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RESEARCH ARTICLE

Drought stress during maize flowering may cause kernel abortion by inhibition of plasma membrane H^+-ATPase activity

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Abstract

Background: Drought stress during flowering of maize (*Zea mays* L.) frequently results in decreased kernel setting, leading to grain yield depressions. Plasma membrane (PM) H^+-ATPase was identified as a key enzyme responsible for supply of assimilates to the developing maize kernels shortly after pollination. The activity of this enzyme was strongly inhibited under salt stress, pointing to an involvement in kernel abortion.

Aims: This study aimed to determine whether also drought stress causes inhibition of PM H^+-ATPase in developing maize kernels shortly after pollination, leading to diminished hexose uptake and finally kernel abortion. The key questions are as follows: What are the limiting factors for grain yield production of maize plants facing drought? Are physiologically relevant parameters, quantified at flowering, reflected by yield determinants at maturity?

Methods: Maize plants were cultivated using the container technique, and drought stress was imposed during 3 weeks bracketing flowering compared to well-watered conditions throughout the entire growth period. The developing kernels were harvested 2 days after pollination, and PM vesicles were isolated and purified using two-phase partitioning.

Results: Water deficit caused a significant decrease in grain yield at maturity (−35%), which was determined by a reduced kernel number (−42%). Source limitation in the developing kernels under stress could be excluded. Acid invertase activity was unaffected by water deficit. Hexose availability was also no limiting factor for kernel setting and development. However, V_{max} of in vitro hydrolytic activity of PM H^+-ATPase was significantly decreased in the developing maize kernels under drought stress and the maximal pH gradient at the PM was also significantly reduced. The observed inhibiting effects on PM H^+-ATPase were mainly of quantitative nature, as a lower number of proton pumps was present in the kernel PM. Qualitative changes of the enzyme (activation energy E_a, Michaelis constant K_m) due to drought were not observed.

Conclusions: The lower pH gradient probably decreased the proton-driven transport of hexoses by carriers into the cytosol of the kernel cells, leading to kernel starvation and eventually contributing to kernel abortion.

KEYWORDS
acid invertase, hexose transport, pH gradient, source-sink relations, water deficit, *Zea mays*

1 | INTRODUCTION

Maize (*Zea mays*) is one of the most important crops worldwide, ranking first in grain production. Based on climate change, abiotic stresses occur more frequently, and maintaining or increasing maize yields is a great challenge (Lesk et al., 2016; Lobell et al., 2011). For European cropping systems, climate change combined with the intensification of drought stress is predicted to cause yield losses of grain maize by 20% by the year 2050 (Webber et al., 2018). Drought stress is often accompanied by high temperatures, which drive nonlinear increases in water vapor pressure deficit of the air. This results in a considerably higher transpiration and thus crop water demand, making water an increasingly limiting factor for plant growth and development (Ort & Long, 2014; Webber et al., 2018). For maize in the USA, drought stress was identified as the probable underlying mechanism of negative yield responses to high temperature (Lobell et al., 2013). Additional reasons for more frequent occurrences of drought stress in recent years are increased water requirements caused by higher plant stand densities and the much higher water use of modern maize cultivars in comparison to their older counterparts (Hammer et al., 2009; Ort & Long, 2014).

The grain yield potential of maize, which usually produces one mature cob per plant, is determined by two parameters: kernel number per cob and single kernel weight. Changes in one or both of these determinants have profound effects on the final grain yield at maturity. The kernel number per cob depends on kernel setting at or shortly after anthesis, whereas the individual kernel weight is determined by the rate and the duration of grain filling. Kernel abortion was identified as the main cause for maize yield depressions under drought stress (Hütsch et al., 2015; Setter & Parra, 2010; Zinselmeier et al., 1995). This strengthens the fact that not only the total amount of available water is decisive for yield development but also its distribution throughout the entire growth period. Sufficient water supply during the reproductive phase is particularly important for kernel set and yield performance (Andrade et al., 1999; Dolferus et al., 2011; Otegui et al., 1995; Passioura & Angus, 2010; Yang & Grassini, 2014; Zhang et al., 2014).

Kernel setting was limited by reduced sink activity under salt stress, which is strongly determined by the activity of two enzymes, acid invertase and plasma membrane (PM) H^+-ATPase (Hütsch & Schubert, 2017; Jung et al., 2017). During the days around pollination, apoplastic loading of hexoses into ovaries is essential to maintain kernels (Andersen et al., 2002; Mäkelä et al., 2005; McLaughlin & Boyer, 2004; Schussler & Westgate, 1994, 1995; Zinselmeier et al., 1995, 1999). The hexoses in the developing kernels are needed for biosynthesis of cell structures, metabolites, hormones, and for energy supply. They may also decrease the osmotic potential and increase the pull of water into the endosperm; an increased turgor pressure is essential for cell expansion (Chourey et al., 2006). There are no symplastic connections between the parental and the daughter cells, thus the apoplastic pathway is compulsory (Tang & Boyer 2013). Sucrose, transported to the maize cob via phloem, has to be hydrolyzed by acid invertase activity, which not only supplies hexoses for the import into ovaries but also prevents the retrieval of sucrose by the phloem (Hütsch & Schubert, 2017). For both of these reasons, acid invertase may be regarded as a key enzyme to establish sink strength in maize kernels shortly after pollination (Cheng et al., 1996; Chourey et al., 2006; Miller & Chourey, 1992). According to Zinselmeier et al. (1995) and Setter et al. (2001), drought stress is responsible for the reduced supply of hexoses to the maize ovaries, which is caused by insufficient hydrolysis of sucrose by apoplastic acid invertase activity (Roitsch & González, 2004). The resulting kernel abortion can be partly compensated by an increased kernel weight (Hütsch et al., 2015; Hütsch & Schubert, 2017), yet full compensation can only be achieved with a sufficient high kernel number.

Although under drought stress acid invertase activity was strongly inhibited in developing maize kernels shortly after pollination, concomitantly a significant increase in hexose concentrations was observed (Hütsch et al., 2015). Thus, the delivery of hexoses by acid invertase activity did obviously not limit kernel setting. Other factors must have contributed to the observed differences. The hexoses must be translocated from the apoplast into the cytoplasm of the developing kernels via H^+-cotransport, mediated by carriers localized in the PM (Bihmidine et al., 2013). This transport is driven by the pH gradient, which is established by the PM H^+-ATPase (Sondergaard et al., 2004; Zhao et al., 2000).

Under salt stress, we were the first to show that maize kernel PM H^+-ATPase activity was inhibited, pointing to a strong involvement of H^+-ATPase in kernel abortion (Jung et al., 2017). The inhibited enzyme activity probably reduced the pH gradient at the PM, limiting the energization of hexose carriers, which resulted in the measured accumulation of hexoses in the apoplast. The reduced hexose uptake supposedly diminished the energy status of storage cells, leading to reduced kernel fresh weight 2 days after pollination (2 DAP) and a lower kernel number at maturity (Jung et al., 2017). A pH increase in the apoplast may also reduce in vivo acid invertase activity by shifting the pH toward less favorable conditions.

As long as ion toxicity can be excluded under salt stress, drought and salinity both cause osmotic stress to the plants and induce similar changes/reactions in plant metabolism (Hütsch et al., 2015; Munns, 2011). Thus, we hypothesize that also drought stress causes inhibition of PM H^+-ATPase in developing maize kernels. In the present study, drought stress was imposed for 3 weeks bracketing flowering. PM H^+-ATPase activity as well as other physiologically relevant parameters such as acid invertase activity and sugar concentrations were determined in the kernels 2 days after controlled pollination. It was also evaluated whether measurements at kernel setting were reflected by yield determinants at maturity. To our knowledge, this is the first study of drought stress effects on the possible relationship between the activity of PM H^+-ATPase in maize kernels and kernel abortion.

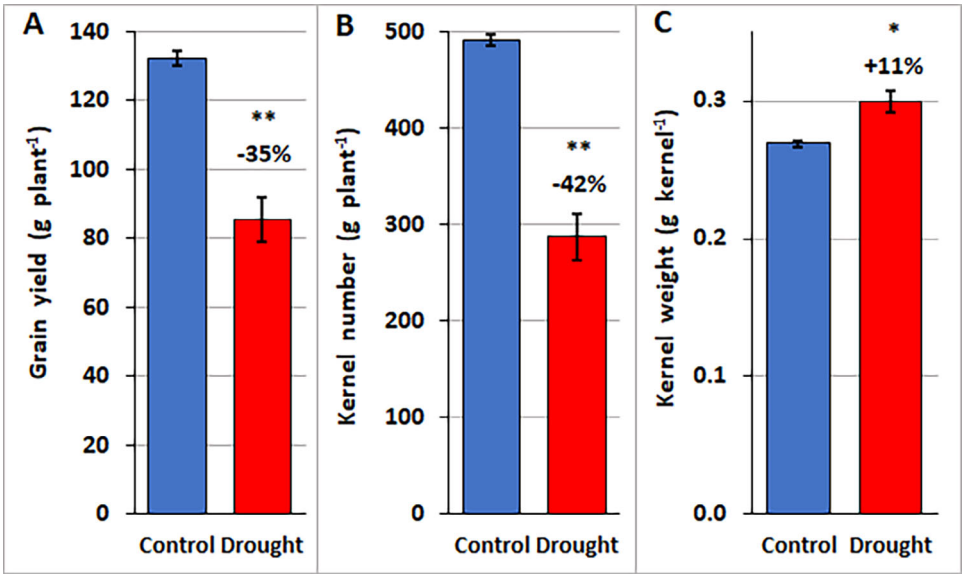

FIGURE 1 Effect of drought stress on grain dry matter yield (A), kernel number (B), and single kernel dry weight (C) of maize cv. Amadeo, harvested at maturity; data show means of six replicates ± standard errors (SEM); differences in percentage between control and stress are given and, if significant, indicated by $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$.

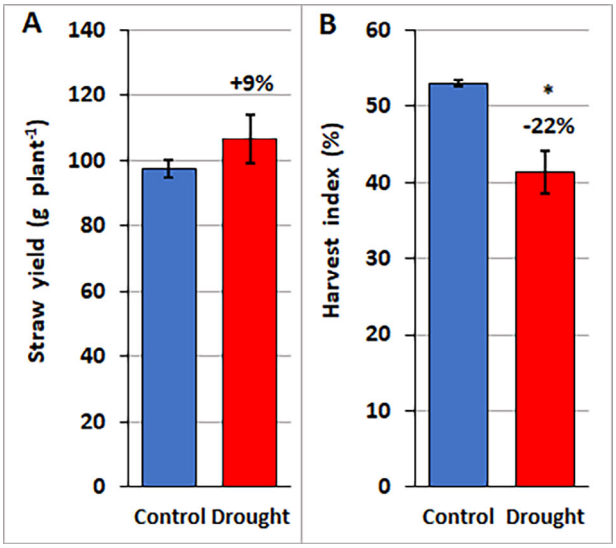

FIGURE 2 Effect of drought stress on straw dry matter yield (A) and harvest index (B) of maize cv. Amadeo, harvested at maturity; data show means of six replicates ± standard errors (SEM); differences in percentage between control and stress are given and, if significant, indicated by $^{*}p \leq 0.05$.

2 | MATERIALS AND METHODS

2.1 | Plant cultivation and harvests

Maize plants (*Z. mays* L. cv. Amadeo) were cultivated using the container technique (Hütsch et al., 2015; Hütsch & Schubert, 2021; Tscharn et al., 2022). Large 120-L plastic containers were filled with 145 kg of an air-dry Luvisol subsoil (loamy sand: 25.4% clay, 40.1% silt, and 34.6% sand; pH_{CaCl2} 7.1) in four increments: three layers with 30 kg soil each, and a topsoil layer (approx. 0–30 cm), which was fertilized with a compound fertilizer and micronutrients according to Hütsch et al. (2015). Each soil layer was moistened immediately after filling. The whole soil depth was 0.8 m. Two different treatments were set up: control and drought stress. The containers were repeated four times for the intermediate harvest ($n = 4$) and six times for the harvest at physiological maturity ($n = 6$), and in each container four plants were grown. In total, 20 containers were prepared.

On 28 May 2018, maize was sown with nine seeds per container, and 10 days later the number of plants was reduced to four per container and water content was adjusted to 50% maximum water-holding capacity (WHC). During the whole vegetation period water supply was recorded for each container and adjusted daily to the desired WHC. Additional compound fertilizer was applied to the maize plants when appropriate. The plants grew in the vegetation hall of the experimental station of the Institute of Plant Nutrition in Giessen under near natural conditions. The containers were set up in a completely randomized design and their position was changed at least once a week. The average daily temperatures during vegetation ranged from 13 to 33°C. As the highest temperatures occurred at flowering time of the maize plants, silking was very fast and coincided for control and drought stress treatments.

In the drought treatment, the plants were allowed to develop under the same conditions as control plants until approximately 2 weeks before start of flowering; thereafter, water supply ceased until a WHC between 25% and 30% was reached 1 week later. Thus, the plants could acclimate to drought conditions during this week 25%–30% max. WHCs were kept for 3 weeks in total, 1 week before and 1 week after the flowering time, which lasted about 1 week. After the drought stress period, WHC was again adjusted to 50%.

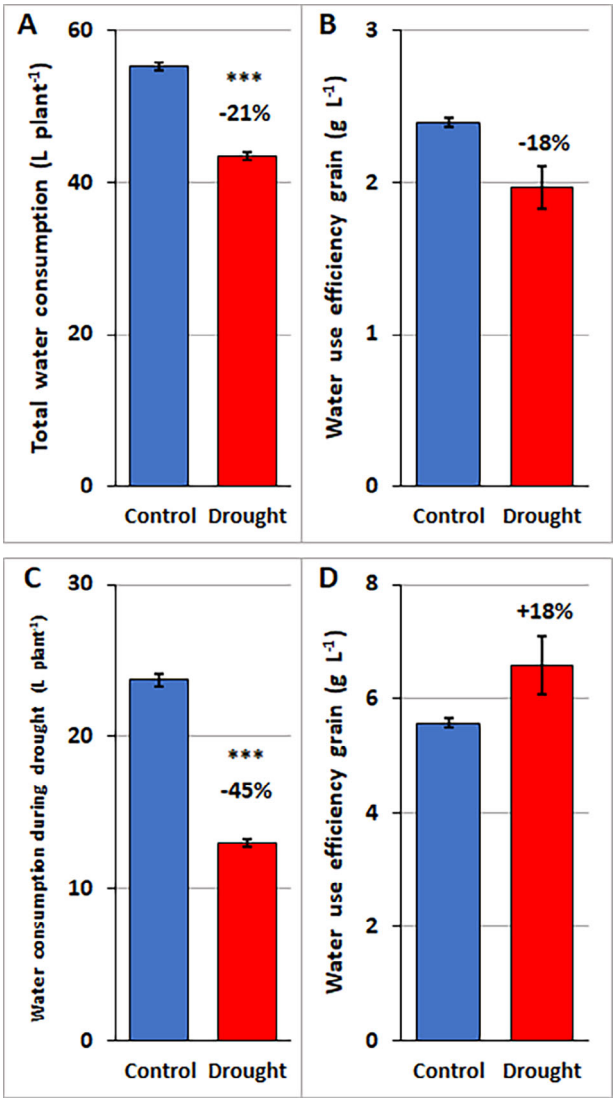

FIGURE 3 Effect of drought stress on total water consumption (A), WUE_{grain} (B), water consumption during 3 weeks of drought stress (C), and WUE_{grain} calculated with water consumed during 3 weeks drought (D) of maize cv. Amadeo; data show means of six replicates ± standard errors (SEM); differences in percentage between control and stress are given and, if significant, indicated by $^{***}p \leq 0.001$.

For the intermediate harvest, uncontrolled pollination was prevented by covering the developing cobs with parchment paper bags. The major cob of each plant was hand-pollinated with fresh pollen from unstressed donor plants 5 days after first silk appearance, which is considered to be the time with best receptivity (Cárcova et al., 2000). Pollen from separately cultivated, unstressed control plants was used to minimize possible drought stress effects on pollination and fertilization. Just prior to pollination silks were cut 1 cm above the tips of the husk leaves. For each treatment two harvests were conducted: An intermediate harvest 2 d after pollination (2 DAP), which was 60 days after sowing (60 DAS) for both treatments, and a harvest at physiological maturity (120 and 121 DAS).

Plants were harvested according to Hütsch et al. (2015). At the intermediate harvest (2 DAP) maize plants had been exposed to drought stress (25%–30% max. WHC) for 9 days. Shoots were cut at the base, and plant height was measured. The cob was separated from the stalk, husks were removed, and kernels were cut off from the rachis with a knife and immediately frozen in liquid N_2. Fresh weight of vegetative shoots, cobs, and kernels was recorded. Shoots were cut into small pieces and dried at 80°C to determine dry weights. For laboratory analyses, the frozen kernels were ground under liquid N_2 using mortar and pestle and stored at −80°C until further analysis. Therefore, enzyme activities and assimilate concentrations were determined in a mixture of maternal tissue as well as of endosperm and embryonic tissue (Hütsch et al., 2015; Jung et al., 2017; Mäkelä et al., 2005; McLaughlin & Boyer, 2004; Zinselmeier et al., 1995, 1999).

At harvest of the mature maize plants, straw, cob, and grain dry weight (80°C drying), kernel number per cob, and single kernel weight were determined.

2.2 | Sugar analyses

A subsample (5 g) of frozen kernels, ground in liquid N_2, was dried at 80°C for 48 h. Sucrose, glucose, and fructose were analyzed in 200 mg dry weight of pulverized samples. Samples were extracted with 30 mL double-deionized water in a shaking water bath at 60°C for 30 min. The extracts were filled up to 50 mL with double-deionized water, filtered, and stored at −20°C until enzymatic sugar determination was conducted with UV test kits (Boehringer Mannheim/R-Biopharm). Each kernel sample was extracted in duplicate prior to sugar analysis. The sugar concentrations were calculated and are given on a fresh weight basis for better comparisons with other data determined at the intermediate harvest. Concentrations should not be confused with contents, which give the amount of sugar in a whole plant or in a plant organ, depending on the material analyzed.

2.3 | Acid invertase enzyme extraction and activity measurements

Enzyme extraction and incubation for the determination of acid invertase (EC 3.2.1.26) activity were performed according to Zinselmeier et al. (1999) modified by Hütsch et al. (2015). Frozen and ground samples were extracted by HEPES-buffer (pH 7.2). After centrifugation, the supernatant was frozen in liquid N_2 and stored at −80°C. All extracts were desalted with Econo-Pac 10 DG columns (BIO-RAD). For determination of acid invertase activity, desalted extracts were mixed with Na-acetate (pH 4.8) and sucrose. Incubation was carried out at 30°C for 30 min. Generated glucose was determined with UV test kits (Boehringer Mannheim/R-Biopharm) and activity rates were calculated (μmol glucose g^{-1} fresh weight min^{-1}).

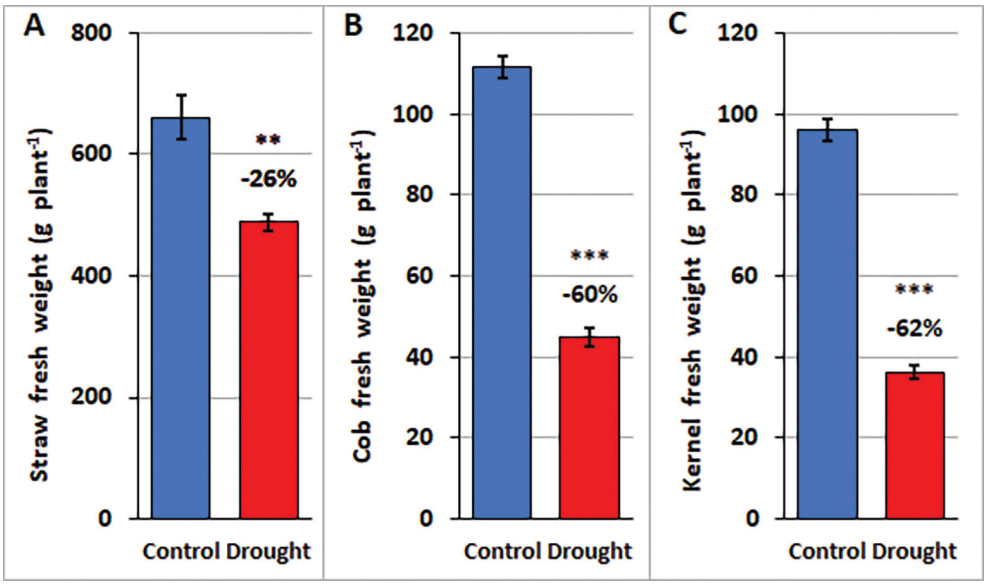

FIGURE 4 Effect of drought stress on straw fresh weight (A), cob fresh weight (B), and kernel fresh weight (C) of maize cv. Amadeo, 2 days after pollination; data show means of four replicates ± standard errors (SEM); differences in percentage between control and stress are given and, if significant, indicated by $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

2.4 | Plasma membrane H^+-ATPase measurements

Detailed description of isolation of PM vesicles from maize kernels and measurements of hydrolytic and pumping activity of PM H^+-ATPase (EC 7.1.2.1) in these vesicles can be obtained from Jung et al. (2017) and Tscharn et al. (2022).

2.4.1 | Isolation of plasma membrane vesicles

PM vesicles were isolated from frozen kernel samples. The material was homogenized according to Briskin and Poole (1983), De Michelis and Spanswick (1986), and Galtier et al. (1988). Phase partitioning of membranes was performed with a polymer concentration of 6.2% (Hanstein et al., 2011; Zörb et al., 2005). Quantification of proteins was done with the method of Bradford (1976). Vesicle aliquots were stored in liquid N_2. Vesicle purity was confirmed using specific ATPase inhibitors (Gallagher & Leonard, 1982; Hanstein et al., 2011; Yan et al., 1998).

2.4.2 | Kinetic assays of hydrolytic PM H^+-ATPase activity

In order to determine the Michaelis constant (K_m) and maximum reaction velocity (V_{max}) of the PM H^+-ATPase, the hydrolytic activity of each sample was measured for nine different ATP concentrations in each assay. The reactions took place at 30°C for 30 min and the protein concentration in the assays was always 0.02 µg µL^{-1}. The hydrolytic activity assays were conducted in the presence of 10 mM Mg according to Hanstein et al. (2011). K_m and V_{max} were determined by transformation of activity data according to Lineweaver–Burk. For determination of the activation energy E_a, the hydrolytic activity was measured with a substrate concentration of 5 mM ATP at two different temperatures, 20 and 30°C. E_a was calculated with the Arrhenius equation.

2.4.3 | PM H^+-ATPase pumping activity

The formation of a pH gradient across the PM of inside-out vesicles was measured as the quenching of absorbance by acridine orange (AO) at 492 nm (ΔA_{492}) (Yan et al., 1998). The amount of vesicle protein in the assays was 30 µg. Proton transport was characterized using the following parameters: Active transport was quantified as initial rate during the first minute after addition of Mg-ATP. The maximum pH gradient was determined as the maximum quenching of absorbance (ΔA_{492}), and passive proton transport was determined as initial rate of proton efflux for 1 min after addition of vanadate, the specific inhibitor of PM H^+-ATPase.

2.4.4 | Western blot of isolated vesicles

The abundance of PM H^+-ATPase was measured according to Zörb et al. (2005) with modifications after separation of isolated vesicle proteins (2 µg) by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). After separation with SDS–PAGE, samples were transferred onto polyvinylidene difluoride (PVDF) membrane filters (0.2 µm, Macherey-Nagel) using a semi-dry blotting system. The "PM H^+-ATPase (rabbit antibody)" (AS07260, Agrisera), diluted 1:5000, was used as first antibody for the detection of PM H^+-ATPase. The "14-3-3, general regulatory element" (AS122119, Agrisera), diluted

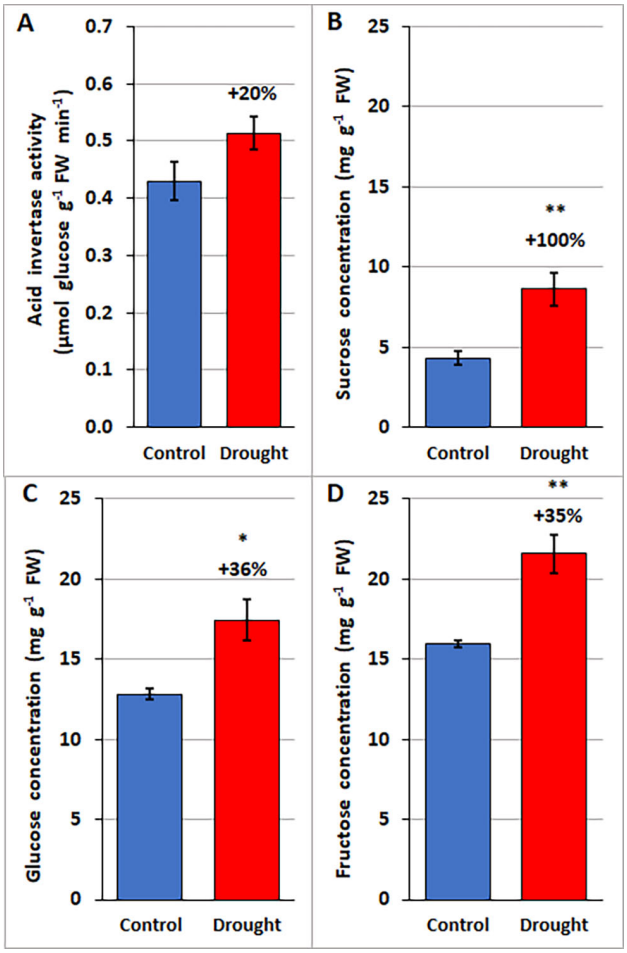

FIGURE 5 Effect of drought stress on acid invertase activity (A), concentrations of sucrose (B), glucose (C), and fructose (D) in kernels from maize cv. Amadeo, 2 days after pollination; data show means of four replicates ± standard errors (SEM); differences in percentage between control and stress are given and, if significant, indicated by $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$.

1:2000, was chosen for the detection of 14-3-3 protein. Alkaline-phosphatase-conjugated anti-rabbit IgG (AS09607, Agrisera), diluted 1:4000, was used as secondary antibody. The H^+-ATPase and 14-3-3 protein immunoreactive bands were quantified densitometrically with the software ImageJ 1.51k (National Institutes of Health). There were four (control) or three (drought stress) biological replicates for each treatment. Western blotting was repeated in two independent experiments.

2.5 | Calculation of efficiency parameters and statistical analysis

Harvest index (HI), which characterizes the efficiency of assimilate allocation to the grain, and water-use efficiency of grain (WUE_{grain}) were calculated according to the following equations (grain yield stands for grain dry matter at maturity):

$$HI = \textit{grain yield}/\text{total aboveground biomass at physiological maturity}, \quad (1)$$

$$WUEgrain = \text{grain yield}/\text{water consumption during the whole vegetation}, \quad (2)$$

$$WUEgrain\ \text{during drought} = \text{grain yield}/\text{water consumption during 3 weeks of drought}. \quad (3)$$

The four plants per container were combined and considered as one biological replicate. Means ± standard errors were calculated from biological replicates as indicated in the figure legends ($n = 3$, $n = 4$, or $n = 6$ per treatment). After Student's *t*-test (Microsoft Office Excel 2010), significant differences between control and drought stress treatment are given and indicated by $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$.

3 | RESULTS

3.1 | Grain yield at maturity and its determinants

Drought stress during 3 weeks around flowering caused a significant reduction in maize grain yield by 35% (Figure 1A). This yield depression was strongly determined by a reduced kernel setting, as the kernel number per plant was decreased by 42% (Figure 1B). The cobs remained smaller under drought stress and kernel abortion mainly occurred in the apical part. However, the remaining kernels showed an improved grain-filling in the drought stress treatment in comparison to the control, as the single kernel weight was significantly enhanced by 11% (Figure 1C). Thus, maize cv. Amadeo was able to partly counteract the detrimental effect of drought on kernel setting with better grain filling. In order to identify why the kernel number was reduced at maturity, possible physiological reasons for kernel abortion were investigated in detail 2 DAP (Sections 3.3–3.5).

The straw yield at maturity was statistically unaffected by drought (Figure 2A). The HI, which is calculated as ratio of grain yield to total aboveground biomass at physiological maturity, is an indicator of the relative investment of plant resources in reproductive plant parts and can be used as a measure of reproductive efficiency. Drought stress caused a significant decrease of the HI by 22% (Figure 2B), mainly resulting from grain yield reduction (Figure 1A).

3.2 | Water consumption and water-use efficiency

The water consumption during the whole vegetation period of drought-stressed maize plants was significantly reduced by 21% in

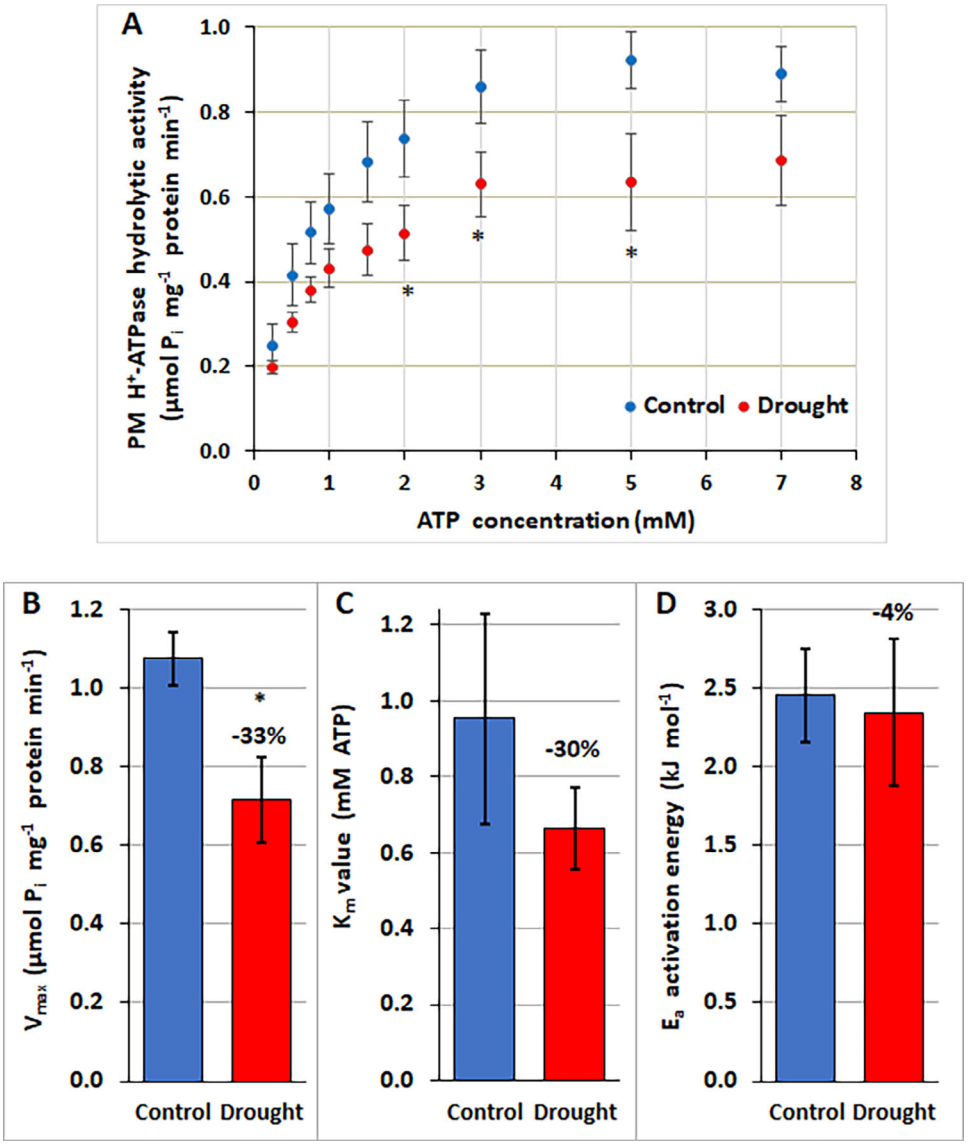

FIGURE 6 Effect of drought stress on plasma membrane (PM) H^+-ATPase hydrolytic activity (A), on kinetic parameters: catalytic rate constant V_{max} (B), Michaelis constant K_m (C), and activation energy E_a (D) of isolated PM vesicles from kernels of maize cv. Amadeo, 2 days after pollination; data show means of four replicates ± standard errors (SEM); differences in percentage between control and stress are given and, if significant, indicated by $^*p \leq 0.05$.

comparison to control plants (Figure 3A). Water-use efficiency, calculated as ratio of grain yield to total water consumption, showed a non-significant decrease under drought stress by 18% (Figure 3B). During the 3 weeks of water deficit with 25%–30% max. WHC, water consumption was even more reduced by 45% (Figure 3C). If WUE_{grain} was calculated with water consumed during 3 weeks of drought, it increased non-significantly by 18% under stress conditions (Figure 3D).

3.3 | Vegetative growth and kernel development (2 DAP)

At the intermediate harvest, 2 days after controlled pollination, plant height was significantly reduced by 12% in the drought stress treatment in comparison to the control (not shown). Similarly, the straw fresh weight was decreased by 26% under drought (Figure 4A). At this growth stage, drought showed a strong negative impact on cob fresh weight and kernel fresh weight with reductions of 60% and 62%, respectively (Figure 4B,C). This points to a high stress intensity.

3.4 | Kernel sugar concentrations and acid invertase activity (2 DAP)

Drought stress did not significantly affect the in vitro acid invertase activity in the maize kernels 2 DAP in comparison to the control (Figure 5A). The concentration of sucrose, the main

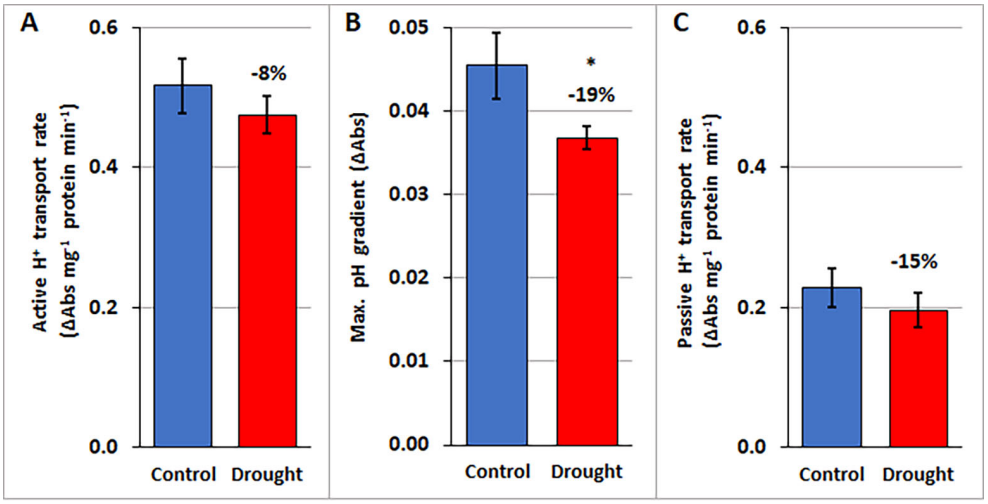

FIGURE 7 Effect of drought stress on plasma membrane (PM) H^+-ATPase active H^+ transport (influx) (A), maximal pH gradient (B), and passive H^+ transport (efflux) (C) of isolated PM vesicles from kernels of maize cv. Amadeo, 2 days after pollination; data show means of four replicates ± standard errors (SEM); differences in percentage between control and stress are given and, if significant, indicated by $^*p \leq 0.05$.

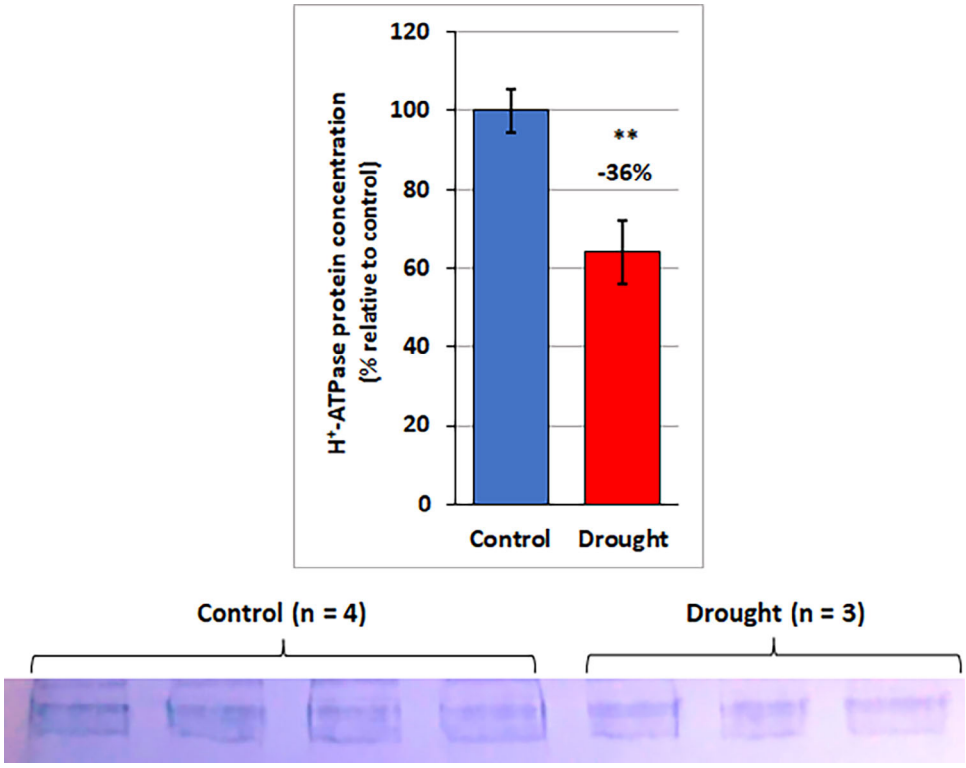

FIGURE 8 Effect of drought stress on total abundance of H^+-ATPase protein of isolated plasma membrane (PM) vesicles from kernels of maize cv. Amadeo, 2 days after pollination; data show means of four (control) or three (drought) replicates ± standard errors (SEM); difference in percentage between control and stress is given and significance is indicated by $^{**}p \leq 0.01$.

transport metabolite of assimilates in the phloem, was doubled in the kernel tissue of the stress treatment (Figure 5B). The hexose concentrations glucose and fructose were also significantly enhanced under drought stress by 36% and 35%, respectively (Figure 5C,D).

3.5 | Kernel PM H^+-ATPase activity (2 DAP)

As the PM H^+-ATPase is important for the uptake of hexoses into the kernels, kernel PM vesicles were isolated to determine the PM H^+-ATPase activity in vitro.

3.5.1 | Hydrolytic activity of the PM H^+-ATPase and activation energy

The hydrolytic activity of the PM H^+-ATPase was characterized by a kinetic study of the enzyme, which allowed to calculate the parameters V_{max} and K_m after transformation according to Lineweaver–Burk. The measured activities fitted well to these transformations with values of r^2 ranging from 0.937 to 0.996 (Figures S1 and S2). V_{max} describes the maximum turnover rate of the substrate Mg-ATP at the enzyme binding sites and K_m characterizes the affinity of the enzyme to its substrate. 2 DAP the hydrolytic activity of PM H^+-ATPase was significantly lower in the developing maize kernels under drought stress in comparison to the control with a reduction of V_{max} by 33% (Figure 6A,B). No significant drought-stress effect was observed for the affinity of the isolated PM H^+-ATPase enzyme to its substrate (K_m value; Figure 6C). There was no difference in activation energy E_a between both treatments (Figure 6D).

3.5.2 | H^+ transport and pH gradient

The pH gradient of isolated PM vesicles is established by the PM H^+-ATPase, which is measured as the quenching of absorbance by AO at 492 nm (ΔA_{492}), after the addition of Mg-ATP as substrate. The active H^+ transport rate (influx of inside-out vesicles), quantified as initial rate during the first minute after substrate addition, was not affected by drought stress (Figure 7A). The maximal pH gradient was significantly decreased in the drought-stress treatment by 19% compared to the control (Figure 7B). The passive H^+ transport rate (efflux), a measure for the leakage of protons out of the isolated PM vesicles, was not altered by the stress treatment (Figure 7C).

3.5.3 | Abundance of H^+-ATPase protein and 14-3-3 protein

The total abundance of H^+-ATPase protein of the isolated PM vesicles was assessed by Western-blot analyses. Drought stress significantly decreased the PM H^+-ATPase protein relative to the control treatment by 36% (Figure 8). The abundance of 14-3-3 protein was not significantly affected by drought (Figure S3).

4 | DISCUSSIONS

4.1 | Assimilate availability in developing maize kernels under drought stress

In the present study, plant height (not shown) and straw fresh weight (Figure 4A) were significantly reduced under drought stress by 12%** and 26%**, respectively, in comparison to the control treatment at 2 DAP. Although stress (25%–30% max. WHC) was imposed rather late, 1 week before start of flowering, it still had profound effects on vegetative growth. The smaller plants were characterized by a reduced leaf area and thus a decreased photosynthetic capacity, although the transpiration rate per leaf area was probably only slightly reduced under drought stress (Hütsch et al., 2015; Li et al., 2018). The transpiration rate can be used as an indirect measure for CO_2 assimilation (James et al., 2008; Rahnama et al., 2010). In maize, carbon assimilation during kernel setting and grain filling is compulsory for good kernel development, as no assimilate storage occurs in the culm and thus remobilization of reserves to feed the kernels is not possible (Cliquet et al., 1990; Schussler & Westgate, 1994). However, even under drought stress carbon assimilation did not limit kernel setting and early development, because significant accumulation of sucrose as the major transport metabolite in phloem was found in the developing kernels 2 DAP (Figure 5B; Hütsch et al., 2015). These results are in agreement with studies of Oury et al. (2016), who suggested no carbon deficiency in ovaries under moderate water deficit; the amount of sugars tended to be even higher than in ovaries of well-watered plants (Turc & Tardieu, 2018). Other experiments under soil water shortage (Andersen et al., 2002; Nuccio et al., 2015) or osmotic stress due to salt (Henry et al., 2015; Jung et al., 2017) support these results and suggestions. Thus, under moderate water deficits occurring at flowering time, sufficient carbon assimilates are obviously available in the developing kernels, and source limitation can be excluded as reason for kernel abortion (Hütsch et al., 2015; Hütsch & Schubert, 2017; Oury et al., 2016; Turc & Tardieu, 2018).

However, these results are in contradiction to observations that abortion in maize is linked to carbon starvation in young ovaries, as sucrose feeding could partly reverse the effect of water deficit on abortion (Boyle et al., 1991; Mäkelä et al., 2005; McLaughlin & Boyer, 2004; Zinselmeier et al., 1995, 1999). All the experiments with sucrose perfusion followed a common protocol causing a drastic reduction in photosynthesis, resulting in the limitation of kernel setting and development by carbon availability (Turc & Tardieu, 2018). When severe drought stress is imposed, for example, by complete withholding water for up to 14 days (Yang et al., 2018), the photosynthetic activity is strongly hampered either because of stomata closing or due to an additional decrease in photosynthetically active leaf area resulting from accelerated senescence (Li et al., 2018; Sade et al., 2018). Under natural drought scenarios a complete lack of available water for several weeks is rather unlikely. Instead, soil water contents of 25%–35% max. WHC, as imposed in our studies, are quite reasonable (present study and Hütsch et al., 2015).

4.2 | The role of PM H^+-ATPase in kernel establishment and grain yield performance

The significant grain yield decrease by 35%** in the drought-stress treatment of the present study was determined by a lower kernel number (−42%**; Figure 1A,B). Thus, the conditions at kernel setting

were decisive for the grain yield at maturity. At the intermediate harvest, 2 DAP, the reductions in cob and kernel fresh weight due to drought stress were even more pronounced (−60%*** and −62%***, respectively; Figure 4B,C). Water shortage during flowering (25%–30% max. WHC) was particularly detrimental to maize kernel development, whereas less severe drought stress in our previous study (35% max. WHC from 4 weeks after sowing until maturity) caused a significant reduction in kernel fresh mass by only 20%, and grain yield at maturity was reduced by 24% or less (Hütsch et al., 2015). However, in both experiments, yield performance was not source- but sink-limited. Acid invertase activity as one key enzyme determining sink strength was either significantly inhibited in vitro or remained unchanged under drought stress, whereas the concentrations of produced hexoses were significantly increased or not different to the control treatment (Figure 5A–D; Hütsch et al., 2015). Thus, in both studies, which were conducted during 3 years with three different maize cultivars (Pioneer 3906 and Fabregas in 2011 and 2012 [Hütsch et al., 2015], Amadeo in the present study of 2018), the availability of hexoses in the developing kernels was not decreased under drought stress pointing to no limitation by acid invertase activity.

However, in the present study, we demonstrate that another key enzyme for sink activity, the PM H^+-ATPase, was inhibited in the developing maize kernels by drought stress. The in vitro hydrolytic activity was reduced along with a significant decrease in the maximum reaction velocity V_{max} (Figure 6A,B). Decreases in V_{max} of hydrolytic activity were accompanied by a significant reduction in the maximal pH gradient under drought stress (Figure 7B). These results were achieved by using the same protein concentrations and amounts in the assays of control and stress treatment.

One factor that may explain the difference in the maximum pH gradient is the total protein abundance of the PM H^+-ATPase, which was significantly reduced under drought stress in comparison to the control (Figure 8). This indicates that a lower number of proton pumps was present in the kernel PM of the drought-stress treatment shortly after pollination (2 DAP). The PM H^+-ATPase is autoinhibited and requires activation by posttranslational modification. Phosphorylation of a specific threonine residue in the C-terminal end creates a binding site for 14-3-3 proteins, which stabilizes the upregulated state of the pump in which autoinhibition is released (Falhof et al., 2016; Fuglsang & Palmgren, 2021). For our study, it was found that 14-3-3 proteins were not limiting in the reaction medium of the isolated PM vesicles (Figure S3). Thus, quantitative differences in the number of proton pumps were responsible for the reduced maximal pH gradient under drought (Figure 7B). The lower turnover rate (V_{max}) of the substrate Mg-ATP is also a result of the decreased number of PM H^+-ATPases, as a lower number of Mg-ATP binding sites is available in the stress treatment. Qualitative changes of the PM H^+-ATPase resulting from drought stress were not observed, as the activation energy E_a and the K_m value as parameter for the affinity of the enzyme to its substrate were not significantly different to the control (Figure 6C,D).

The restrictions of physiological processes at flowering are reflected in the strongly decreased kernel number at maturity as the cause of grain yield reductions under drought stress. One possibility to attenuate drought stress intensity during flowering is a reduction in vegetative shoot growth, as a large shoot biomass needs a lot of water, which is no longer available for the particularly sensitive time of kernel setting. The supply of assimilates to the maize cob is most likely not a limiting factor for grain yield performance. Kernel setting per se could probably be improved by stimulation of the PM H^+-ATPase activity by elicitors (Falhof et al., 2016; Havshøi & Fuglsang, 2022; Hütsch & Schubert, 2022). The knowledge of key physiological processes, which affect drought stress resistance of maize, can be used in breeding programs leading to a more sustainable crop production.

In conclusion, the results are in strong accordance with the effects found under salt stress (Jung et al., 2017). The hexose uptake into the cytosol of the sink cells was most likely reduced due to a lower pH gradient energizing the transport by carriers (Figure 7B; Sondergaard et al., 2004). The lower hexose availability in endosperm and embryo of the developing kernels probably caused abortion, which resulted in the reduction of kernel number at maturity (Figure 1B).

5 | CONCLUSION

Under drought stress, carbon assimilate availability and invertase activity did not limit maize kernel setting and development. However, in vitro PM H^+-ATPase hydrolytic activity was significantly inhibited, confirming our results previously obtained under salt stress. The maximal pH gradient at the PM was also significantly lower, pointing to an impaired hexose transport into the cytosol of the kernel cells.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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