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Drought stress response in maize: molecular, morphological and physiological analysis of tolerant and sensitive genotypes

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Abstract

The aim of this work was to develop a physiological method based on transpiration in combination with molecular methods, i.e., *dehydrin* gene expression analysis, for identifying the reactions of maize (*Zea mays* L.) plants that display different levels of tolerance to drought stress during the generative growth stage. Drought stress was induced in two genotypes, 2087 and 2637, by four irrigation treatments. The rate of transpiration and the expression of the *dehydrin* genes *ZmDHN1* and *ZmDHN2* were dependent on genotype and duration and intensity of stress. The yield components were affected by the level of *dehydrin* gene expression and transpiration rate. Compared with genotype 2637, genotype 2087 a) maintained higher transpiration intensity, even under strong drought stress conditions, b) exhibited an earlier onset and a higher level of expression both at a lower stress intensity and during the initial phases of the stress reaction, c) showed higher values of yield components, and d) was characterized by a lower water-use efficiency of cob yield. Drought tolerance is of increasing importance and is one of the breeding targets in maize. However, traditional breeding methods have numerous limitations. The simultaneous use of new molecular genetic techniques and physiological methods could therefore help to elucidate the genetic and physiological basis of plant responses to drought stress and provide more accurate evaluation for screening parental breeding material.

Introduction

In plants, reaction to drought stress at the cellular level involves induction of the synthesis of a wide range of proteins, including dehydrins (*DHN*s; Late Embryogenesis Abundant, D11 family). The functions of *DHN*s have been studied in relation to cellular protective mechanisms, mainly cell membrane stabilization under drought stress conditions (Hanin et al., 2011; Graether and Boddington, 2014). The activation of *dehydrin* expression has been shown to occur immediately in response to stress and is also part of the acclimatization processes that increase stress tolerance (Vítámvás et al., 2010). Increased expression of *DHN* genes is generally correlated with the acquisition of tolerance to abiotic stresses such as drought (Lopez et al., 2003; Suprunova et al., 2004; Guo et al., 2009). Maize is particularly sensitive to water stress during the reproductive stages, the early maturity phase and grain filling (Zinselmeier et al., 2002). Several DHN sequences have been identified (Alexandrov et al., 2009; Li and Cao, 2016) or predicted, but only the previously known genes *ZmDHN1* and *ZmDHN2* have been studied in high detail. The activation, structure and function of these genes, as well as their promoter regulatory activity, have been studied in plants grown in different conditions (Koag et al., 2003; Capelle et al., 2010; Xing et al., 2011). Additionally, the assessment of *ZmDHN* gene expression may be useful as an indicator of stress intensity (Klimešová et al., 2017) and for testing and comparing the sensitivity of maize genotypes to drought stress (Xing et al., 2011; Gullì et al., 2015).

At the physiological level, the rate of transpiration is considered to be indicative of the water status of a plant as well as the relative water content (RWC) or water potential (Meinzer and Grantz, 1991; Jones, 2007). Changes in transpiration, which are strongly dependent on meteorological variables, are induced in accordance with the plant's growth stage (Pivec et al., 2009), the water content in the soil (Jiang et al., 2016) and the water potential of the leaves (Li et al., 2002) or stems (Blanco-Cipollone et al., 2017).

Transpiration is closely related to sap flow in xylem ves-

sels (Kučera et al., 1977; Miner et al., 2017). Therefore, methods for the evaluation of sap flow can be used to measure either the water consumption of plants (Bethenod et al., 2000) or stomatal conductivity. Sap flow measurements are often used as a reference method for crop model testing (e.g., Heinlein et al., 2017), to model evapotranspiration (Han et al., 2018; Bo et al., 2017) or for refinement of crop coefficients (for maize, e.g., Hou et al., 2014).

The aim of the work was to verify the possibilities of selected methods to distinguish and describe different intensities of stress reactions in maize plants with two contrasting genotypes growing under conditions of long-term water shortage. To test this presumption, a two-genotype experimental design is sufficient. Taking into account previously published results, we can conclude that a one-year vegetation period is also sufficient.

Materials and Methods

Plant material and growing conditions

The experiment was conducted using two maize (*Zea mays* L.) genotypes. The genotypes (breeding lines) were designated "2087" and "2637" and exhibited different levels of drought sensitivity. Long-term pre-experiment field observations showed that the difference in sensitivity to drought was manifested by different durations of green leaf area, onset of leaf rolling, plant height and dry matter yield. The screening of breeding material for drought sensitivity was performed by the CEZEA breeding company (CEZEA–šlechtitelská stanice, a.s., Čejč, Czech Republic).

The pot experiments were conducted in natural climate conditions in terms of natural day length, air temperature and humidity, but with controlled irrigation (specified in Středová and Středa, 2015), a factor that has a dominant effect on both plant transpiration and the expression of protective genes.

The plants were maintained under four different watering regimes beginning at phase BBCH 40 (Meier, 1997). Condition A, the control, involved 90% of the available water holding capacity (AWHC); condition B represented mild stress, at 50% AWHC; condition C represented moderate stress, at 25% AWHC; and condition D represented severe stress, at 15% AWHC. Six maize plants were planted in each container with a volume of 200 dm^3 and dimensions of 0.73 × 0.54 × 0.51 m.

Evaluation of traits

The cob yield, plant height, stem diameter and harvest index (HI) as well as the green biomass yield and dry matter yield of whole plants were evaluated for all of the plants in each experimental treatment at the stage of full maturity (BBCH 89). The harvest index was calculated by dividing the dry weight of the cobs by the dry weight of the entire plant. The water-use efficiency of cob yield (WUEc) was calculated based on the amount of water consumed by the plant in a generative period (BBCH 50–89) and the cob yield: WUEc = dry matter yield of cobs per plant (g) / sum of water transpired by the plant (kg).

Sap flow measurements

Transpiration was monitored through continuous measurements of xylem sap flow. An EMS62 sap flow system (EMS Brno, Brno, Czech Republic), which uses the "stem heat balance" method (Kučera et al., 1977), was employed to measure xylem sap flow. The sap flow values [$kg.h^{-1}.plant^{-1}$] were provided at 10-min intervals. Only diurnal sap flow values were included in the analyses. Two plants from each condition were measured between BBCH 50 and BBCH 89.

Meteorological variables

The relative air humidity [%] and air temperature [°C] were measured at 10-min intervals using HOBO U23 Pro V2 sensors (Onset Computer Corporation, Bourne, USA) with an accuracy of ± 0.21°C. Global solar radiation [$W.m^{-2}$] and photosynthetically active radiation (PAR) [$\mu mol.m^{-2}.s^{-1}$] were measured at 10-min intervals using MinikinRTi and QTi sensors, respectively (EMS Brno, Czech Republic). The soil moisture content [%] was measured at 15-min intervals using VIRRIB automatic electromagnetic sensors (AMET Velké Bílovice, Czech Republic).

Data processing and statistical analysis

The experimental data of sap flow were processed using Mini32 software (EMS Brno, Czech Republic) and statistically analysed using SW OriginPro 2017 (OriginLab Corporation, Northampton, MA, USA) and STATISTICA 10 software (StatSoft Inc., Tulsa, USA). The analyses performed included correlation analysis, variance analysis, consequent testing using Tukey's HSD test and confidence interval calculations.

Analyses of dehydrin gene expression

Plant biomass was sampled to assess the expression levels of the selected genes on four dates: period I, 2 weeks of drought stress (BBCH 63–65); period II, 4

weeks of drought stress (BBCH 73–75); period III, 6 weeks of drought stress (BBCH 80–83); and period IV, 7 weeks of drought stress (BBCH 83–85).

Total RNA was isolated from 100 mg of leaf disc tissue taken from the second youngest leaf. The conditions for RNA isolation, cDNA synthesis and evaluation of the normalized relative gene expression (NRE) of *ZmDHN1* and *ZmDHN2* were the same as those used by Klimešová et al. (2017). The ubiquitin gene was amplified with specific primers and served as a reference gene (Gómez-Anduro et al., 2011). The stability of the expression of this gene under our experimental conditions and at different developmental stages was assessed using BestKeeper software (Pfaffl et al., 2004). Gene expression was calculated using the Pfaffl method (2001). The results are presented as gene expression levels relative to the value of the internal calibrator. The expression of each gene was calculated with its own calibrator, and both evaluated lines had a common calibrator for the specific gene. The *ZmDHN1*/*ZmDHN2* gene expression levels of the first samples of the control treatment of genotype 2637 were considered 1. The values presented in the graph are the averages of three independent samples that were measured twice ± SD (standard deviation).

Results and Discussion

Course of sap flow

The transpiration (sap flow) of maize plants was monitored from the beginning of flowering until full maturity. Sap flow rates were influenced by meteorological conditions (data not shown), phenological phase and irrigation regime. The highest average hourly values of sap flow in the diurnal part of the day were observed during flowering and at the beginning of grain filling in all conditions (6.78 $g.h^{-1}.plant^{-1}$), with maximal values of 32.78 $g.h^{-1}$ for genotype 2087 and 20.32 $g.h^{-1}$ for genotype 2637. The strongest correlations between sap flow and meteorological conditions, especially global radiation, was observed in the treatments with relatively high soil moisture. Compared with the control (90% AWHC), with the increasing intensity and duration of drought stress, the correlations weakened. Soil moisture thus became the main factor limiting transpiration. The stress induced by moisture scarcity reported by various authors significantly differs. Wu et al. (2011) set the margin of decrease of maize hybrid transpiration at 80% AWHC. According to Matejka et al. (2005), drought stress in maize occurs at asoil water content of 58.2% AWHC. Sadras and Milroy (1996) reported that the beginning of the linear decrease in plant transpiration occurs at 40–25% AWHC. The changes in sap flow were also affected by the genotype (Fig. 1). Drought stress at the wilting point (condition D) caused a significant decrease in sap flow in both genotypes; nevertheless, genotype 2087 transpired 62% more water than did genotype 2637.

Although genotype 2087 was characterized as presenting higher values of sap flow in most irrigation regimens than those presented by genotype 2637, the significant decrease in the transpiration of genotype 2087 (compared with that of the control) observed under all of the tested stress conditions (B, C and D)

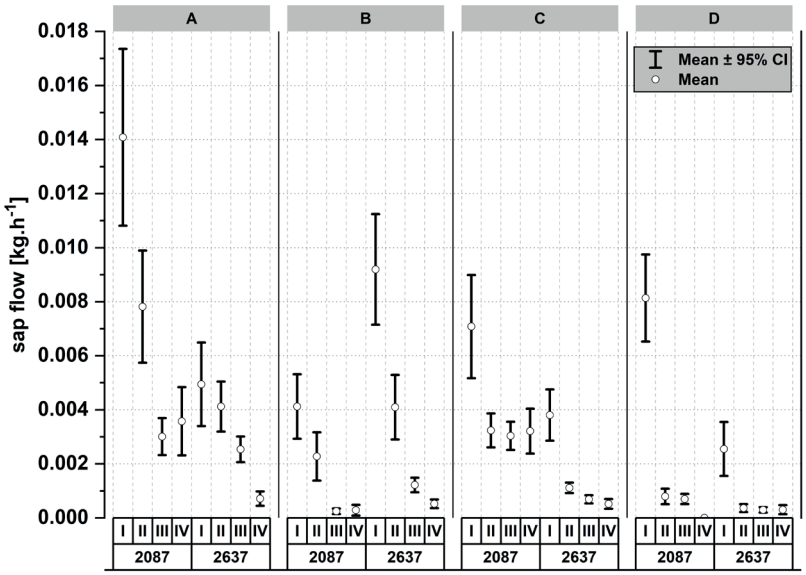

Fig. 1 Average hourly sap flow rates per plant ($kg.h^{-1}$) during the diurnal part of the day of the study periods (I–IV) in two maize genotypes (2637 and 2087) grown in different irrigation treatments (A, B, C, D). Period I, 2 weeks of drought stress (BBCH 63–65); period II, 4 weeks of drought stress (BBCH 73–75); period III, 6 weeks of drought stress (BBCH 80–83); and period IV, 7 weeks of drought stress (BBCH 83–85)

as early as the flowering stage demonstrates that this genotype a) is more sensitive to water shortages and b) has the ability to conserve water, and it maintains less intense transpiration until the end of the ripening stage, which has a positive impact on biomass yield.

ced only 51% and 27% of the yield achieved by the control, respectively, and 32% and 27% of the yield of genotype 2087, respectively. A strong decrease in cob yield as a consequence of drought during the flowering stage (Zinselmeier et al. 2002, Doorenbos and Kassam 1979) and the early stages of maturity is a well-known

Table 1 - Morphological and yield characteristics of plants at harvest (average values across all plants, n = 12)

Morphological or yield characteristic	Genotype	Value	Significance of difference
Cob weight (g)	2637	24.01	a
	2087	37.90	a
Dry matter weight (g)	2637	69.26	a
	2087	89.30	a
Green matter weight (g)	2637	143.51	a
	2087	225.90	b
Weight of dry matter without cobs (g)	2637	45.25	a
	2087	51.40	a
Harvest index	2637	0.28	a
	2087	0.40	b
Height of plants (cm)	2087	149.08	a
	2637	189.67	b
Diameter of stems (cm)	2637	1.13	a
	2087	1.35	b
Length of leaves (cm)	2087	67.77	a
	2637	72.50	b
Number of leaves	2637	8.67	a
	2087	9.67	b

Note: Statistically significant different means are indicated by different letters

Effects of genotype and environment on the morphological and yield characteristics of plants (Table 1)

The effects of the genotype and watering regime as well as the interaction of these factors on the morphological and yield characteristics of the plants were confirmed. Compared with genotype 2087, genotype 2637 presented a 37% lower grain yield on average in all conditions. The phenotypes of genotype 2637 plants changed, as the plants became substantially taller and had a smaller stem diameter and longer and fewer leaves. In addition, cob yield was considerably affected by water shortage, primarily in the case of genotype 2637. The plants in conditions C and D produ-

phenomenon of maize and is caused by a decrease in grain number (Salter and Goode 1967), restricted development of cobs or prolonged anthesis-silking intervals (Bolanos and Edmeades 1993).

The efficiency of cob production per unit of transpired water is expressed as WUEc for the generative period (Table 2). The cob yield in the control (A) was 32% lower in genotype 2637 than in genotype 2087. However, genotype 2637 was 20% more efficient in water use (WUEc) (12.1 g cob.l^{-1} water used for genotype 2637 in contrast to 9.6 g cob.l^{-1} for genotype 2087).

From a practical breeding point of view, the transpiration intensity had a positive influence on cob yield, which was related to increased water uptake (effective use of water (EUW)). Therefore, an increase in the

Table 2 - Total amount of transpired water, average weight of cobs and WUEc (water-use efficiency of cob yield) per maize plant during the generative vegetation phase (BBCH 50-89).

Genotype	Condition	Amount of water transpired per plant (kg)	Weight of cobs (g)	WUEc
2087	A	4.26	41.09	9.64
	B	0.96	37.08	38.45
	C	2.56	44.50	17.36
	D	1.22	28.93	23.62
2637	A	2.30	27.90	12.11
	B	2.26	46.10	20.38
	C	1.08	21.46	19.87
	D	0.52	7.74	14.87

yield of maize genotypes with a high yield potential can be achieved only by maximizing water gain from the environment (Lopes et al., 2011) through EUW (Blum, 2009). The increasedwater uptake and relatively hightranspiration intensity wereassociatedin both genotypes with the so-called "water wasting" in thewell-watered environment. In this treatment(A), the WUE was the smallest, but at the same time, the highest cob yield was found.Similarly, Condon et al. (2002) related the high yield of barley and wheat to a low WUE.

The plants of both genotypes produced cobs more efficiently under drought stress, especially under the mild stress of condition B compared withthe control (A). Under conditions of drought stress, compared with genotype 2637, genotype 2087 achieved a higher cob yield (up to 73% higher). The causes of the higher EUW observed for genotype 2087, which exhibited higher yields under drought conditions than under well-watered conditions, manifesting as a relatively low WUE and relatively high transpiration intensity, have not yet been determined. However, the causes could be related to the characteristics of the root system (Cseresnyés et al., 2014; Leitner et al., 2014). Increasing EUW could be a suitable strategy for "rain-fed" conditions during the vegetation period, when water supplies in the soil are replenished after a dry period. Hence, high-yielding maize genotypes under drought stress show a relatively high transpiration intensity (Blum, 2009)

Evaluation of DHN gene activity

The activity of the *dehydrin* genes in the leaves was evaluated using the means of the NRE values. Each gene was evaluated separately. The NRE values for the tested genes are presented in Fig. 2. The common attributes of the two genes were activation by drought, similar expression dynamics under stress conditions (1st sampling), and wide expression variability within individual plants. It was nevertheless obvious that the two genes were regulated in a slightly different way. In a study by Klimešová et al. (2017), a difference between the initial expression levels of these genes was observed under well-watered conditions (the NRE of *ZmDHN1* was 102$^{\times}$ higher than the NRE of *ZmDHN2*). It has been widely reported that the *ZmDHN1* gene is activated by drought (Badicean et al., 2011), but the high NRE of *ZmDHN1* in the 2nd through the 4th samplings in the control treatment (condition A) may indicate that *ZmDHN1* is more sensitive to other abiotic environmental stress factors than is *ZmDHN2*. It is well known that *dehydrin* genes are activated by transcription factors that are regulated not only by ABA but also by ethylene, and they could be involved in the response to various environmental stresses (Jia et al., 2006). Moreover, under drought stress, *dehydrin* gene expression may be negatively influenced by high air humidity (Wójcik-Jagła et al., 2012), to which experimental plants were exposed during periods II-IV.

At the first sampling (14 days of stress, period I), multiple increases in the NRE of the *ZmDHN1* gene were observed under conditions of 25% AWHC (C) and 15% AWHC (D), and differences between the two genotypes were observed in these conditions (C and D). For genotype 2637, a 104$^{\times}$ increase in NRE was observed in condition C and a 105$^{\times}$ increase in condition D compared with the expression of the common internalcalibrator. For genotype 2087, under conditions C and D, a 103$^{\times}$ and 104$^{\times}$ increase in NRE was observed compared with the expression of the common internal calibrator.

Two weeks later (at the 2nd sampling, period II), a distinct increase in *ZmDHN1* gene expression was observed in the plants of both genotypes in the control treatment and condition B. In both cases, the gene expression level was higher in genotype 2087 than in genotype 2637, albeit not significantly. In the next set of samples taken 14 days later (at the 3rd sampling, period III), a decrease in gene expression was observed in the control conditions (A). Under mild stress conditions (B), the expression level of *ZmDHN1* in plants of the sensitive genotype (2637) was similar to that in condition A. In the tolerant genotype (2087), the expression of this gene increased by 102$^{\times}$ compared with that of the control. Under more severe stress conditions (treatments C and D), an increase in this gene was observed in genotype 2637. In the next period (4th sampling, period IV), a 10$^{\times}$ increase in NRE was observed only under severe stress in the tolerant genotype (2087). However, in the sensitive genotype (2637), relatively high levels of gene expression were maintained under both intermediate and severe levels of stress.

For the *ZmDHN2* gene, a generally lower sensitivity to drought was observed because the activation of *ZmDHN2* expression is more often related to the impact of cold temperatures (Xing et al., 2011). In the tolerant genotype (2087), an increase in expression was detected only under severe stress (treatments C and D). In the sensitive genotype (2637), an increase in *ZmDHN2* gene expression was noted under control conditions up to the 4th sampling (102$^{\times}$) as well as under the B treatment conditions in the 3rd sampling (by approximately 10$^{\times}$). Under more severe stress conditions (C and D), relatively high expression levels were detected at the 1st sampling. In the last (4th) sampling, the largest differences in the NRE of the *ZmDHN2* gene were observed. However, the level of expression in the tolerant genotype (2087) increased only under severe stress (by 102$^{\times}$ compared with the control). In the sen-

sitive genotype (2637), the level of expression of this gene increased even in plants grown under the conditions of the control treatment (by $102^{\times}$ compared with the expression of the internal calibrator).

Based on the results reported by Suprunova et al. (2004), Fracasso et al. (2016), Lopez et al. (2013) and on the generally accepted hypothesis, we expected that DHN gene expression would be higher (at least in some phases) in plants of the more drought-tolerant. This hypothesis was not unambiguously confirmed by the results of our experiment. A higher NRE of the studied genes in the more drought-tolerant genotype (2087) was observed only in the 1st and 2nd sampling groups under well-watered or low-stress conditions. Under more severe drought conditions (conditions C and D), a higher NRE was observed in the plants of the more sensitive genotype (2637) than the less sensitive one. The level of NRE showed a clear dependence on the intensity of stress, but it is evident that evaluating the relationships between the expression levels of *dehydrin* genes and the level of tolerance to abiotic stress can be rather difficult. There are issues related to using the NRE parameter or evaluating expression at the level of transcription because the regulation of expression is a dynamic process. Gene activity may fluctuate (Solařová et al., 2016), particularly during long-term stress, even though the accumulation of protein remains high during stress and decreases after the stress subsides (Ganeshan et al., 2009). However, a number of works confirm positive correlations of DHN-gene expression with a level of tolerance to drought, and for this reason, their expression can be used as a marker of stress (Guo et al. 2009).

The type of stress reaction may influence the course of stress. For example, Kosová et al. (2015) observed more *dehydrin* accumulation after the onset of one-shot stress than after a gradual onset of stress conditions. Similar differences could be observed at the level of transcription. The intensity and duration of stress had an impact on the expression levels of both genes. Based on these observations, it can be concluded that the differences in tolerance to abiotic stress depend on the genotype and are most often manifested during the initial stress phase. However, it is advisable to compare these parameters with the plant damage intensity caused by drought (Barnaby et al., 2013).

Relations between the expression levels of DHN genes and morphological and production characteristics

The correlations between the levels of expression of both *dehydrin* genes and the selected yield components were evaluated using the Pearson correlation coefficient (r). The values of the correlation coefficients in Table 3 indicate strong correlations of the drought-activated levels of DHN gene expression with the values of yield components. All of the correlations were negative, which indicated that the expression increased with the level of damage.

Genotypic differences in the expression of DHN genes, sap flow and morphological and production characteristics

In period I, both the expression of DHN genes and the level of transpiration were generally highly dependent on stress intensity and genotype. A significant correla-

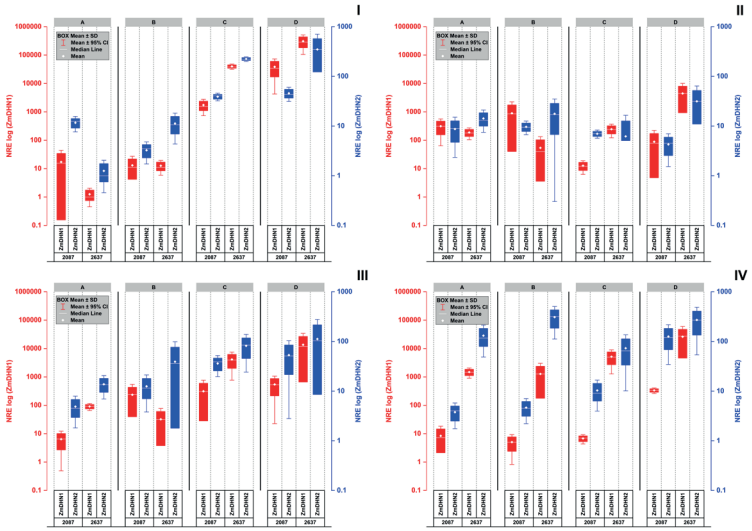

Fig. 2 - Evaluation of the normalized relative expression (NRE) of the *ZmDHN1* (red colour) and *ZmDHN2* (blue colour) genes in two contrasting maize genotypes, 2637 and 2087, cultivated under four different intensities of drought on four sampling dates (figure part I), 2 weeks of drought stress (period I); figure part II, 4 weeks (period II); figure part III, 6 weeks (period III); figure part IV, 7 weeks (period IV). The logarithms of the NRE values are presented as the averages of 3 independent samples measured 2 times ± SDs and ± confidence intervals with a 95% level of confidence.

Table 3 - Correlations (r values) between the levels of DHN gene expression on four sampling dates (periods I–IV) and the selected biometric and yield parameters (average of both genotypes across the A, B, C and D conditions; n = 8). Period I, 2 weeks of drought stress (BBCH 63–65); period II, 4 weeks of drought stress (BBCH 73–75); period III, 6 weeks of drought stress (BBCH 80–83); and period IV, 7 weeks of drought stress (BBCH 83–85)- NRE: normalized relative gene expression.

Period	NRE	Dry matter weight	Weight of the cob and grain	Green matter weight	Harvest Index
I.	ZmDHN1	-0.872**	-0.841**	-0.692	-0.778*
	ZmDHN2	-0.897**	-0.855**	-0.820*	-0.899**
II.	ZmDHN1	-0.811*	-0.773*	-0.615	-0.674
	ZmDHN2	-0.568	-0.528	-0.565	-0.401
III.	ZmDHN1	-0.863**	-0.826*	-0.715*	-0.774*
	ZmDHN2	-0.840**	-0.778*	-0.834**	-0.836**
IV.	ZmDHN1	-0.555	-0.454	-0.689	-0.411
	ZmDHN2	-0.223	-0.316	-0.627	-0.229

Note: * statistical significance at $p \leq 0.05$; ** statistical significance at $p \leq 0.01$

tion between transpiration intensity and the expression of DHN genes was observed only for genotype 2637.

In the ripening period (period III), the expression of both genes showed greater dependence on the level of drought stress in the sensitive genotype (2637). The lower sap flow intensity in the 2637 genotype was more strongly correlated with the expression of the DHN genes. The greatest differences in the reactions to stress between the individual genotypes were observed during this stage. There was a distinctly negative correlation between the expression of both genes and the evaluated morphological and yield characteristics (particularly for the harvest index) in the tolerant genotype (2087). However, these strong correlations were not observed in the sensitive genotype (2637).

Conclusion

Based on the evaluated parameters, we were able to distinguish the stress reactions of both genotypes. Transpiration intensity evaluated by sap flow data and water-use efficiency of cob yield showed that genotype 2087 employs a "stress-avoidance strategy", while genotype 2637 employs a "drought-escape strategy" mainly under severe drought stress. The tolerant genotype (2087) used in this experiment maintained fully turgid tissues and provided a balanced biomass yield in the control treatment, even under severe drought stress. Furthermore, compared with genotype 2637, this genotype had a conclusively higher harvest index, cob yield and biomass but a lower height in. It can be assumed that this genotype employed the ability to apply the strategy of "stress avoidance", probably by the use of effective molecular and physiological mechanisms. The sensitive genotype (2637) showed typical symptoms of drought stress – curling of leaves and a faster onset of senescence of older leaves. This genotype had relatively hightranspiration intensity under mild drought stress. Nevertheless, under strong stress, the low sap flow values of this genotype were accompanied by a low cob yield. The probable strategy of the genotype was "drought escape" and contributed to faster transpiration intensity and, thus, growth under conditions of mild stress. The expression levels of the *dehydrin* genes *ZmDHN1* and *ZmDHN2* increased with the intensity and duration of drought stress and were dependent on the damage caused to the plants. The results show that the methods used could be applicable for the characterization of stress reactions of parent material for breeding drought-tolerant maize hybrids adapted to water-limiting conditions. Unlike traditional breeding methods, these methods allow prompt screening of genotypes for drought tolerance.

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