16 ± 0.726	29.90 ± 3.07	*
NC1	109.67 ± 1.80	118.74 ± 0.60	120.81 ± 2.67
NC4	106.54 ± 2.50	93.48 ± 5.31	115.24 ± 1.53
NC8	99.79 ± 7.17	95.53 ± 6.03	94.26 ± 5.73
NC11	117.98 ± 9.51	121.63 ± 5.42	118.63 ± 3.25
NC12	89.08 ± 1.35	151.08 ± 1.37	133.20 ± 1.86
NC13	109.00 ± 3.46	144.80 ± 2.07	179.33 ± 4.39
NC14	109.58 ± 4.18	116.08 ± 1.68	102.87 ± 5.09
GC3	96.02 ± 3.85	104.53 ± 1.13	100.03 ± 0.84
GC4	102.67 ± 0.57	138.47 ± 4.93	123.34 ± 2.13
GC5	98.28 ± 1.35	114.21 ± 5.19	90.67 ± 3.28
GC6	105.00 ± 1.93	152.33 ± 3.65	131.16 ± 1.54
GC8	97.44 ± 6.58	197.17 ± 1.49	137.38 ± 2.8
GC9	107.99 ± 1.94	143.74 ± 3.60	117.25 ± 1.69
GC16	108.27 ± 0.58	113.94 ± 2.02	103.41 ± 1.03
NS14	103.88 ± 2.16	146.19 ± 0.403	108.67 ± 0.88
NS22	110.38 ± 1.82	144.27 ± 7.85	44.13 ± 4.02
GS1	100.39 ± 0.72	115.33 ± 2.79	102.90 ± 1.43
GM12	98.81 ± 5.71	196.67 ± 4.65	140.16 ± 1.12
GM21	100.17 ± 0.79	143.11 ± 6.06	70.57 ± 3.71
GM24	108.03 ± 1.6	205.12 ± 2.68	155.63 ± 1.4
Bereshneh1	100.54 ± 1.64	77.80 ± 4.22	62.50 ± 1.27
Bereshneh2	104.94 ± 2.48	44.80 ± 3.58	51.80 ± 3.46
Chamsohrabkhani	101.85 ± 7.63	110.67 ± 1.41	73.03 ± 1.47
Delkhan1	106.07 ± 1.34	81.47 ± 1.12	70.07 ± 2.32
Delkhan2	111.00 ± 2.08	72.00 ± 3.79	42.00 ± 3.00
Homayjan1	87.60 ± 2.58	42.20 ± 2.31	42.07 ± 2.60
Homayjan2	97.02 ± 1.79	81.77 ± 1.58	51.47 ± 0.52
Homayjan3	95.99 ± 5.56	63.00 ± 2.95	160.00 ± 12
Homayjan-Bereshneh	99.22 ± 0.73	74.15 ± 4.87	67.00 ± 1.80
Komehr1	106.29 ± 4.32	110.82 ± 1.99	68.83 ± 3.51
Komehr2	92.13 ± 3.13	47.40 ± 1.25	30.53 ± 3.43
Khosroshirin	112.82 ± 1.02	72.87 ± 2.44	42.27 ± 5.76
Margon	101.24 ± 1.13	83.23 ± 0.91	32.73 ± 2.70
Pirsabzali	116.87 ± 5.37	73.87 ± 1.92	42.53 ± 2.10
Shirmohamadi	100.33 ± 1.73	81.27 ± 1.16	84.67 ± 2.95
Sheshpir	109.69 ± 5.07	74.27 ± 1.65	90.33 ± 1.85
Tangheh Ardakan	113.33 ± 2.16	39.43 ± 1.77	88.53 ± 1.43

Data are presented as means ± SE

W wild-type including GW and NW are presented 'Grassland' and 'Numan' cultivars, respectively. *Rd29A-IPT* transgenic including: from NC1 to GM24, which GC and NC: 'Grassland' and 'Numan' cultivars generated from transgenic callus; GM Grassland and Numan cultivars generated from transgenic meristem; GS and NS generated from 'Grassland' and 'Numan' cultivars generated from transgenic seed. Local accessions include: from Bereshneh1 to Tangheh Ardakan

*WT plants that did not recover. LSD value = 0.17

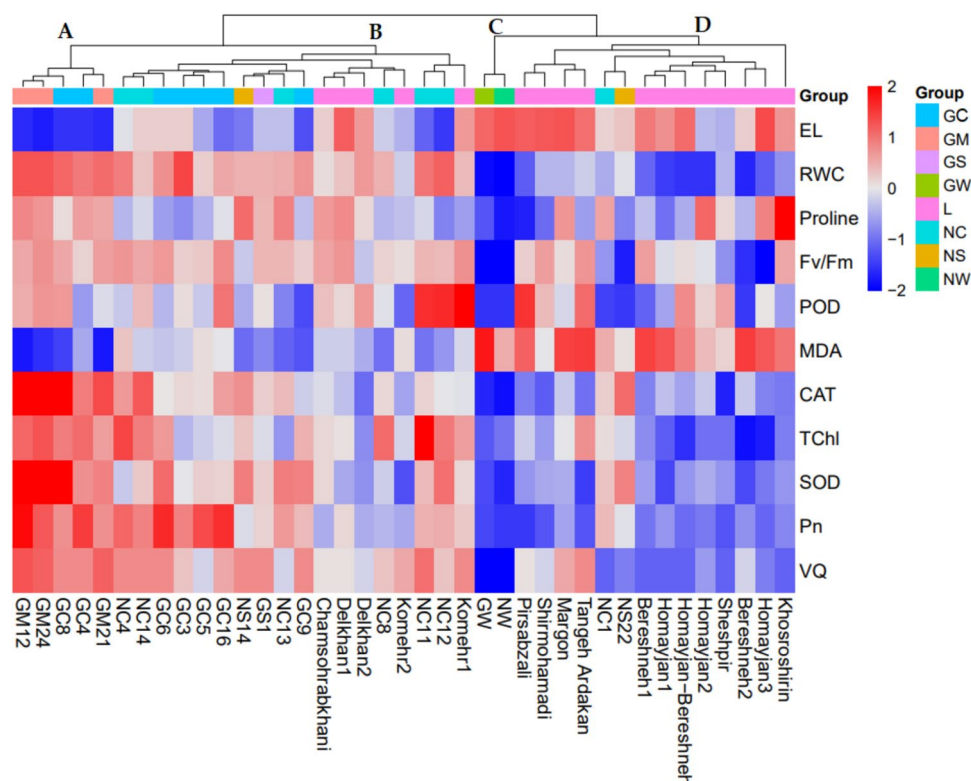


Fig. 2 Clustered heat map of wild-type, *Rd29A-IPT* transgenic, and local accessions of perennial ryegrass based on visual quality (VQ) and physio-biochemical traits at 10 days of drought treatment. The dark color of the value bar corresponds to high values of VQ and physio-biochemical traits. The rectangular brackets connect different samples (GC and NC: 'Grassland and 'Numan' cultivars generated from transgenic callus; GM: 'Grassland and 'Numan' cultivars gener-

ated from transgenic meristem; GS and NS generated from 'Grassland and 'Numan' cultivars generated from transgenic seed, W wild-type, L local accessions) with similar traits. Based on the grouping by the rectangular brackets, the samples were categorized into four groups including: **A** drought tolerant, **B** moderate drought tolerant, **C** moderate drought sensitive, and **D** drought sensitive

(EL and MDA) strongly associated with PC2. Notably, SOD, CAT, and Pn were aligned in the same direction in the biplot, indicating their collective influence on plant response to drought. In addition, these traits were located close to genotypes GM12 (20), GM24 (22), GC8 (14), GC4 (11), and GM21 (21) and were recognized as the superior genotypes in drought stress. These results confirmed the heat map clustering results. In contrast, EL and MDA were opposite to these traits and strongly associated with PC2. Consequently, choosing genotypes with high values for PC1 and PC2 would result in the selection of drought-tolerant genotypes with superior turf quality, efficient photosynthetic systems, increased water retention in leaf tissue, and enhanced antioxidant defense mechanisms during drought conditions. The vector length from traits to the origin represents their variability and contribution. POD enzyme activity had the largest contribution in Komehr 1 (32).

Correlation analysis was performed using VQ and physio-biochemical data at 10 days drought stress (Fig. 4). Correlation analysis based on the result of 10 days drought stress

showed that Fv/Fm, RWC, and TChl were strongly positively correlated with VQ with respective correlation coefficients of 0.91, 0.86, and 0.8. This result showed that these characteristics are good indicators for turf performance under drought stress in perennial ryegrass. Also, there was a significant negative correlation between SOD, CAT, and IPA with EL and MDA above 0.7.

RT-PCR analysis

RT-PCR analysis was only conducted on transgenic and WT plants exposed to drought stress. RT-PCR products of both *eEF-1 α* (114 bp) as reference gene and *IPT* gene (200 bp) as target genes are shown in Fig. 5. Based on the RT-PCR results, the expression of the reference gene (*eEF-1 α*) occurred in all transgenic and WT plants (Fig. 5A). *IPT* gene expression in all transgenic plants was confirmed with 200 bp, except for GC9 (3) and NS22 (18). *IPT* expression was observed in GM12 (10), GM24 (14), GC8 (12), GC6 (16), NC12 (11), NC14 (13), NS14 (17), and GC3 (7).

Fig. 3 Biplot display of visual quality (VQ) and physio-bio-chemical traits of wild-type, *Rd29A-IPT* transgenic, and local accessions under drought stress. 1: GW; 2: NW; 3: NC1; 4: NC4; 5: NC8; 6: NC11; 7: NC12; 8: NC13; 9: NC14; 10: GC3; 11: GC4; 12: GC5; 13: GC6; 14: GC8; 15: GC9; 16: GC16; 17: NS14; 18: NS22; 19: GS1; 20: GM12; 21: GM21; 22: GM24; 23–24: Bereshneh1, 2; 25: Chamsorabkhani; 26–27: Delkhan 1, 2; 28–30: Homayjan 1, 2, 3; 31: Homayjan- Bereshneh; 32–33: Komehr 1, 2; 34: Khosroshirin; 35: Margon; 36: Sheshpir; 37: Pirsabzali; 38: Shirmohamadi; 39: Tanghe Ardakan. GC and NC: 'Grassland' and 'Numan' cultivars generated from transgenic callus; GM: 'Grassland' and 'Numan' cultivars generated from transgenic meristem; GS and NS generated from 'Grassland' and 'Numan' cultivars generated from transgenic seed, W wild-type

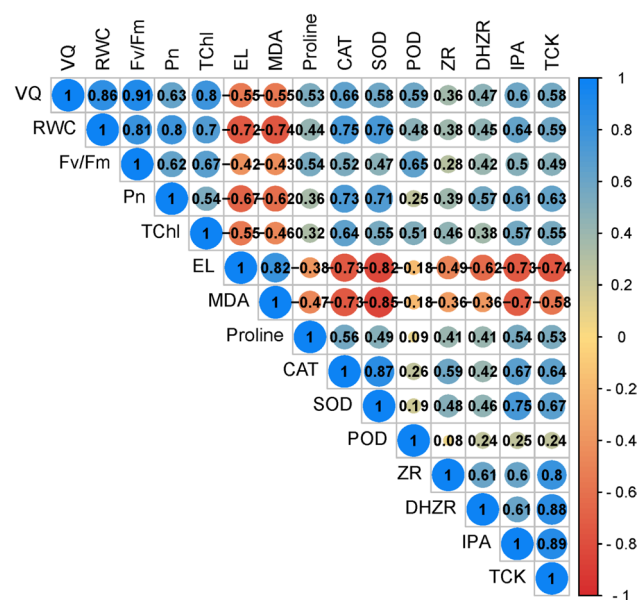
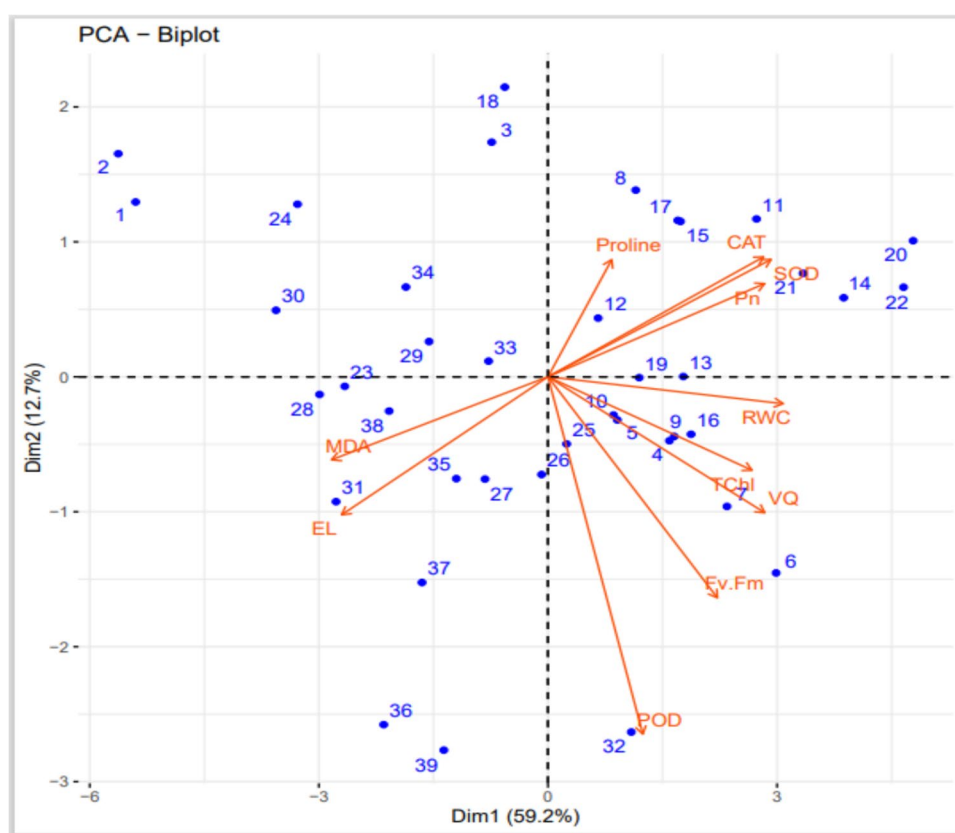


Fig. 4 Correlation between visual quality (VQ), relative water content (RWC), photochemical efficiency (Fv/Fm), net photosynthesis rate (Pn), total chlorophyll content (TChl), electrolyte leakage (EL), malondialdehyde (MDA), proline, catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), zeatin riboside (ZR), isopentenyl adenosine (IPA), dihydrozeatin riboside (DHZR), and total cytokinin (TCK) at 10 days of drought stress for wild-type, transgenic, and local accession of perennial ryegrass

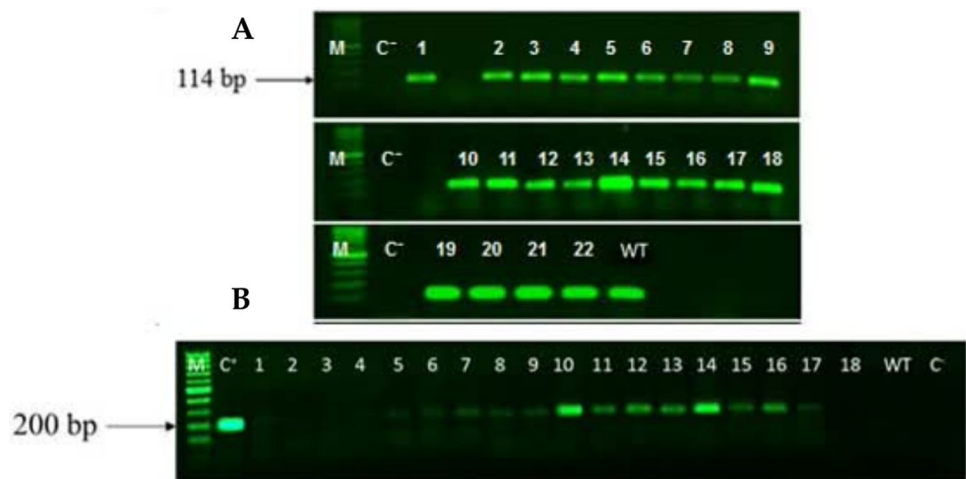
Discussion

A subtle variation in leaf color and area was observed in this research. Some *IPT* transgenic plants in the 'Numan' cultivar showed narrow, dark green leaves and more compact crowns in comparison with other transgenic plants such as NC4 and NC8 (Fig. 1). Furthermore, based on observational scoring it seems that there was a close relation between leaf area and color of the leaf with drought stress among transgenic plants.

RT-PCR results indeed confirmed *IPT* expression in transgenic lines already proven via PCR and DNA-blot tests. In addition, transgenic lines exhibited higher drought tolerance with greater maintenance of endogenous CK content in leaves that resulted in improved important physiological characteristics such as RWC, photosynthetic rate, and antioxidant activities and decreased EL and MDA in leaves. Gene expression is mostly affected by the efficiency of the promoter used.

Recently, studies on *IPT* gene expression were focused on using inducible promoters such as *SAG12* a senescence-associated promoter, and *SARK* promoter a senescence-associated receptor protein kinase in various plant transformations (Merewitz 2011a, 2012a, b; Peleg et al. 2011; Robson et al. 2004; Rivero et al. 2007; Qin et al. 2011). The use of inducible and senescence-activated promoters to control *IPT* expression has emerged as a promising strategy to improve

Fig. 5 RT-PCR analysis of transgenic and wild-type plants with specific primers of reference gene *eEF-1 α* (114bp) from perennial ryegrass for transgenic (1–22) and WT plants (A) and with specific primers of *IPT* gene (200bp), *M*: marker, mol wt. (10kb), *C*⁺ positive control (Plasmid), 1–18 transgenic lines, WT non-transgenic plant, *C*[−] water (B)



drought tolerance in various grass species (Merewitz 2010; Peleg et al. 2011; Ijaz et al. 2025). Previous research indicated applications of the *RD29A* promoter as more beneficial than the CaMV 35S. Research indicates that the presence of a dehydration-responsive element (DRE) in the *RD29A* promoter facilitates the swift activation of the *RD29A* gene in response to high salinity, drought, or low-temperature stress. This finding implies that the prompt expression of this gene is independent of the influence of intracellular abscisic acid (ABA) (Su et al. 2022).

In our study, *RD29A* promoter as an inducible stress promoter was effective. We found excellent results using *RD29A* promoter under drought stress with expression of *IPT* gene in perennial ryegrass transgenic plants compared to WT plants. In addition, it is reported that introns can increase gene expression in transgenic plants, especially in the case of crop improvement (Emami et al. 2013; Laxa 2017). UBQ10 intron was also used in the *IPT* gene construct to enhance its expression during drought stress (Esmaeili et al. 2019).

Previous studies on creeping bentgrass suggested that *IPT* transgenic plants containing senescence or inducible promoters had better turf quality (TQ) compared to transgenic ones with constitutive promoters. Although, significant variation was observed among the *IPT* lines (Merewitz et al. 2011a). Our results are following previous findings. Transgenic lines have exhibited varying responses to drought stress. These inconsistencies may stem from differences in the genomic insertion sites of the transgene, which can influence the expression of neighboring endogenous genes (Ghandour et al. 2023). Additionally, somaclonal variation arising during in vitro plant tissue culture may introduce genetic and epigenetic changes, further contributing to variations in transgene expression patterns (Bairu et al. 2011).

Compared to wild-type plants, several transgenic lines showed improvements in total chlorophyll content, RWC, and photosynthesis rate. Moreover, significant differences in physiological traits were observed among various perennial

ryegrass accessions under well-irrigated conditions, potentially attributed to genetic variations as reported in other turfgrass species (Taleb et al. 2023). This aligns with observations in other transgenic perennial ryegrass studies. For instance, Liebao et al. (2008) demonstrated that perennial ryegrass 'Accent' lines, engineered to overexpress *CBF3-DREB1 A*, exhibited increased total Chl content after 16 days of drought stress. Similarly, Patel et al. (2015) reported improved drought tolerance in *LpHUB1*-overexpressing perennial ryegrass, characterized by enhanced turf quality, turgor maintenance, and increased Chl content.

Similarly, *SAG12-IPT* and *HSP18.2-IPT* transgenic lines of creeping bentgrass exposed to drought stress significantly maintained higher total Chl and RWC than WT plants (Merewitz et al. 2010, 2012a). In another experiment with *SARK-IPT* transgenic rice total Chl content was maintained at about 69–75% compared to WT plants after 5 days in the dark (Peleg et al. 2011). Additionally, *SARK-IPT* transgenic maize plants exhibited higher RWC and delayed senescence compared to wild-type plants under drought conditions (Muruo et al. 2023).

In a similar study, the TR1 and TR4 transgenic lines expressing *SARK-IPT* displayed a delayed senescence characteristic. Importantly, these genetic modifications resulted in both reduced yield penalties under water stress and increased yield under optimal watering showed a water-deficit-tolerant phenotype in different environments (Bez nec et al. 2021).

However, present results demonstrated that leaf samples of most *IPT* transgenic plants maintained higher total Chl content and RWC than WT plants under drought stress. No significant differences occurred in the case of RWC of the leaf samples among WT plants in both cultivars under the well-irrigated condition. However, some transgenic lines including NC13, GC16, and GC4 showed higher RWC compared to other transgenic lines under well-irrigated conditions.

Most plants were returned to conditions before drought stress after 14 days of recovery treatment except wild-type plants and some perennial ryegrass accessions based on important physiological traits (Table 4), similar to the reported effect of *IPT* expression in other species (Merewitz et al. 2010, 2011a; Peleg et al. 2011; Muruo et al. 2023). They reported that *IPT* transgenic plants exposed to drought stress exhibited higher RWC than in WT plants. In addition, these results are similar to Chang et al. (2016) on creeping bentgrass under drought stress. They concluded that the higher leaf RWC in *IPT* transgenic plants and those treated with exogenous CK was closely associated with an increase in leaf total CK. Therefore, CK has an important role in maintaining water relations and carbon metabolism from root to shoot.

Similarly, the Fv/Fm ratio and performance index (PI) and leaf area were significantly decreased in forage grasses including *Festuca pratensis* (L.), *Festulolium braunii* [(K. Richt.) A. Camus], *L. multiflorum* (Lam.), *Festuca arundinacea* (Schreb.), and *L. perenne* (L.) in response to drought than in the well-irrigated condition. The most and least drought tolerance were observed in *F. pratensis* and *L. perenne*, respectively (Fariaszewska and Staniak 2015). In addition, some investigations showed similar results on different hybrids of *Festulolium* and *L. perenne* under drought conditions (Yu et al. 2013).

This is noteworthy, these plants remarkably increased photochemical efficiency after the recovery stage. Photochemical efficiency decreased during drought stress in most transgenic plants except GM12 which showed an increase of about 2.5% in comparison to the well-irrigated condition (Table 5). According to the results, the Fv/Fm ratio significantly increased during recovery even more of well-irrigated conditions in GM12 and Komehr1. Merewitz et al. (2011a, 2012b) indicated that the higher Fv/Fm in *SAG12-IPT* plants resulted in having cells more hydrated (RWC) while upholding qualitative parameters under drought stress. Similar results were achieved in this experiment too. The plants that were able to maintain good performance, higher chlorophyll, and RWC in leaves had higher photochemical efficiency resulting in better tolerance to drought stress. Therefore, the Fv/Fm ratio can be an index to distinguish drought-tolerant plants among various species and varieties. Drought stress reduced CO₂ absorbance and resulted in reduced electron transfer in PSII (Huang and Wang 2005). Bi et al. (2016) indicated that heat-tolerant genotypes such as PI578718 of tall fescue had healthy PSII systems under drought and heat stress compared to sensitive genotypes such as PI234881.

The significantly higher photosynthetic rate in transgenic lines particularly under drought stress revealed that these plants could have a better photosynthetic adaptation to nominate them as more drought-tolerant plants. In addition, Komehr1 as a local accession exhibited higher

photosynthesis than other local accessions under drought stress. Our results revealed that some accessions such as Komehr1 have a high potential to be introduced as a drought tolerant perennial ryegrass cultivar. These findings are the same as previous studies by Merewitz et al. (2010, 2011a, b). They stated that most of the transgenic lines had lower ABA contents in leaves and roots than the non-transgenic ones. Higher ABA content in leaves of WT plants may induce stomatal closure and cause limited photosynthesis during drought stress. On the other hand, they reported that the improved drought tolerance along with the increase in CK-to-ABA ratio in *SAG12* and *HSP-IPT* plants suggests that CK may have key roles in regulating drought stress response in creeping bentgrass through changing the accumulation of CK and its balance with ABA (Merewitz et al. 2010).

Xu et al. (2013) found that higher stability of drought-tolerant cultivar of Kentucky bluegrass ‘Midnight’ subjected to short periods of water stress resulted in significantly higher Pn, higher glyceraldehyde phosphate dehydrogenase (GADPH) activity, and Rubisco transcript level. Soon after re-watering, this cultivar experienced higher Rubisco activation. Similarly, transgenic maize *SARK-IPT* plants under water-deficit stress had photosynthetic rates at the normal range. Transgenic plants showed a negligible decrease in grain count per plant and other grain-related traits (Oneto et al. 2016).

A shortage of water may furthermore undermine thylakoid membrane stability and inhibit photosynthetic regulation (Chaves et al. 2009). *TaFBA1* expression under *RD29A* promoter led to higher Pn of transgenic plants in comparison with WT when water-deficit stress affected the plants, thereby compensating for the loss by greater amounts of chlorophyll in transgenic plants (Kong et al. 2016).

Although several CKs occur in plants, grasses mainly contain zeatin riboside (ZR) and isopentenyl adenine (iPA) as CKs, which regulate plant mechanisms against stresses (Xu et al. 2009; Hai et al. 2020).

Increases in total CK occurred in response to heightened levels of iPA since *IPT* catalyzes the reaction product. During drought stress, the delivery of cytokinins typically declines from roots to shoots (Hai et al. 2020; Nguyen et al. 2021). In previous studies, it was demonstrated that iPA and ZR markedly declined under drought stress in some plant species including Kentucky bluegrass and creeping bentgrass (DaCosta and Huang 2007b; Merewitz et al. 2011a; Zhang et al. 2019). In a report, Man et al. (2011) indicated that leaf ABA increased under drought stress whereas IAA and ZR decreased in *F. arundinacea* (Schreb.) cultivars. ‘Van Gogh’ (relatively drought-tolerant) had higher ABA, ZR, and IAA in comparison to ‘AST7002’ (relatively drought-sensitive). The application of ABA alone to *Helianthus annuus* L. plants resulted in a decline in transpiration rate; however, when ABA and ZR were applied together,

the effects of ABA on transpiration rate were significantly reduced, even when ZR concentrations were 100 times less than that of ABA (Hansen and Dörffling 2003). This suggests that even small changes in cytokinin concentration or delivery to leaves can have drastic effects on the physiological responses of plants to environmental stresses.

In water-stressed plants, decreased CK content and accumulation of ABA lead to an increased ABA/CK ratio. The decrease in CK levels increased apical dominance. In combination with ABA dynamics in the stomata, they assist in countering drought stress (O'Brien and Benková 2013; Zhang et al. 2019). Research showed that transgenic creeping bentgrass overexpressed *SAG12-IPT* and exhibited high levels of *IPT* expression in leaf samples that experienced drought stress, thereby corresponding to higher levels of indigenous ZR and iPA in leaf samples (Merewitz et al. 2010, 2011a; Xu et al. 2016).

Similarly, all transgenic plants manifested greater iPA contents compared to the WT plants on the 10 th day of drought treatment. However, the leaf iPA content of the GM21 transgenic line was higher than other plants. Although leaf ZR levels were less than iPA and DHZR under drought stress the maximum value of them was found in GM24. As shown in Table 7, the total CK content of GC4 leaves exhibited a marked increase of about 49.7% in comparison to WT plants.

Havlova et al. (2008) indicated that severe drought stress markedly stimulated cytokinin oxidase/dehydrogenase (CKX) activity in lower leaves of *Nicotiana tabacum* L., associated well with the decrease in bioactive CK levels. This suggests that a gradient of bioactive CKs in favor of upper leaves is established during drought stress, which might be beneficial for the preferential protection of these leaves.

Similarly, *Arabidopsis thaliana* L., 35S-CKX, and DEX-*IPT* plants experienced suppressed growth because of increases in ABA and a decline in auxin levels and active cytokinins. In contrast, *SAG-IPT* plants showed higher levels of active cytokinin content (more than fourfold) while drought stress progressed in intensity compared to the control with an absence of stress (Cao et al. 2018).

After rewatering for 14 days, proline content declined in all plants, except for WT plants. However, this reduction was less than the well-irrigated plants. The higher proline content of transgenic plants vs WT plants may have reflected a rise in endogenous cytokinins.

Merewitz et al. (2012a, b) stated that increases in proline among *SAG12-IPT* plants can assist in osmotic regulations and relevant stress-related defense responses that constitute plant resistance against drought-mediated stress. In particular, the CK 6-benzylaminopurine (BAP) postpones leaf senescence, followed by increases in proline values (Alvarez

et al. 2008). Nonetheless, past results are contradictory regarding the effectiveness of BAP and other CK compounds in promoting genes that synthesize proline (Hu et al. 1992).

Dobra et al. (2010) observed that tobacco plants expressed a transgene that increased indigenous proline values to control the adverse effects of drought, compared to non-transgenic plants that had smaller proline values. Similarly, drought responses in 35S-CKX plants were associated with decreases in growth rates, small transcription levels of the dehydration response gene *AtRD29B*, and the proline biosynthesis gene *AtP5 CS1* (Prerostova et al. 2018). Interactions among cytokinins and proline molecules were relevant to enhancements in carbohydrate components, e.g., fructose, glucose, mannitol, and sucrose (Kishor et al. 2005). It was also stated that CKs are linked to ABA homeostasis in drought stress (Hai et al. 2020). It has also been stated that drought-tolerant cultivar 'Van Gogh' of *Festuca arundinacea* (Schreb.) had greater turfgrass quality rating, LWC, proline, ABA, and ZR content compared to drought-sensitive cultivar 'AST7002' under drought stress conditions. Therefore, the proline, ABA, and ZR content are associated with drought tolerance (Man et al. 2011). However, to date, it is not clear that the accumulation of proline is directly related to the increase in CK level, whether it results from higher carbohydrate content or manifests an osmotic response (Merewitz et al. 2012a, b). Therefore, further research can be valuable to understand the association between high CK levels and proline accumulations in *IPT* transgenic plants.

According to the results, higher indigenous cytokinins corresponded positively with greater levels of drought resistance in perennial ryegrass as previously stated in the case of bentgrass species when assessing shoot elongations and physiological mechanisms (DaCosta and Huang 2007b; Merewitz et al. 2011a). Proline functions not only as an osmoprotectant, a molecular chaperone, a pH buffer, and a source of carbon and nitrogen during recovery but also as an important compound decreasing reactive oxygen species (ROS) concentrations in both direct and indirect ways (Szabados and Savoure 2010). It has been proved that proline plays the dual role as an osmoprotectant and ROS scavenger which can protect chloroplasts under drought stress (Vanková et al. 2012).

Proline also seems to play an important role during recovery from drought stress. In the present study, some transgenic and local accessions of perennial ryegrasses including NC11, GC5, and Bereshneh 2 had higher proline content under the recovery condition in comparison to well-irrigated conditions. While rehydration is quickly perceived by the plant, as reflected in the fast down-regulation of dehydrin encoding gene expression, the P5 CSA transcript level and free proline content remain high in tobacco leaves (Vanková et al. 2012).

Previous reports indicated the expression profiles of genes involved in proline biosynthesis in *Zea mays* L. because of inadequate water availability (Cao et al. 2018). All the DEGs (differently expressed genes) in the proline biosynthesis pathway were elevated within the first 96 h, except for ornithine decarboxylase and 2 ALDH (aldehyde dehydrogenase) homologs. These findings suggested that proline synthesis in maize was increased by water scarcity. The degree of cell membrane injury during water stress could be estimated through the measurement of EL from cells or tissues (Blum and Ebercon 1981). In most local accessions, leaf EL percentage significantly increased. Under drought stress the lowest leaf EL was recorded for Komehr1 as 35.68% (Table 10). In addition, it also demonstrated good impacts of CK and N on physiological traits. Application of exogenous CK along with high N in 'PennA- 4' creeping bentgrass caused lower EL under drought conditions (Chang et al. 2016).

The ultimate consequence of drought stress is stomatal closure, which downregulates the CO₂ flow into the leaves, thus exhausting electron acceptors NADP and transporters to NADP, and mediating ROS production (Koffler et al. 2014). MDA is regarded as an end-product of lipid peroxidation and defines membrane-linked damages (Ayala et al. 2014).

Despite leaf MDA content that decreased in all plants in response to rewatering, the MDA content of most plants did not recover fully as well-irrigated controls, while for NC14, GC3, GM12, GM21, and GM24 transgenic lines and Komehr1 and Cham Sohrabkhani accessions, the MDA was significantly lower than those of the well-irrigated condition after 14 days of rewatering. These results indicate that these species have more ability to maintain the cell membrane stability and to recover the damaged cell membranes.

According to the results, the highest reduction (57%) in leaf MDA content was found in the NC1 transgenic line after rewatering. In WT plants, leaf MDA content showed a marked increase of about 7–tenfold after the drought stage compared to the well-irrigated condition (Table 10). It has been proven in previous reports that turfgrass species exposed to drought conditions remain higher MDA than control plants (Chang et al. 2016; Duo et al. 2018; Tatari et al. 2018). Our current results are in agreement with previous findings as stated above. On the other hand, *IPT* overexpression in some turfgrasses such as creeping bentgrass resulted in lower MDA content in the leaves, associated with an increase in antioxidant activities that led to higher endogenous CKs (Merewitz et al. 2012a; Xu et al. 2016). Additionally, leaf MDA levels significantly increased in non-transgenic creeping bentgrass with decreased water soil content. However, *SAG-IPT* creeping bentgrass showed low leaf and root MDA under drought stress (Merewitz et al. 2012a). It is proposed that overexpression of *TaMYB30-B* in *Arabidopsis* leads to improved

drought tolerance via integrative regulatory mechanisms in stress-driven genetic triggers that modify specific physiological attributes, including proline, MDA, and soluble sugar contents (Zhang et al. 2012).

RD29A-TaFBAI transgenic *Nicotiana tabacum* L. plants accumulated less reactive oxygen species, maintained smaller MDA contents, and regulated membrane leakage under water-deficit stress, thus reflecting increased antioxidant enzyme activity and upregulated gene expressions (Kong et al. 2016).

Drought stress responses of antioxidant enzymes may vary with stress duration and severity, tissue or organ types, as well as plant species and cultivars (DaCosta and Huang 2007a). SOD and CAT activities decreased in the leaves of three bentgrass (*Agrostis* spp.) species exposed to severe drought stress (DaCosta and Huang 2007a). The activity of SOD increased while that of CAT and POD decreased in leaves of Kentucky bluegrass and tall fescue when only surface soil drying was simulated (Fu and Huang 2001). A Positive correlation between antioxidant activity and drought tolerance has been reported in different turfgrass species, although the specific enzymes imparting drought tolerance may differ between species or cultivars.

Xu et al. (2011) discovered that the activities of ascorbate peroxidase (APX) and CAT in Kentucky bluegrass leaves correlate with drought tolerance under drought stress. Additionally, the enzymes glutathione reductase (GR), monodehydroascorbate reductase (MHAR), and dehydroascorbate reductase (DHAR) were linked to enhanced drought tolerance, while CAT, POD, and APX also played roles in recovery after re-watering.

In our research, CAT and SOD activity became higher compared to POD activity under drought stress, especially in transgenic plants. It was also found that higher SOD and CAT antioxidant enzyme activities under drought stress are associated with increases in CK content, especially iP_A. Correlation analysis also confirmed these results. There was a significant positive correlation between iP_A with SOD ($r = 0.76$) and CAT ($r = 0.67$) antioxidant enzymes.

Moreover, Chang et al. (2016) stated that CK could enhance the activities of isozymes, thus contributing to the enhanced antioxidant mechanism while reducing levels of ROS in leaves that resulted in increasing drought tolerance in creeping bentgrass plants.

In a study, *RD29A-TaFBAI* activity in transgenic lines was significantly more than in WT plants in drought conditions. Greater mRNA levels increased the protein constituents for antioxidant enzyme activity in transgenic lines (Kong et al. 2016). The synthesis and accumulation of ROS increases under stress conditions, and ROS can damage biological membranes and organic molecules.

Creeping bentgrass that overexpresses an *Agrobacterium IPT* gene regulated by the senescence-activated promoter SAG12 showed significantly reduced ROS levels when subjected to drought stress. This was associated with increased activities of antioxidant enzymes such as SOD, CAT, APX, and dehydroascorbate reductase (DHAR) compared to non-transgenic plants (Xu et al. 2016).

The activities of POD and SOD in maize leaves were significantly higher under drought stress than under CK conditions, and their activities decreased to normal levels 3 days after re-watering (Cao et al. 2018). These results indicated that CAT and SOD activities in most plants did not return to their normal levels however, POD activity was decreased after 14 days of rewatering. It appears that CAT and SOD activities have more effect than POD activity in drought adaptation and recovery.

Conclusion

It is concluded that improvements in major morphological and physiological characteristics were made by enhancements in indigenous CKs and thus drought tolerance in transgenic lines. In addition, the results of RT-PCR of transgenic and WT plants exposed to drought stress revealed more *IPT* transgenic plants with an increase in its expression in transgenic plants compared to WT plants. Among transgenic plants, GM12, GM24, GC8, GC6, NC12, NC4, NC14, NS14, and GC3 had acceptable performance during drought stress and recovery. Higher drought tolerance was not only affected in enhancing endogenous CK but also *RD29A* and *UBQ10* introns are important factors in increasing *IPT* expression which led to drought tolerance of transgenic plants. Additionally, drought tolerance and recovery screening of local accessions revealed that many possess potential for molecular breeding and direct cultivation in semi-arid green spaces. Notably, Komehr1 and Cham Sohrabkhani exhibited higher RWC, photosynthetic rate, Fv/Fm, CK content, and antioxidant enzyme activity, along with lower EL and MDA content. These results are also valuable for selecting drought-tolerant species, identifying candidate genes for drought tolerance, and expanding germplasm sources in future studies.

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Author contributions SE performed the experiments, analyzed data, and wrote the manuscript; HS assisted with writing and revising the manuscript. GK read and revised the manuscript. All authors have read and agreed on the final version of the manuscript.

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Data availability The data that support the findings of this study are available from the corresponding authors, upon reasonable request.

Declarations

Conflict of interest All the authors declare no conflicts of interest.

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