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The effect of freezing temperature on physiological traits in sunflower

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ABSTRACT

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This study was conducted to identify the physiological mechanisms associated with the resistance and tolerance of young sunflower plants to freezing temperatures. The effect of overnight temperature –3°C on the maximal quantum efficiency of PSII (F_v/F_m), the relative electrolyte leakage (REL) and the osmotic potential (Ψ_π) was determined in five genotypes of sunflower: C33, C98, C124 and C148 were chosen from the population of recombinant inbred lines (RILs) based on contrasted responses to low temperature, and a wild genotype 2603 that was chosen for its ability to maintain activities in cold conditions. The night temperature –3°C over the course of 10 h caused an immediate significant decrease of F_v/F_m in C33, C98, C124 and C148. In the case of genotype C98, the effect of this freezing temperature was manifested by a significant increase of REL. Significant changes of Ψ_π, as a reaction to the effect of freezing temperatures, were not found in any of the monitored genotypes. The measurements of the physiological traits after 5 days of regeneration indicated the renewal of integrity of cellular structures and an increase of PSII reaction centre efficiency in all monitored genotypes. From the point of view of tolerance or sensitivity, the wild genotype 2603 showed itself as tolerant towards the tested freezing temperature, displaying insignificant differences with control plants in all monitored traits. Genotype C98 appears to be the most sensitive from the monitored set, with evident changes in two traits signalling frost damage.

Keywords: *Helianthus annuus* L.; cold acclimation; chlorophyll fluorescence; early sowing

Sunflower is one of the most widely cultivated oil crops in the world. Sunflower is grown in a number of countries on so-called marginal soils, often in semi-arid conditions where almost every year an abiotic stress of one kind or another is present, acting as a limiting factor on crop production. However, of all field crops, sunflower is best able to withstand stress conditions (Škorić 2009).

One of the significant factors reducing yield is drought (Alahdadi et al. 2011), despite the fact that sunflower is relatively tolerant against it (Killi et al. 2017). The critical period of ontogeny development of the sunflower is flowering and grain filling (Hewezi et al. 2006). There are multiple ways to prevent the negative effects of drought in critical developmental stages, for example, breeding and growing tolerant genotypes (Adiredjo et al. 2014), application of growth regulators (Hussain et al. 2013) and early sowing enabling the avoidance of the drought period (Allinne et al. 2009, Houmanat et al. 2016).

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Early sowing is mainly connected to the risk of cold or freeze stress in the early stages of development of sunflower. Cold stress, which includes both chilling and freezing injuries, modifies gene expression and plant metabolism with consequent effects on many biological functions (Janmohammadi et al. 2015). There is a disruption of plasmatic membrane (Steponkus 1984), changes of protein synthesis (Duman and Wisniewski 2014) and limited breathing, photosynthesis and carbon fixation (Liu et al. 2012). Freeze damage is not caused by the actual freeze temperature but by the formation of ice crystals (Beck et al. 2007). The formation of ice crystals in the extracellular space thus causes the drainage of water from protoplast into the extracellular space and the subsequent dehydration of cells. Although dehydration causes the fading of tissues, it prevents the adverse formation of ice in the protoplasts (Scott 2008). Despite this, the dehydration of protoplasts remains the main cause of freeze damage. The two distinct strategies taken by plants to combat low temperature stress are avoidance and tolerance. Stress avoidance entails preventing the freezing of sensitive tissues. A more elaborate avoidance strategy involves supercooling, in which endogenous ice nucleation is prevented by inhibiting the formation of ice nucleators (Janská et al. 2010). Balbuena et al. (2011) studied sunflower cold acclimation changes in freezing; susceptible and tolerant lines were investigated using a label-free comparative proteomic approach. They stated that cold-responsive proteins were mostly involved in metabolism, protein synthesis, energy and defense processes and that tolerant lines have different proteome responses to cold acclimation. Tetreault et al. (2016) examined variation in cold acclimation capacity and freezing tolerance among three natural populations (Texas, Kansas, and Manitoba) of the perennial sunflower species. Freezing tolerance was the highest in plants from the northernmost latitude under both non cold-acclimated and cold- acclimated experimental conditions. The plants from all populations retained the ability to increase freezing tolerance through the process of cold acclimation.

The aim of the research was to identify the physiological mechanisms of resistance and tolerance of young sunflower plants to overnight freezing temperatures. This should contribute to the stabilization of cultivation in areas with the possibility of early spring frosts.

MATERIAL AND METHODS

Plant material and growth design. Four genotypes of sunflower (C33, C98, C124, C148) were chosen from the population of recombinant inbred lines (RILs) based on contrasted responses to low temperatures in field conditions (Allinne et al. 2009) and a wild genotype 2603, that was chosen for its ability to maintain activities in cold conditions. Genotypes C33, C98, C124 and C148 are the results of crossbreeding between the parent genotypes RHA266 and PAC2. These genotypes are F_8 generation obtained using the Single Seed Descent method. Genotype RHA266 is the result of crossbreeding between wild sunflower (*Helianthus annuus* L.) and genotype Peredovik (originally from Russia). Genotype PAC2 is the result of crossbreeding between genotype HA61 and the wild prairie sunflower (*Helianthus petiolaris* Nutt.). The parent genotype PAC2 may be considered relatively resistant to freeze stress, while genotypes C124 and C148 are rather sensitive to freezing (Hejnák et al. 2014). The sensitivity and physiological response of other used genotypes to freezing temperatures is not known.

The experiments were based in the French National Institute for Agricultural Research (INRA) in Toulouse (France). Achenes were pre-germinated in a thermostat Memmert ICP 800 (Memmert GmbH., Schwabach, Germany) on wet filter paper at a temperature of 23°C for 4 days. They were then planted into cultivation containers (diameter 8.0 cm, height 11.7 cm); the cultivation substrate was a mixture of clay (50%), peat (40%) and sand (10%). The number of experimental plants was 24 from each genotype (always one plant in one cultivation container), where 12 plants of each cultivar were stressed group (S) and 12 plants formed the control group (C). All of these sunflower plants were grown in the phytotron under following conditions: photoperiod 14 h day (photon flux density of 108 μmol/m^2/s)/10 h night, temperature 23°C during day/18°C during night and the relative air humidity 63%. Control plants were grown under these conditions throughout the experiment. Stressed plants were grown under the same conditions to the BBCH phase 16–18 (6–8 fully developed leaves). At this phase, the plants were transferred to the growth chamber (Snijders Scientific b. V., Tilburg, Netherlands) and one-time overnight for 10 h exposed to a freezing temperature of –3°C. The

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measurement of the selected characteristics and extraction of samples for their identification was performed in 6 stressed (S1) and 6 control plants (C1) immediately after the effect of the freezing temperature. The remaining 6 stressed plants were transferred back to the phytotron where they were left to regenerate. After five days of regeneration, further measurements took place in the group of 6 stressed (S2) and 6 control plants (C2).

Chlorophyll fluorescence. The chlorophyll fluorescence parameters – minimum (F_0) and maximum (F_m) were measured on the fully developed leaves of the 3rd or 4th pair of leaves by the fluorometer PAM-2000 (WALZ, Heinz Walz GmbH., Effeltrich, Germany) with 1 s excitation pulse (660 nm) and saturation intensity 8000 μmol/m^2/s after 20 min dark-adaptation of the leaves. The maximal quantum efficiency of PSII was calculated as F_v/F_m ($F_v = F_m - F_0$).

Relative electrolyte leakage (REL). REL was measured using the conductometer (WTW LF 95, probe type TetraCon 96, WTW GmbH. & Co. KG., Weilheim, Germany). One leaf disc with a diameter of 2 cm was removed from a fully developed leaf (3rd or 4th pair of leaves), rinsed with demineralised water and then was left to float in a tube (30 mL, with cap) with 10 mL demineralised water in ambient temperature for 24 h. Then the 1st measurement of conductivity (REL1) was realised and it meant a relative quantity of electrolytes passing through a membrane. After REL1 measurement the tubes were placed into autoclave (20 min, 121°C) to damage cells and to release all electrolytes. 24 h after the autoclave treatment the 2nd measurement (REL2) was realised and it corresponded to total conductivity. The results are expressed as a ratio of relative amount of released electrolytes (REL1) to the total amount of released electrolytes (REL2) in %.

Osmotic potential. Osmotic potential at full turgor was measured on expressed sap of frozen and thawed leaves using 10 μL aliquots placed in a vapour pressure osmometer (VAPRO® 5520, Wescor, Inc., Logan, USA) calibrated with manufactured solutions (Allinne et al. 2009). For osmotic potential measurement were used small pieces of the same leaves that were used for the determination of REL. They were placed into the tubes with 5 mL of demineralised water and left in 4°C for 24 h to fully saturate with water. Then the leaves were transferred into syringes and put into the freezer (–15°C). The osmometer determined the value of osmotic potential Ψ_π (mmol/kg); this value can be converted in MPa.

Statistical analysis. A statistical evaluation of the experiment was made using the analysis of variance (ANOVA) and the values obtained were compared in further detail, using the Tukey's test at the significance level $P < 0.05$. All data (C1, S1, C2, S2) for each genotype group were analysed together. Statistical analyses were performed using Statistica 9.0 CZ for MS Windows software (Tulsa, USA).

RESULTS AND DISCUSSION

Chlorophyll fluorescence. Figure 1a identifies the effect of –3°C temperature on the maximum quantum efficiency of PS II (F_v/F_m) in the monitored genotypes. A significant decrease of the F_v/F_m values in stressed plants immediately after the effect of the freezing temperature (S1), occurred in genotypes C33, C98, C124 and C148 compared to control plants (C1). In genotype 2603 the decrease of F_v/F_m values in stressed plants S1 was not significant, compared to control plants (C1). The largest decrease of F_v/F_m was identified in the C148 genotype. In this genotype, the decrease of the F_v/F_m was statistically significant compared to the C33 and 2603 genotypes. The F_v/F_m ratio following a saturating light pulse represents a measure of the potential photochemical efficiency of PSII electron transport. Lower F_v/F_m value indicates that a proportion of PSII reaction centres is damaged or inactivated; this phenomenon, termed as photoinhibition, commonly observed in plants under stress (Sharma et al. 2015). Hejnák et al. (2014) stated that the temperature of –3°C did not have an evident effect on the F_v/F_m ratio in the monitored sunflower genotypes, unlike –5°C where there was an evident decrease of F_v/F_m values in genotypes C148 and C124. In sunflower, the long-term low temperature exposure induced a reduction of growth capacity as indicated by the reduction of F_v/F_m and a reduction of dry matter accumulation (Allinne et al. 2009).

Stressed plants after five days of regeneration (S2) were able to increase their F_v/F_m value to a level equivalent to the values measured prior to the stress effect and the measured F_v/F_m values in all monitored genotypes did not statistically

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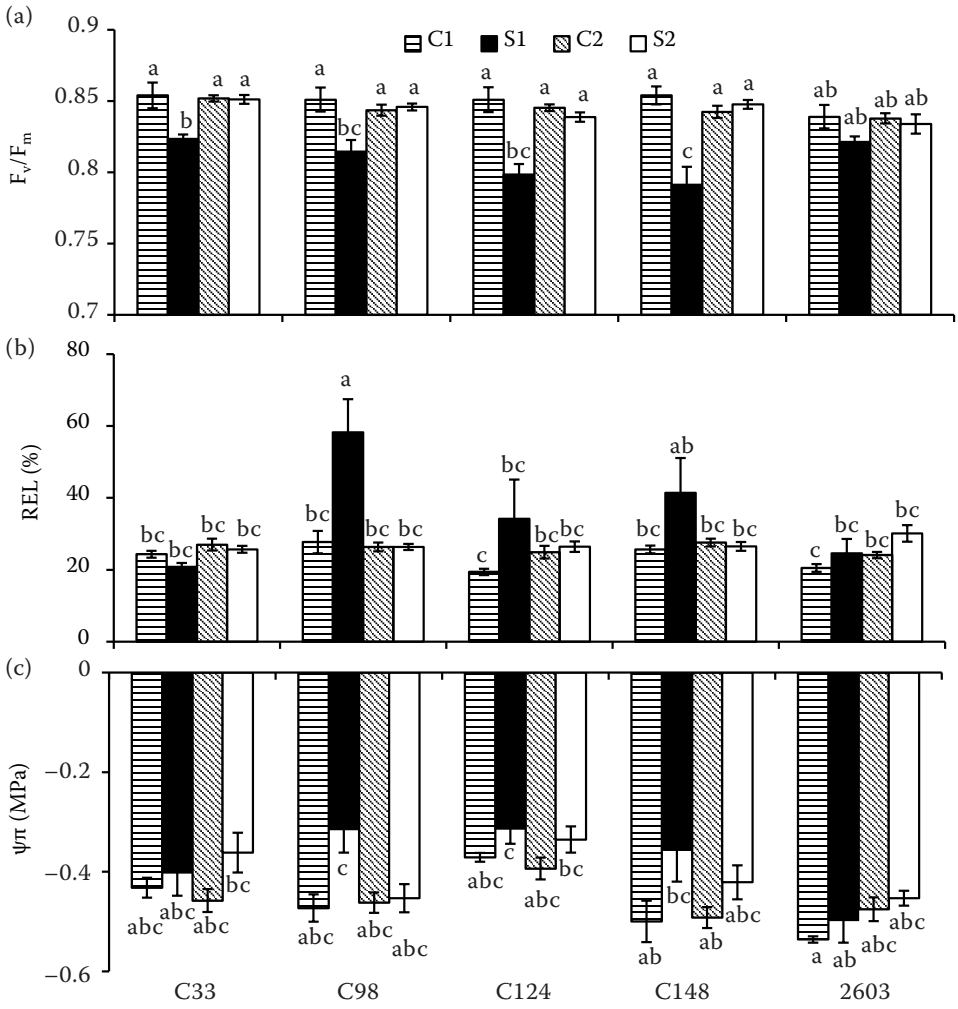

Figure 1. Maximum quantum efficiency of PSII (F_v/F_m), relative electrolyte leakage (REL) and osmotic potential (ψ_π) of the monitored genotypes of sunflower. Values are the means ± standard error (n = 6); α = 0.05 (Tukey's test); values sharing the same letters are not significantly different. C1 and S1 – control and stressed plants; immediately after the night effect of the freezing temperature –3°C. C2 and S2 – control and stressed plants; after five days of regeneration

differ from control plants after five days of regeneration (C2).

Relative electrolyte leakage. Abiotic stresses induce cell membrane injury, leading to intracellular ion efflux. Electrolyte leakage measurements can reflect the change of ion exosmosis, and determine the cell damage level (Bykova and Sage 2012). The increased values of REL in stressed plants immediately after the effect of the freezing temperature –3°C (S1), compared to the control plants (C1), occurred in all monitored genotypes, aside from C33 (Figure 1b). However, only for genotype C98, this increase is statistically significant and the REL value was 58%. In the case of genotypes 2603, C124 and C148, the increased REL was statistically insignificant. Many studies use 50% electrolyte leakage as the critical viability threshold, although many plants perish after suffering more than 30% electrolyte leakage (Peixoto et al. 2015). Comparing the individual genotypes, obviously, the highest values of REL were measured in stressed plants immediately after the effect of the freezing temperature –3°C (S1) in the C98 genotype (Figure 1b). Hewezi et al. (2006) recorded higher values of REL after exposing sunflower plants to night temperatures of –3.8, –4.8 and –5.8°C. Hejnák et al. (2014) cited the high stability of REL values in a set of tested sunflower genotypes after exposure to –3°C freezing night temperature, as opposed to –5°C, where the results showed that

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physiological parameters were more sensitive to this freezing temperature. Allinne et al. (2009) also stated the increase of REL in sunflower plants in field conditions exposed to low temperatures. According to Xin and Browse (2000) the increase of REL corresponds to the primary damage of plant tissues and breakage in cell membranes.

The values of REL measured in stressed plants after five days of regeneration (S2) were not statistically different from control plants after five days of regeneration (C2). This indicates the recovery of membrane integrity in the regeneration phase that includes tissue thawing, cellular rehydration, restoration of cell structure, and resumption of cellular activities (Li et al. 2008).

Osmotic potential. The decrease of values of osmotic potential, due to the increased concentration of osmotically-active substances (Mahajan and Tuteja 2005) is one of the strategies of cold tolerance, leading to the protection of structural integrity of cellular membranes and proteins (Kosová et al. 2007). As apparent from the values of osmotic potential stipulated in Figure 1c, none of the monitored genotypes showed statistically significant change in stressed plants immediately after the effect of the freezing temperature (S1) compared to control plants (C1). Similarly, the values of osmotic potential measured in stressed plants after five days of regeneration (S2) were not statistically different from the control plants after five days of regeneration (C2). Hejnák et al. (2014) also stated that in the genotypes of sunflower (C120, C124, C148 and PAC2) after the application of a freezing temperature of –3°C, no statistically evident changes of osmotic potential compared to the control group C were recorded. According to Allinne et al. (2009) a decrease of the osmotic potential indicates an increase of the intracellular osmolyte concentration in sunflower genotypes in response to low temperature exposure. The decrease of the leaf osmotic potential is reported e.g. Centinari et al. (2016) and Bilska-Kos et al. (2017).

When comparing between the individual genotypes, the lowest values of osmotic potential were found in the genotype 2603 immediately after the effect of the freezing temperature in control plants (C1) and stressed plants (S1). Compared with these, significantly higher values of osmotic potential were found in some stressed plants of genotypes C33, C98, C124 and C148. The highest values of osmotic potential were identified in stressed plants immediately after the effect of the freezing temperature (S2) on genotypes C98 and C124 (Figure 1c).

The night freezing temperature of –3°C over the course of 10 h caused a decreased function of the PSII reaction centre in sunflower genotypes C33, C98, C124 and C148, as shown by an immediate decrease of F_v/F_m values. In the case of genotype C98, the effect of this freezing temperature was manifested by a significant increase of values of relative electrolyte leakage, due to the damage to the integrity of cellular structures. Statistically significant changes of the osmotic potential, as a reaction to the effect of freezing temperature, were not found in any of the monitored genotypes.

On the fifth day of regeneration after the effect of freeze temperature (S2), a renewal of integrity of cellular structures and an increase of PSII reaction centre efficiency were recorded in all monitored genotypes, as evident from the insignificant differences in monitored parameters compared to control plants (C2). From the point of view of tolerance or sensitivity of the evaluated genotypes of the sunflower, wild genotype 2603 is the most tolerant to the freezing temperature of –3°C, having shown insignificant differences in all monitored parameters compared to control plants after exposure to freezing temperature. Genotype C98 appears to be the most sensitive from the monitored set, showing evident changes in two parameters, signalling damage due to freeze temperature.

REFERENCES

Adiredjo A.L., Navaud O., Muños S., Langlade N.B., Lamaze T., Grieu P. (2014): Genetic control of water use efficiency and leaf carbon isotope discrimination in sunflower (*Helianthus annuus* L.) subjected to two drought scenarios. Plos One, 9(7): e101218.

Alahdadi I., Oraki H., Khajani F.P. (2011): Effect of water stress on yield and yield components of sunflower hybrids. African Journal of Biotechnology, 10: 6504–6509.

Allinne C., Maury P., Sarrafi A., Grieu P. (2009): Genetic control of physiological traits associated to low temperature growth in sunflower under early sowing conditions. Plant Science, 177: 349–359.

Balbuena T.S., Salas J.J., Martínez-Force E., Garcés R., Thelen J.J. (2011): Proteome analysis of cold acclimation in sunflower. Journal of Proteome Research, 10: 2330–2346.

doi: 10.17221/214/2017-PSE

Beck E.H., Fettiq S., Knake C., Hartiq K., Bhattarai T. (2007): Specific and unspecific responses of plants to cold and drought stress. Journal of Biosciences, 32: 501–510.

Bilska-Kos A., Solecka D., Dziewulska A., Ochodzki P., Jończyk M., Bilski H., Sowinski P. (2017): Low temperature caused modifications in the arrangement of cell wall pectins due to changes of osmotic potential of cells of maize leaves (*Zea mays* L.). Protoplasma, 254: 713–724.

Bykova O., Sage R.F. (2012): Winter cold tolerance and the geographic range separation of *Bromus tectorum* and *Bromus rubens*, two severe invasive species in North America. Global Change Biology, 18: 3654–3663.

Centinari M., Smith M.S., Londo J.P. (2016): Assessment of freeze injury of grapevine green tissues in response to cultivars and a cryoprotectant product. Hortscience, 51: 856–860.

Duman J.G., Wisniewski M.J. (2014): The use of antifreeze proteins for frost protection in sensitive crop plants. Environmental and Experimental Botany, 106: 60–69.

Hejnák V., Němcová L., Matějovič M., Martinková J., Hnilička F., Skalický M., Grieu P. (2014): Physiological responses as influenced by night freeze stress at the beginning of vegetative growth of sunflower. Research on Crops, 15: 473–480.

Hewezi T., Léger M., El Kayal W., Gentzbittel L. (2006): Transcriptional profiling of sunflower plants growing under low temperatures reveals an extensive down-regulation of gene expression associated with chilling sensitivity. Journal of Experimental Botany, 57: 3109–3122.

Houmanat K., EL Fechtali M., Mazouz H., Nabloussi A. (2016): Evaluation and selection of promising sunflower germplasm under early winter planting conditions. African Journal of Agricultural Research, 11: 4610–4618.

Hussain S., Ahmad M., Qaisrani S.A., Iqbal J., Subhani M.N., Nadeem S.M., Atta S., Ibrahim M. (2013): Improvement of drought tolerance in sunflower (*Helianthus annuus* L.) by foliar application of abscisic acid and potassium chloride. Pakistan Journal of Nutrition, 12: 345–352.

Janmohammadi M., Zolla L., Rinalducci S. (2015): Low temperature tolerance in plants: Changes at the protein level. Phytochemistry, 117: 76–89.

Janská A., Maršík P., Zelenková S., Ovesná J. (2010): Cold stress and acclimation – What is important for metabolic adjustment? Plant Biology, 12: 395–405.

Killi D., Bussotti F., Raschi A., Haworth M. (2017): Adaptation to high temperature mitigates the impact of water deficit during combined heat and drought stress in C3 sunflower and C4 maize varieties with contrasting drought tolerance. Physiologia Plantarum, 159: 130–147.

Kosová K., Vítámvás P., Prášil I.T. (2007): The role of dehydrins in plant response to cold. Biologia Plantarum, 51: 601–617.

Li W., Wang R., Li M., Li L., Wang C., Welti R., Wang X. (2008): Differential degradation of extraplastidic and plastidic lipids during freezing and post-freezing recovery in *Arabidopsis* thaliana. The Journal of Biological Chemistry, 283: 461–468.

Liu Y.F., Qi M.F., Li T.L. (2012): Photosynthesis, photoinhibition, and antioxidant system in tomato leaves stressed by low night temperature and their subsequent recovery. Plant Science, 196: 8–17.

Mahajan S., Tuteja N. (2005): Cold, salinity and drought stress: An overview. Archives of Biochemistry and Biophysics, 444: 139–158.

Peixoto M.M., Friesen P.C., Sage R.F. (2015): Winter cold-tolerance thresholds in field-grown *Miscanthus* hybrid rhizomes. Journal of Experimental Botany, 66: 4415–4425.

Scott P. (2008): Physiology and Behaviour. New York, John Wiley and Sons, 305.

Sharma D.K., Andersen S.B., Ottosen C.O., Rosenqvist E. (2015): Wheat cultivars selected for high F_v/F_m under heat stress maintain high photosynthesis, total chlorophyll, stomatal conductance, transpiration and dry matter. Physiologia Plantarum, 153: 284–298.

Steponkus P.L. (1984): Role of the plasma membrane in freezing injury and cold acclimation. Annual Review of Plant Physiology, 35: 543–584.

Škorić D. (2009): Sunflower breeding for resistence to abiotic stresses. Helia, 32: 1–16.

Tetreault H.M., Kawakami T., Ungerer M.C., Levy C. (2016): Low temperature tolerance in the perennial sunflower *Helianthus maximiliani*. The American Midland Naturalist, 175: 91–102.

Xin Z., Browse J. (2000): Cold comfort farm: The acclimation of plants to freezing temperatures. Plant, Cell and Environment, 23: 893–902.

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