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RESEARCH ARTICLE

Auxin application to maize plants at flowering increases abundance and activity of plasma membrane H^+-ATPase in developing maize kernels

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Background: The kernel number of maize (*Zea mays* L.) at maturity is mainly determined at the time around pollination. Kernel abortion frequently occurs during this period, 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.

Aims: This study aimed at stimulating PM H^+-ATPase activity in the kernels by in vivo application of the auxin indole-3-acetic acid (IAA) to maize plants at flowering, leading to an improved hexose uptake and finally to a better kernel set.

Methods: Maize plants were cultivated under well-watered conditions using the container technique. IAA was applied to unstressed maize plants twice, 2 days before controlled pollination and at pollination (application rate per plant: 1.9 mL of 1.5 mM IAA). The developing kernels were harvested 2 days after pollination, and PM vesicles were isolated and purified using two-phase partitioning.

Results: The in vitro hydrolytic activity of the PM H^+-ATPase was significantly stimulated by 22% due to in vivo IAA application (control: 0.99 ± 0.05, IAA treatment: 1.21 ± 0.03* μmol inorganic phosphate mg^{-1} protein min^{-1}). V_{max} was significantly increased by IAA treatment, whereas K_m was reduced. The maximal pH gradient (ΔA_{492}) at the PM was increased by 10% (control: 0.071 ± 0.002, IAA treatment: 0.078 ± 0.002*). IAA caused a significant increase of PM H^+-ATPase abundance in the vesicles. Concentrations of sucrose and hexoses as well as acid invertase activity in the kernels were unaffected by IAA treatment. However, at maturity kernel numbers per cob were significantly decreased causing grain yield reductions of 19%.

Conclusions: Increased PM H^+-ATPase activity could not be translated into grain yield improvements. Probably the auxin application occurred too early during kernel development. As cytokinins play a key role during pollination, auxin application at this stage may have disturbed the phytohormone balance, causing disruption of cell division and a rather early onset of cell extension due to increased IAA concentrations. In further studies, it

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should be tested if applications of cytokinin at flowering or of IAA at a later growth stage have positive impacts on kernel set.

KEYWORDS
enzyme kinetics, grain yield, indole-acetic acid, kernel number, pollination, *Zea mays*

1 | INTRODUCTION

Maize grain yield is mainly determined by kernel number per cob at maturity (Borrás & Gambín, 2010), as only set kernels can be filled and contribute to single kernel weight, the second yield determinant (Hütsch & Schubert, 2022). Under typical field conditions, only 60%–65% of all initiated kernels give rise to completely developed grains (Gustin et al., 2018). Reasons for low kernel numbers are defective ovaries, failure of pollination, and abortion of fertilized ovaries as a consequence of insufficient kernel development (Gustin et al., 2018).

Kernel setting may be determined by source or sink limitation. As a C_4 plant, maize is very efficient in nitrogen and carbon assimilation as well as in water consumption. Even under stress conditions such as salinity or drought, sucrose as the main transport metabolite was not limiting kernel development at flowering (Hütsch et al., 2015; Jung et al., 2017). Thus, impaired kernel set predominantly results from sink limitation rather than source limitation (Hütsch & Schubert, 2017, 2022; Hütsch et al., 2015; Jung et al., 2017). Sucrose is transported in the phloem from the source leaves to the developing maize kernels as main sinks during flowering. After release into the sink apoplast, sucrose is hydrolyzed by acid invertase into the hexoses glucose and fructose. This irreversible cleavage generates a gradient of sucrose, which is necessary to establish sink activity, and it prevents retrieval of sugars in the phloem. Since no symplastic connections between mother cells and kernels are present, an apoplastic pathway for phloem unloading is compulsory (Tang & Boyer, 2013). Glucose and fructose are transported into the kernel cytoplasm by hexose carriers using H^+ symport, energized by the plasma membrane (PM) H^+-ATPase (Bihmidine et al., 2013; Hütsch & Schubert, 2017, 2022; Jung et al., 2017; Sondergaard et al., 2004; Zhao et al., 2000). Hexoses are needed for synthesis of various cell components and thereby they are indispensable for kernel development (Andersen et al., 2002; Mäkelä et al., 2005; McLaughlin & Boyer, 2004; Schussler & Westgate, 1994; Zinselmeier et al., 1995, 1999). In addition, the established H^+ gradient and thus decrease in apoplastic pH favors activity of acid invertase, which has a pH optimum between 4 and 5 (Roitsch & González, 2004). Thus, PM H^+-ATPase could be an important enhancer of sucrose utilization and sink activity, improving kernel development (Hütsch & Schubert, 2017, 2022; Jung et al., 2017).

Another important function of PM H^+-ATPase is the promotion of cell extension by apoplastic acidification, which was described by Cleland (1971) and Hager et al. (1971) with the acid-growth theory. The apoplastic pH decrease to values between 4.5 and 6, caused by ATPase activity, leads to an activation of expansins (Cosgrove, 2005), which are cell-wall loosening enzymes (McQueen-Mason & Cosgrove, 1994). Expansins are proposed to bind cellulose microfibrils and disrupt non-covalent bonds between polysaccharides in the cell wall (Cosgrove, 2005). After disruption of bonds, a turgor-driven displacement and reassociation of cell wall components can occur and cell extension is initiated (Yennawar et al., 2006). The required turgor is also enhanced by the PM H^+-ATPase-driven proton gradient. The hyperpolarized PM leads to the opening of inward-rectifying K^+ channels, resulting in K^+ and water uptake into the cells, which increases the turgor (Hedrich et al., 1995). This cell extension is not restricted to vegetative growth; β-expansins can also promote sexual reproduction by enhancing pollen tube growth to enable better pollination (Cosgrove et al., 1997). Taken together, PM H^+-ATPase is an important promoter of kernel development by enhancing sucrose hydrolysis and sink activity, hexose uptake into the kernel cytoplasm, and endosperm expansion by promoting cell extension.

The PM H^+-ATPase is regulated by various factors including light, phytotoxins, stress, and hormones (Serrano, 1989). The present study focuses on the effect of the phytohormone auxin on kernel development. Auxin was first identified in 1934 as indole-3-acetic acid (IAA) (Kögl et al., 1934), which is the most abundant endogenous auxin in plants (Simon & Petrášek, 2011). Translocation of auxin takes place in the phloem by mass flow, or via polar transporters through membranes (Zazímalová et al., 2010). The latter mechanism is unique in phytohormone transport, as only auxin has the ability to be transported that way (Zazímalová et al., 2010). Because of the low apoplastic pH, auxin is protonated under normal conditions and thus able to diffuse through membranes into the cytoplasm. Intracellular auxin is mostly deprotonated and its efflux depends on the PIN-FORMED (PIN) carrier proteins and ATP-binding Cassette Subfamily B transporters (Cho & Cho, 2013; Gälweiler et al., 1998). Due to a well-organized synthesis and polarity of transporters, auxin transport is highly coordinated allowing the hormone to act in specific cells (Friml, 2003). Thus, the resulting auxin gradients may regulate cell and plant development (Tanaka et al., 2006). The PIN-mediated auxin transport is also crucial for correct kernel development in maize, regarding embryogenesis and endosperm development (Forestan & Varotto, 2010).

Auxin is known to enhance PM H^+-ATPase activity (e.g., Cleland, 1995; Falhof et al., 2016; Hager et al., 1971, 1991; Jahn et al., 1996). Furthermore, ATPases can be activated by 14-3-3 proteins (Fuglsang et al., 1999). Since 14-3-3 proteins are phospho-binding regulators, the ATPase has to be phosphorylated at its threonine residue at the C-terminal end in order to get activated. Phosphatase-mediated changes in PM H^+-ATPase phosphorylation were identified as important determinants of auxin-regulated growth (Haruta et al., 2015).

Phosphorylation of Thr-947 was stimulated in response to IAA (Takahashi et al., 2012). Spartz et al. (2014) proposed a mechanism for auxin-mediated PM H^+-ATPase activation involving SAUR proteins. *SAUR* genes represent the largest family of auxin-responsive genes, and several are rapidly induced following auxin treatment (Ren & Gray, 2015). SAUR overexpression results in both increased PM H^+-ATPase activity and Thr-947 phosphorylation. These effects seem to be mediated by SAUR regulation of a family of protein phosphatases that dephosphorylate Thr-947; SAURs inhibit their activity. If the phosphatase is inactivated, the PM H^+-ATPase remains in its phosphorylated form allowing for 14-3-3 binding and enzyme activation (Spartz et al., 2014). Other possible mechanisms by which auxin enhances proton flux into the apoplast could be higher expression of PM H^+-ATPase genes (Frías et al., 1996) or inhibition of PM H^+-ATPase endocytosis (Paciorek et al., 2005).

The aim of the present study was to support kernel setting and development and thus presumably increase grain yield of maize by enhancing sink activity and cell extension growth. The key enzyme, which is presumed to promote both, is the PM H^+-ATPase. To enhance the activity of PM H^+-ATPase, the phytohormone auxin (IAA) was applied on maize plants in vivo. Because kernel number at maturity is mainly determined at time around pollination (Gustin et al., 2018), IAA was first applied 2 days before pollination and second on the pollination day. Our first hypothesis is as follows: (1) Auxin application has a positive impact on PM H^+-ATPase activity, which was tested by measuring hydrolytic activity of PM H^+-ATPase and H^+-pumping activity in membranes from maize kernels harvested 2 days after controlled pollination (2 DAP). In addition, the way in which PM H^+-ATPase activity is regulated was investigated. Thus, we second hypothesize that (2) auxin increases PM H^+-ATPase activity via a direct hormone–enzyme interaction. For this purpose, IAA was applied in vitro to isolated PM vesicles of formerly untreated maize plants. Gene expression and signal transduction are absent in those vesicles due to their specific isolation. Another possibility, how ATPase activity could be increased, is the upregulation of ATPase synthesis, leading to our third hypothesis: (3) Auxin increases the abundance of PM H^+-ATPase in developing maize kernels. This was tested by quantification of the PM H^+-ATPase enzyme via western blot analysis of the PM vesicles. Finally, we hypothesize that (4) after auxin application at flowering, kernel setting is improved leading to an increase in grain yield.

2 | MATERIALS AND METHODS

2.1 | Plant cultivation and harvests

The study was conducted in a vegetation hall at the experimental station of the Institute of Plant Nutrition in Giessen, Germany, near natural conditions during the vegetation period of 2020. A soil-culture experiment was set up in large 120-L plastic containers (140 kg soil, depth: 80 cm) as described in detail in Hütsch and Schubert (2021). On May 25, 2020, maize (*Zea mays* L cv. Pioneer 3906) was sown with nine seeds per container, and 10 days later the number of plants was reduced to four per container. Water content was adjusted to 60% maximum water-holding capacity during the entire vegetation period by water applications at least twice daily, and water supply was recorded for each container. The containers were set up in a completely randomized design and their position was changed at least once a week. Compound fertilizer was applied prior to sowing and during vegetation according to the requirement of maize plants grown with optimal nutrient supply (Hütsch & Schubert, 2021). The average daily temperature during the vegetation period ranged from 10 to 29°C with a mean of 20.9 ± 0.3°C. For better comparisons of grain yield per plant and of yield components, such as single kernel weight and kernel number per cob, tillers were removed immediately after appearance. Axillary branches did not develop. Seven plants produced a second cob on the main culm, which were not removed but degenerated after pollination of the uppermost major cob. Insecticides against *Oscinella frit* L., European corn borer, and aphids were applied when required.

Uncontrolled pollination was prevented by covering the developing cobs of all plants with parchment paper bags. The major cob of each plant was hand-pollinated with fresh pollen from control father plants 5 days after first silk appearance, which is considered to be the time with best receptivity (Cárcova et al., 2000). Just prior to pollination, silks were cut 1 cm above the tips of the husk leaves. The pollinated cobs were covered again for 1 week, until pollen shed in the vegetation hall was terminated. Two days before controlled pollination (August 3) and immediately after pollination (August 5, 0 days after pollination, DAP), plants of nine containers were treated with the auxin IAA. For this purpose, a 1.5 mM IAA solution plus 0.1% Tween-20 as surfactant was freshly prepared in deionized water, and 7.5 mL were sprayed evenly onto the entire surface (except the covered cobs) of the four plants per container (application rate per plant: 1.9 mL of 1.5 mM IAA). IAA was applied in the morning in order to ensure proper uptake by the maize plants and to minimize the risk of drying of the solution on the leaf surface.

On August 7 (2 DAP), an intermediate harvest of 10 containers (five controls and five with IAA treatment) was conducted according to Hütsch et al. (2015) with some variations. 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 at –80°C until further analyses. Therefore, measurements of enzyme activities and analyses of 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). Fresh weight of vegetative shoots, cobs, and kernels was recorded. Shoots were cut into small pieces and dried at 105°C to determine dry weights.

For control and IAA-treated plants, four containers with four maize plants each were harvested at physiological maturity on October 14 (142 days after sowing, DAS). Plant height, straw dry mass per container, cob dry mass per plant, kernel dry mass (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 for good homogenization, 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.

2.3 | Acid invertase enzyme extraction and activity measurements

Extraction of enzymes 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 with 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 a UV test kit (Boehringer Mannheim/R-Biopharm), and activity rates were calculated (μmol glucose g^{-1} fresh weight min^{-1}).

2.4 | PM 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). In contrast to Jung et al. (2017), the kernels were not ground in liquid N_2, but cut into small pieces and further chopped with a blender (Waring Commercial) during homogenization on ice.

2.4.1 | Isolation of PM vesicles, protein quantification, and vesicle purity

PM vesicles were isolated from kernel samples stored at –80°C. The material was homogenized according to Briskin and Poole (1983), De Michelis and Spanswick (1986), and Galtier et al. (1988), mixing 52 g maize kernels with 200 mL homogenization buffer. The homogenate was filtered, and during subsequent centrifugation the PMs accumulated in the pellet, which was solubilized by resuspension. To separate the PMs from the intracellular membranes, the suspension was added to a two-phase system with a polymer concentration of 6.1% (Hanstein et al., 2011; Zörb et al., 2005). The system consisted of a polyethylene glycol (PEG) phase, where PMs accumulate, and a dextran phase, where intracellular membranes accumulate. The microsomal PMs were purified from cytoplasmic components by further centrifugation steps and resuspension of the resulting pellets. Finally, aliquots of the purified vesicles were stored in liquid N_2 until further analyses. Here, they are ordered as right-side-out vesicles. Contrary to that, PM H^+-ATPase activity analyses were performed with inside-out vesicles, so they had to be turned by the surfactant Brij 58 in later steps.

In order to use the same amount of protein in each sample for the following analyses, the protein concentration of the isolated PM vesicles was determined using the Bradford Assay (Bradford, 1976). As the purified vesicles contain also other enzymes in addition to the PM H^+-ATPase, the purity of the vesicle samples was determined using specific ATPase and unspecific phosphatase inhibitors (Gallagher & Leonard, 1982; Hanstein et al., 2011; Yan et al., 1998). For this purpose, the hydrolytic activity was determined in an assay containing 5 mM ATP and 10 mM Mg by quantification of inorganic phosphate (P_i) that was released during the reaction time of 30 min at 30°C with a pH of 6.5. Prior to incubation, vesicle samples were diluted to a protein concentration of 0.05 $\mu g\ \mu L^{-1}$ with resuspension buffer. Each sample batch was prepared in triplicate. These were the standard conditions for measurements of hydrolytic activity unless otherwise stated.

2.4.2 | Hydrolytic PM H^+-ATPase activity and kinetic assay

The in vivo effect of IAA, when intact maize plants had been treated with this hormone, was determined in vitro at pH 6.5 as the optimum of the PM H^+-ATPase, and at the cytosolic pH 7.5. In addition, the effect of various concentrations of IAA (5, 10, 20, and 30 μM IAA) was tested in vitro by application to PM vesicles, derived from untreated control plants, prior to incubation at pH 6.5. 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 10 different ATP concentrations (0.25, 0.5, 0.75, 1, 1.5, 2, 3, 5, 6, and 7 mM ATP). For these kinetic studies, the vesicle samples were diluted to a protein concentration of 0.04 $\mu g\ \mu L^{-1}$ with resuspension buffer, and the assays contained an ATP regenerating system (pyruvate kinase/phosphoenolpyruvate). Kinetic curves were generated by plotting the hydrolytic activity data against ATP concentrations. For calculation of V_{max} and K_m, the Michaelis–Menten equation was transformed according to Hanes.

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). AO is an uncharged molecule, which enables diffusion into the vesicles. Through active PM H^+-ATPases, protons are pumped into the vesicles and AO is protonated. Caused by this charge, AO is trapped inside and the measured absorption

declines if PM H^+-ATPases are active. Addition of vesicle samples to the assay was adjusted to a protein amount of 80 μg. Proton transport was characterized using the following parameters: Active transport (influx) was quantified as initial rate during 1 and 2 min after addition of Mg-ATP. The maximum pH gradient was determined as the maximum quenching of absorbance (ΔA_{492}), and passive H^+ transport was determined as initial rate of H^+ efflux for 1 and 2 min after addition of vanadate, the specific inhibitor of PM H^+-ATPase.

2.4.4 | Western blot of isolated vesicles

Isolated PM vesicles were diluted to a protein concentration of 1.2 μg μL^{-1} using RIPA buffer (50 mM Tris/HCl, pH 7.4; 150 mM NaCl; 0.5% [w/v] Na-deoxycholate; 0.1% [w/v] SDS; 1% [v/v] Igepal CA-630; 1 mM EDTA; 10 mM NaF; 1 mM Na_3VO_4; 10 μg mL^{-1} aprotinin; 5 μg mL^{-1} leupeptin; 1 mM PMSF) and SDS buffer (250 mM Tris/HCl, pH 6.8; 10% [w/v] SDS; 40% [v/v] glycerol; 15% [v/v] β-mercaptoethanol; 0.1% [w/v] bromophenol blue). The samples were sonicated three times for 20 s each, and subsequently heated to 95°C for 5 min. SDS–PAGE was used to separate proteins based on their molecular weight. After the separation, the proteins were transferred from the gel onto polyvinylidene diflouride (PVDF) membranes, and unspecific binding sites on the membrane were blocked with 5% (w/v) nonfat dry milk in TBS-T (25 mM Tris [pH 7.4]; 137 mM NaCl; 5 mM KCl; 0.7 mM $CaCl_2$; 0.1 mM $MgCl_2$; 0.1% [v/v] Tween 20) for 1 h at room temperature. For the immunoblot, the PVDF membranes were incubated for 16 h at 4°C with 1:2000 diluted primary antibodies (anti-H^+-ATPase IgG-rabbit [AS07260, Agrisera] and anti-14-3-3 IgG-rabbit [AS122119, Agrisera] in 2% [w/v] nonfat dry milk in TBS-T). After primary antibody binding, the membranes were washed in TBS-T. Chemiluminescent signals, provided by the HRP conjugated secondary antibody (AS09602, Agrisera, 1:5000 dilution in 2% [w/v] nonfat dry milk in TBS-T), were detected with the ChemiDoc touch imaging system (Bio-Rad Laboratories) and the use of Western Lightning Plus ECL solution. Coomassie staining was performed for verifying equal protein loading in western blot analysis (Moritz, 2017). Western blotting was repeated in three independent analyses.

2.5 | Statistical analysis

Statistical analysis and data visualization were carried out using "Prism 9" (GraphPad Software). Means ± standard errors (SEM) were calculated from biological replicates as indicated in the figure legends ($n = 5$ and $n = 4$ per treatment for intermediate harvest and at maturity, respectively). The four plants per container were combined and considered as one biological replicate. Comparing two groups of treatments, an unpaired Student's t-test was used. For the statistical comparison of means from more than two independent variables, one-way ANOVA was performed. Statistical significance was referred to a p-value < 0.05 (ns = not significant $p > 0.05$, $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$). Outliers were detected and removed by a two-sided Grubb's test using RStudio (RStudio Inc.).

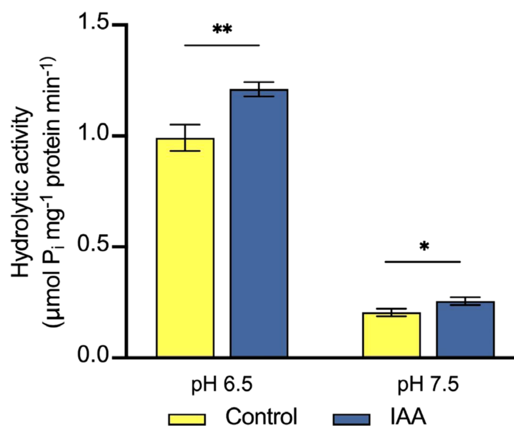

FIGURE 1 Effect of in vivo application of IAA on the in vitro hydrolytic activity of PM H^+-ATPase at two different pH values in the test medium. Maize plants were treated with 1.9 mL of 1.5 mM IAA 2 days before controlled pollination and on pollination day. PM vesicles were isolated from kernels harvested 2 DAP and hydrolytic activity of PM H^+-ATPases was analyzed by photometrical detection of P_i after addition of 5 mM ATP. The reaction took place over a period of 30 min at pH 6.5 and pH 7.5. Statistical analysis was performed using unpaired t-test; data are shown as means ± SEM ($n = 5$; $^*p \leq 0.05$, $^{**}p \leq 0.01$).

3 | RESULTS

Maize plants were harvested 2 DAP, and the effect of in vivo IAA application (at –2 and 0 DAP) on the activity of PM H^+-ATPase in developing kernels was determined using isolated PM vesicles. PM H^+-ATPase enzyme and 14-3-3 proteins were quantified to investigate possible changes in their protein levels due to in vivo IAA treatment. In addition, the effect of IAA application in vitro to isolated vesicles derived from formerly untreated control plants was tested. Furthermore, various sugars were quantified and acid invertase activity was measured in the developing kernels to evaluate the impact of IAA on sink strength. At physiological maturity, a second harvest was conducted to investigate the effects of IAA application on grain yield and its determinants.

3.1 | PM H^+-ATPase activity in developing maize kernels after in vivo application of IAA

3.1.1 | Hydrolytic activity and enzyme kinetics

In order to test whether IAA application to maize plants promotes PM H^+-ATPase activity, ATP hydrolysis was measured by photometric detection of P_i at pH 6.5 and 7.5. With both pH values, a significant increase in hydrolytic activity after IAA treatment was observed (Figure 1). As the hydrolytic activity was about four times higher at

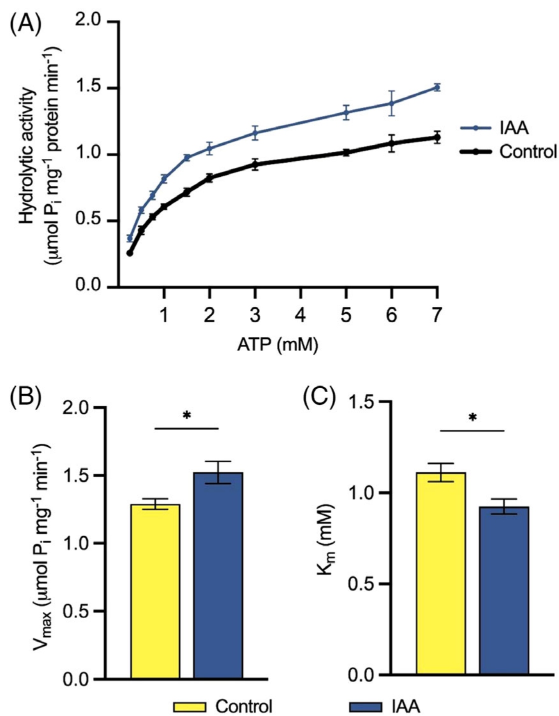

FIGURE 2 Effect of in vivo application of IAA on the in vitro PM H^+-ATPase kinetics. Maize plants were treated with 1.9 mL of 1.5 mM IAA 2 days before controlled pollination and on pollination day. PM vesicles were isolated from kernels harvested 2 DAP and hydrolytic activity of PM H^+-ATPases was analyzed by photometrical detection of P_i after addition of different ATP concentrations. The reaction took place over a period of 30 min at pH 6.5 (A). For calculation of V_{max} (B) and K_m (C), the Michaelis–Menten equation was transformed according to Hanes. Statistical analysis was performed using unpaired *t*-test; data are shown as means ± SEM (control: $n = 4$, IAA: $n = 5$; $^*p \leq 0.05$).

the optimum pH of 6.5 compared to pH 7.5 (Figure 1), all further measurements were performed at the optimum of pH 6.5.

In order to calculate the maximum reaction rate (V_{max}) and the Michaelis constant (K_m), which characterizes the enzyme–substrate affinity, hydrolytic activity was further measured at 10 different ATP concentrations. Every concentration showed a significant increase of PM H^+-ATPase activity after IAA treatment (Figure 2A). V_{max} was significantly increased by IAA treatment, whereas K_m was reduced (Figure 2B,C).

3.1.2 | Proton pumping activity (H^+ transport and pH gradient)

Another parameter for the analysis of PM H^+-ATPase activity is the H^+-pumping activity. The measurement allows a calculation of the maximum H^+ gradient between the intra- and extravesicular space, which results from ATPase activity. By addition of IAA to the maize plants, a significantly higher maximum H^+ gradient was observed (Figure 3C). Furthermore, influx and efflux rates of H^+ were investigated. H^+ influx and efflux were measured at two different intervals after ATP and vanadate addition, respectively. H^+ influx and efflux rates measured within 1 min as well as within 2 min were significantly increased due to IAA treatment (Figure 3A,B).

3.2 | Abundance of PM H^+-ATPase enzyme and 14-3-3 protein

To determine whether application of the auxin IAA to maize plants can enhance the abundance of PM H^+-ATPases in the kernels, western blot analysis was performed using the isolated vesicles. Additionally, the abundance of 14-3-3 proteins was measured. Absolute signal intensity and thus abundance of PM H^+-ATPases were significantly increased by IAA treatment, whereas 14-3-3 protein levels remained unchanged (Figure 4).

3.3 | PM H^+-ATPase activity after in vitro application of IAA

In addition to the previous measurements, hydrolytic activity was also determined when IAA was added in vitro to isolated PM vesicles derived from untreated control plants. This analysis was performed to determine whether IAA has a direct impact on PM H^+-ATPase activity, or whether gene expression or signaling pathways involving other proteins are presuming factors for an activity increase. The hydrolytic ATPase activity was unaffected by in vitro IAA treatment, independent of the IAA concentrations of 0–30 µM in the assay (Figure 5). Furthermore, the maximum H^+ gradient as well as the influx and efflux rates of H^+ showed no significant differences after in vitro IAA application (5, 10, 20, and 30 µM IAA in the assay) compared to the untreated vesicles (results not shown).

3.4 | Kernel sugar concentrations and acid invertase activity

It was presumed that an enhanced PM H^+-ATPase activity could lead to a higher sink activity. To examine this, acid invertase activity and concentrations of sucrose, D-glucose, and D-fructose were measured in the maize kernels at 2 DAP. The invertase activity measurement showed no significant difference between IAA-treated and untreated plants (Figure 6A). Sucrose concentrations were increased by 52% due to IAA application, whereas glucose and fructose concentrations did not show significant differences with respect to the control plants (Figure 6B,C,D).

3.5 | Straw yield, grain yield, and its determinants

At the intermediate harvest (2 DAP), IAA treatment of the maize plants showed no significant effects on plant height, straw fresh weight (FW),

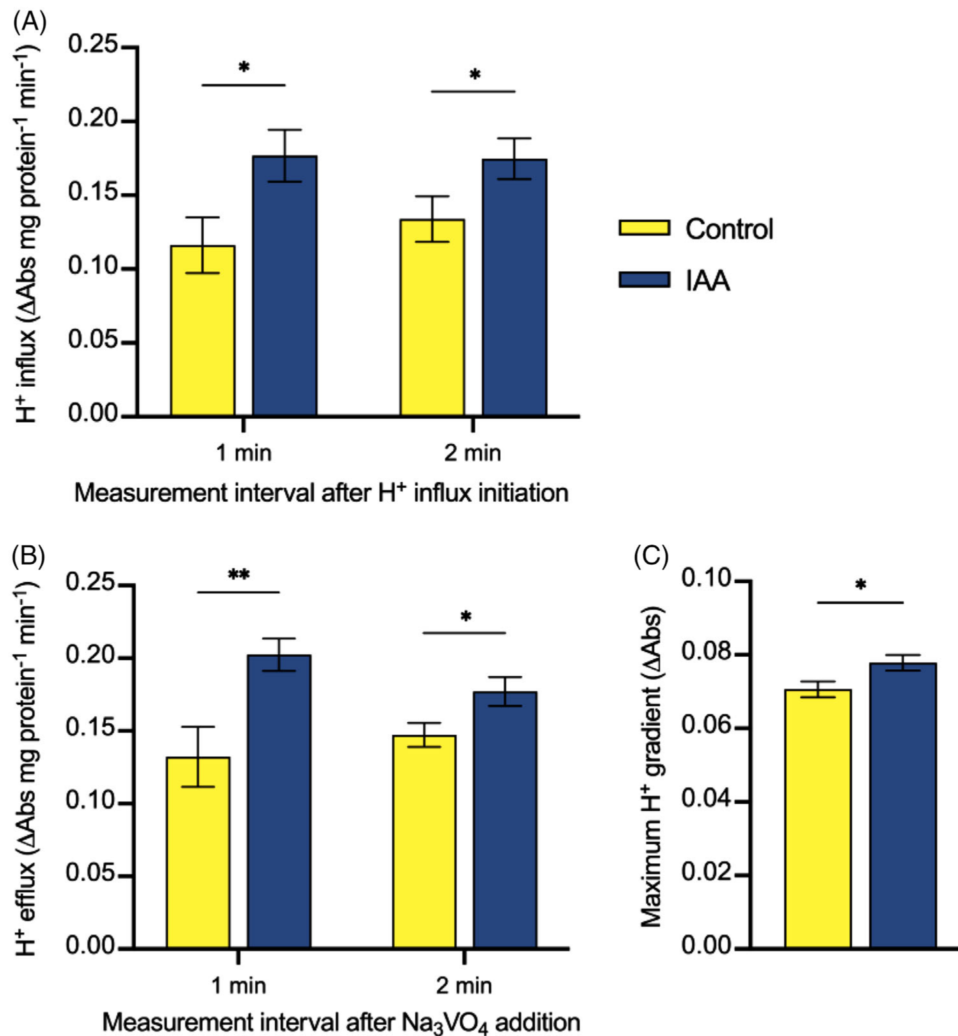

FIGURE 3 Effect of in vivo application of IAA on the in vitro H^+-pumping activity of PM H^+-ATPases. Maize plants were treated with 1.9 mL of 1.5 mM IAA 2 days before controlled pollination and on pollination day. PM vesicles were isolated from kernels harvested 2 DAP and H^+-pumping activity was measured by photometrical detection of AO outside the vesicles. H^+ influx was initiated by addition of ATP (A) and efflux was measured after addition of vanadate (B). H^+ fluxes were measured within 1 and 2 min after initiation. Maximum H^+ gradient was determined by Δ minimum and maximum absorbance at 492 nm (C). Statistical analysis was performed using unpaired *t*-test; data are shown as means ± SEM ($n = 5$; $*p \leq 0.05$, $**p \leq 0.01$).

cob FW, kernel FW, and silk FW as compared with the control plants (Table 1).

At kernel maturity, straw dry weight (DW) was significantly increased by 23% after in vivo IAA application (Figure 7A). In contrast, grain yield of IAA-treated plants was significantly reduced by 19% (Figure 7C). This grain yield depression was solely caused by a smaller kernel number per cob (decrease by 26% after IAA treatment; Figure 7), whereas single kernel weight was not significantly affected by IAA (Figure 7D). At maturity, between one and three barren cobs had developed at the same insertion as the main kernel-carrying cob on 50% of the IAA-treated plants. The untreated control plants produced just one main cob. In addition, intermittent kernel setting was observed after IAA application (Figure 8).

4 | DISCUSSIONS

4.1 | Effect of auxin application on PM H^+-ATPase activity

The auxin IAA was applied on maize plants during flowering in order to prevent kernel abortion and to reach a high kernel number and grain yield at maturity. It was hypothesized that IAA has a positive impact on enzymes that are critical for kernel development, particularly on PM H^+-ATPase activity. Different measurements of PM H^+-ATPase activity were performed using isolated PM vesicles from developing maize kernels harvested 2 DAP. The hydrolytic activity was significantly increased after in vivo IAA treatment of the plants (Figure 1). The

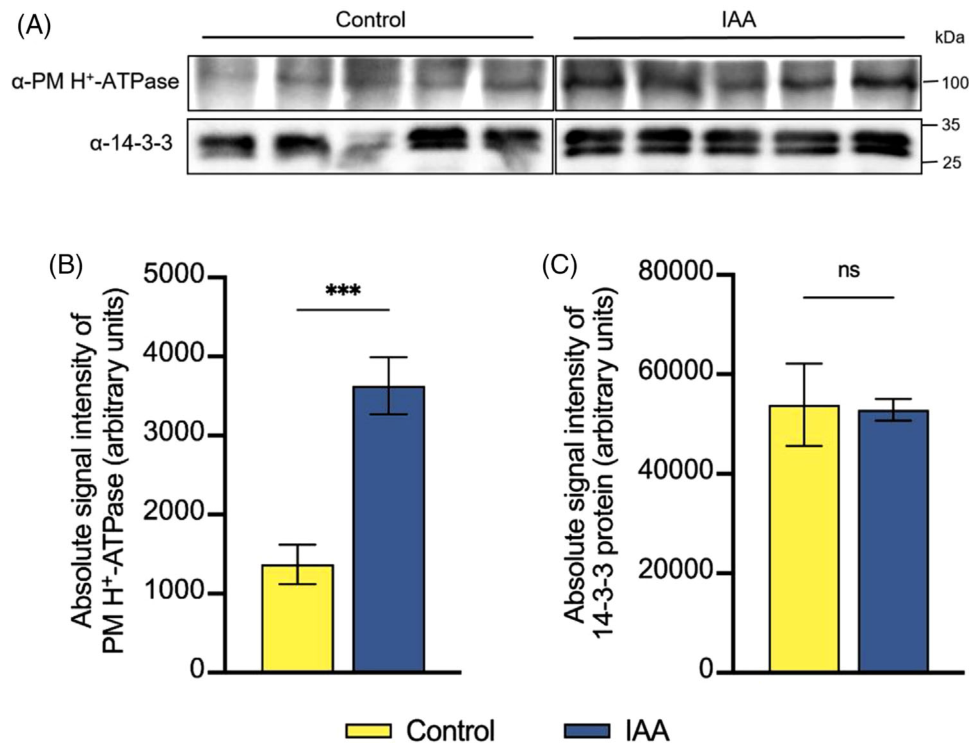

FIGURE 4 Effect of in vivo application of IAA on the abundance of H^+-ATPases and 14-3-3 proteins in the plasma membrane of maize kernels. Maize plants were treated with 1.9 mL of 1.5 mM IAA 2 days before controlled pollination and on pollination day. PM vesicles were isolated from kernels harvested 2 DAP and abundance of PM H^+-ATPase (A, B) and 14-3-3 protein (A, C) was determined via western blot analysis. Statistical analysis was performed using unpaired *t*-test; data are shown as means ± SEM ($n = 5$; ns = not significant $p > 0.05$, ***$p \leq 0.001$).

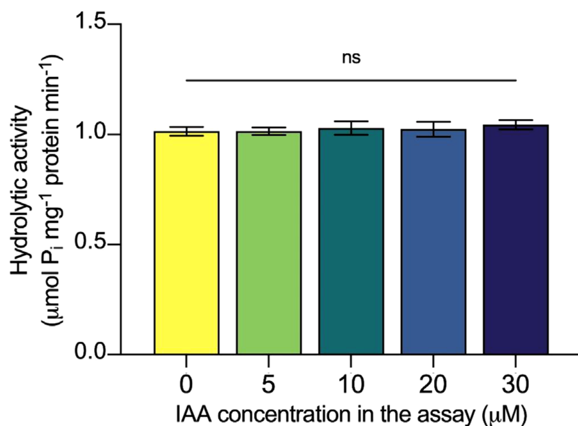

FIGURE 5 Effect of in vitro application of IAA on hydrolytic activity of PM H^+-ATPases. PM vesicles were isolated from maize kernels harvested 2 days after pollination (2 DAP); no IAA treatment of plants prior to harvest (controls). Hydrolytic activity of PM H^+-ATPases was analyzed by photometrical detection of P_i after addition of different IAA concentrations (shown are the concentrations in the assay) and 5 mM ATP. The reaction took place over a period of 30 min at pH 6.5. Statistical analysis was performed using one-way ANOVA; data are shown as means ± SEM ($n = 5$; ns = not significant $p > 0.05$).

kinetic study revealed a decreased K_m after IAA application, indicating a higher affinity of PM H^+-ATPase to its substrate ATP (Figure 2C). In addition to the increased hydrolytic activity and decreased K_m, a higher maximum reaction rate V_{max} also occurred (Figure 2B). Measurements of the H^+-pumping activity showed significant increases of the influx rates and the maximum H^+ gradient after in vivo IAA application (Figure 3A,C). These results are in accordance with the stimulatory effect of IAA on the hydrolytic activity (Figures 1 and 2): If more ATP is dephosphorylated by PM H^+-ATPases, more protons can be transported across the membrane resulting in higher H^+ gradients. H^+ efflux within the first and the second minute after vanadate addition was increased by IAA as well (Figure 3B). H^+ efflux was probably enhanced due to the higher H^+ gradient. If the electrochemical gradient between in- and outside of the vesicles is high, protons have a high force to diffuse out of the vesicles for balancing the gradient. According to the results of the present study, it is concluded that auxin application on maize plants leads to a higher activity of PM H^+-ATPases derived from developing maize kernels 2 days after pollination. Our first hypothesis can thus be accepted.

According to the currently available research articles in the literature, until now only our research group has been successful in isolating PM vesicles from young maize kernels (Jung et al., 2017), and in the present study the stimulatory effect of IAA treatment on H^+-ATPase activity of these vesicles is described. Previous investigations mainly focused on the impact of IAA on elongation growth of sunflower

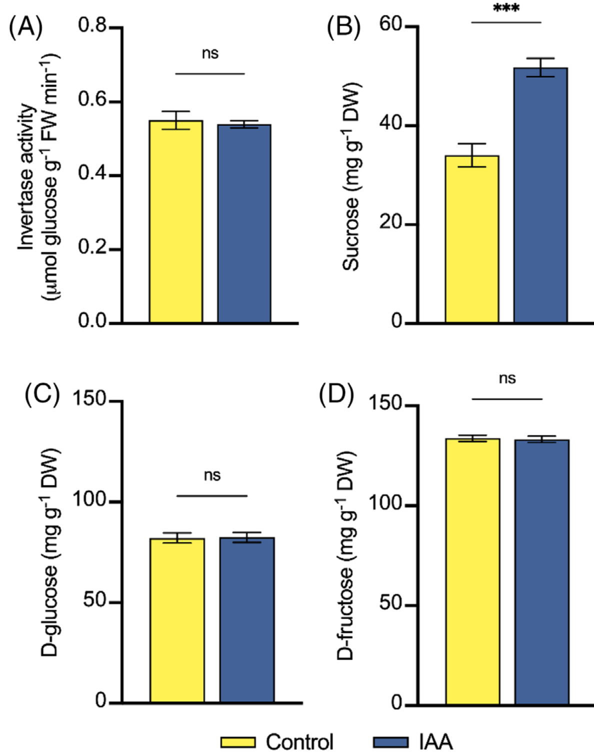

FIGURE 6 Effect of in vivo application of IAA on sugar concentrations and invertase activity in maize kernels. Maize plants were treated with 1.9 mL of 1.5 mM IAA 2 days before controlled pollination and on pollination day. Kernels were harvested 2 DAP and concentrations of sucrose (B), D-glucose (C), and D-fructose (D) as well as acid invertase activity (A) were determined. Statistical analysis was performed using unpaired *t*-test; data are shown as means ± SEM ($n = 5$; ns = not significant $p > 0.05$, ***$p \leq 0.001$).

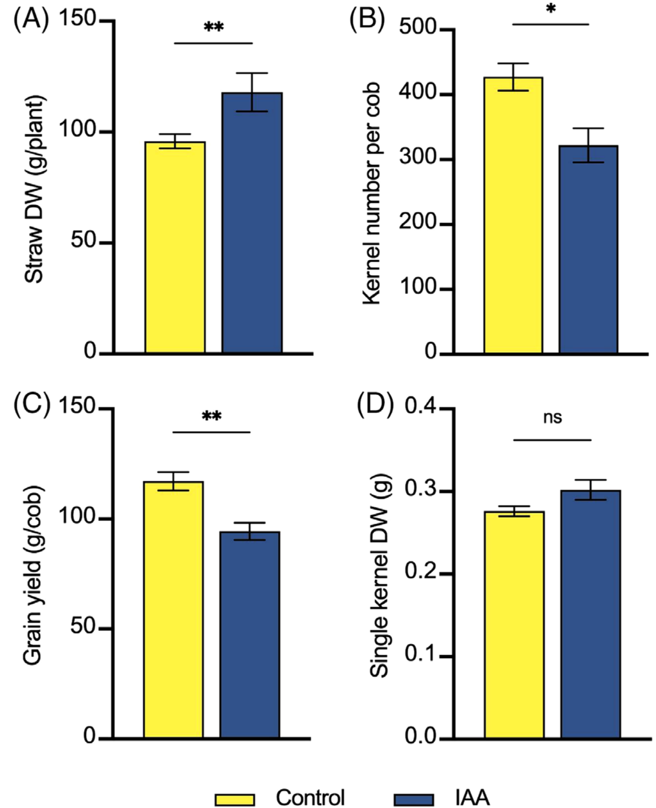

FIGURE 7 Effect of in vivo application of IAA on harvest parameters at kernel maturity. Maize plants were treated with 1.9 mL of 1.5 mM IAA 2 days before controlled pollination and on pollination day. Plants were harvested 54 DAP and subsequently dried. The weight of straw (A), kernel number per cob (B), grain yield (C), and single kernel weight (D) were determined. Statistical analysis was performed using unpaired *t*-test; data are shown as means ± SEM ($n = 4$; ns = not significant $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$).

hypocotyls (Hager, 2003; Hager et al., 1971), *Avena* hypocotyls (Cleland, 1995), or maize coleoptiles (Hager et al., 1991; Jahn et al., 1996; Kutschera & Schopfer, 1985; Kutschera et al., 1987; Polak & Karcz, 2021), whereas direct measurements of PM H^+-ATPase activity were hardly performed. Takahashi et al. (2012) found an activation of PM H^+-ATPase after IAA application to hypocotyl sections of *Arabidopsis*.

4.2 | Mode of action of auxin regulating PM H^+-ATPase activity

Having shown that auxin can enhance PM H^+-ATPase activity, it is still unclear, in which way auxin promotes the enzyme. It was tested whether IAA has a direct impact on the ATPase or whether a change in enzyme abundance occurs. The direct hormone–ATPase interaction was tested in a system that excludes gene expression and other signaling pathways by applying IAA to the isolated vesicles of untreated maize plants. Subsequently, hydrolytic activity and H^+-pumping activity were measured. Since no significant differences were found between treated and untreated vesicles (Figure 5), it is concluded that IAA is not able to work in that system. It is likely that other cellular components are required for ATPase regulation, which corresponds to previous studies on the pathways of auxin function.

TABLE 1 Plant height, straw fresh weight (FW), cob FW, kernel FW, and silk FW of maize cultivar Pioneer 3906 under control conditions and after in vivo application of IAA, determined at the intermediate harvest 2 DAP

	Plant height (cm)	Straw FW (g plant^{-1})	Cob FW (g plant^{-1})	Kernel FW (g plant^{-1})	Silk FW (g plant^{-1})
Control	253.5 ± 3.2	586.0 ± 15.2	72.3 ± 2.5	53.9 ± 1.5	5.94 ± 0.33
IAA	257.6 ± 3.9	619.6 ± 10.6	66.9 ± 3.3	50.2 ± 2.3	5.34 ± 0.21

Note: Plants were treated with 1.9 mL of 1.5 mM IAA 2 days before controlled pollination and on pollination day. Data show means of five replicates ± SEM ($n = 5$). Statistical analysis was performed using unpaired *t*-test, which did not show significant IAA effects ($p > 0.05$) for all given parameters.

FIGURE 8 Effect of in vivo application of the plant hormone indole-acetic acid (IAA) on cob development of maize cultivar Pioneer 3906 at maturity (at 142 days after sowing [DAS] and 70 days after last IAA application [DAA]; IAA dosage per plant: 1.9 mL of 1.5 mM IAA 2 days before pollination and on pollination day)

Fendrych et al. (2016) showed in *Arabidopsis* that cell extension did not occur when auxin was applied in combination with the protein synthesis inhibitor cycloheximide. These results indicate that protein synthesis is crucial for the auxin-mediated pathway of inducing cell extension. Edelmann and Schopfer (1989) obtained similar results in maize coleoptiles. Our second hypothesis that auxin is able to increase PM H^+-ATPase activity via direct hormone–enzyme interactions can therefore be rejected.

For further investigation of the mode of auxin action on PM H^+-ATPases, the enzyme abundance was quantified in the vesicles, showing increased ATPase levels after in vivo IAA treatment (Figure 4A,B). This fits to the previous observations, where an increase of hydrolytic activity and H^+-pumping activity after IAA treatment was measured (Figures 1 and 2). Other studies showed an increase in PM H^+-ATPase abundance after auxin application as well, first detected in maize coleoptiles by Hager et al. (1991). Frías et al. (1996) observed a threefold increase of the PM H^+-ATPase isoform MHA2 mRNA after auxin application to nonvascular parts of maize coleoptiles. Furthermore, this increase of ATPase gene expression was correlated with increased proton extrusion into the apoplast. Both studies support the results of the experiment in the current work; thus, concerning the measurements on developing maize kernels, our third hypothesis is accepted. Although there are strong indications that auxin-induced acid growth requires active transcription and translation (Arsuffi & Braybrock, 2018), contradictory results were also obtained (Cho & Hong, 1995; Jahn et al., 1996; Takahashi et al., 2012). Jahn et al. (1996) treated maize coleoptile segments in vivo with IAA, and found increased H^+-extrusion and elongation rates, whereas no increase in the amount of ATPase molecules was observed.

The abundance of the regulatory protein 14-3-3 was also quantified, as its binding is necessary for activation of the PM H^+-ATPase (Fuglsang et al., 1999; Haruta et al., 2015; Maudoux et al., 2000; Spartz et al., 2014; Takahashi et al., 2012). In contrast to the PM H^+-ATPase abundance, no differences in 14-3-3 protein levels between auxin-treated and untreated plants were observed (Figure 4A,C). This result could imply that ATPase abundance and 14-3-3 protein binding are not coupled. However, it must be taken into account that the used antibodies against 14-3-3 probably did not detect the PM H^+-ATPase bound 14-3-3 protein exclusively, but also further 14-3-3 proteins that regulate other enzymes of the vesicles. Proteins that are also regulated by 14-3-3 are, for example, K^+ channels (Sottocornola et al., 2006), which also occur in PMs and are consequently present in the isolated vesicles that were used for the western blot. Thus, also 14-3-3 proteins that were bound to K^+ channels or to other proteins were probably detected in the assay.

4.3 | Effect of auxin application on kernel development

Kernel number and thus grain yield are predominantly determined at flowering, when upon pollination and fertilization the availability of assimilates is decisive for kernel setting. After IAA treatment, kernel development was neither limited by sucrose availability at 2 DAP (no source limitation) nor by the supply of hexoses due to acid invertase activity (Figure 6). These results are in agreement with previous studies of sugar concentrations in kernels developed under stress conditions (Hütsch et al., 2015, 2020; Jung et al., 2017; Reed & Singletary, 1989). Despite sufficient sugar availability and an increased PM H^+-ATPase activity after in vivo IAA application on maize plants at flowering, the hormone treatment resulted in a significantly decreased kernel number causing grain yield

depressions at maturity (Figure 7). Thus, our fourth hypothesis "Auxin improves kernel setting leading to an increase in grain yield" must be rejected, as auxin rather promoted kernel abortion in this experiment (Figure 8).

However, the question remains why a distinct increase in PM H^+-ATPase activity due to IAA could not be translated into an improved kernel set. Shortly after pollination, developing cereal grains exhibit significant transient increases in two of the major plant hormones, auxins (IAA) and cytokinins (Locascio et al., 2014; Morris, 1997). In maize kernels, cytokinins (zeatin and zeatin riboside) showed maximal concentrations at an early stage (approximately 9–10 DAP), which coincided in time with the peak in endosperm cell division rate (Dietrich et al., 1995; Jameson & Song, 2016; Lur & Setter, 1993a, 1993b; Morris et al., 1993; Rijavec et al., 2011). The enzyme cytokinin oxidase increases shortly after the increase in active cytokinins and is responsible for decreasing cytokinins back to basal levels (Jones & Setter, 2000; Morris, 1997). In contrast, IAA concentration was low at 9 DAP and increased sharply thereafter (Lur & Setter, 1993a). By the time IAA begins to accumulate, cell division has ceased and growth occurs through cell expansion, an effect that is usually attributed to IAA (Jensen & Bandurski, 1994; Morris, 1997).

In addition to their absolute concentrations and timing of their accumulation, the cytokinin-to-auxin ratio and their interaction play an important role in regulating development (Lur & Setter, 1993b). The decrease in cytokinin concentration and the strong increase in auxin concentration at about 10 DAP created a sharp decline in the cytokinin-to-auxin ratio. This ratio is important for the regulation of several developmental processes, such as the start of starch and storage protein accumulation at about 10 DAP (Jones & Setter, 2000; Lending & Larkings, 1989; Ou-Lee & Setter, 1985). Gradients of hormones in the different seed compartments are generated and are important for communication. Correct seed development requires a coordinated cross talk between the seed tissues, and failure in phytohormone communication can cause seed abortion (Locascio et al., 2014).

In our study, the auxin application occurred probably too early during kernel development. As upon pollination the cytokinins play an initial key role, auxin application at this stage might have disturbed the phytohormone balance, causing disruption of cell division (shorter period of mitotic activity and generation of less endosperm cells; Ma et al., 2019) and a rather early onset of cell enlargement due to increased IAA concentrations. In fact, individual kernel weight was nonsignificantly increased by 11% due to IAA application (Figure 7D).

5 | CONCLUSIONS AND OUTLOOK

In the present study, it was shown that in vivo IAA application to maize plants at the time around pollination led to increased PM H^+-ATPase activity, measured in developing kernels harvested 2 DAP. However, this activation of H^+-ATPase did not result in an improved kernel setting, as at maturity the kernel number was reduced causing grain yield depressions. The underlying reason for decreased kernel setting despite enhanced ATPase activity is not yet clear, but it is probable that IAA application disturbed the well-regulated signaling system of plant hormones that is required for a correct kernel development. This regulation involves suitable concentrations of IAA within embryo and endosperm at defined growth stages, and suitable ratios to other phytohormones such as cytokinin. As a consequence, further studies should test later applications of IAA, considering naturally occurring concentration peaks in the kernels (e.g., at start of grain filling stage). Furthermore, applications of cytokinin at flowering should also be tested in order to enhance cell division at the time of kernel setting. In conclusion, there is strong evidence for positive effects of IAA on kernel development, but we still have to figure out how to take advantage of these effects to reach higher grain yields in maize.

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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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