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Reduced expression of selected *FASCICLIN-LIKE ARABINOGALACTAN PROTEIN* genes associates with the abortion of kernels in field crops of *Zea mays* (maize) and of Arabidopsis seeds

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Abstract

Abortion of fertilized ovaries at the tip of the ear can generate significant yield losses in maize crops. To investigate the mechanisms involved in this process, 2 maize hybrids were grown in field crops at 2 sowing densities and under 3 irrigation regimes (well-watered control, drought before pollination, and drought during pollination), in all possible combinations. Samples of ear tips were taken 2–6 days after synchronous hand pollination and used for the analysis of gene expression and sugars. Glucose and fructose levels increased in kernels with high abortion risk. Several *FASCICLIN-LIKE ARABINOGALACTAN PROTEIN* (*FLA*) genes showed negative correlation with abortion. The expression of *ZmFLA7* responded to drought only at the tip of the ear. The abundance of arabinogalactan protein (AGP) glycan epitopes decreased with drought and pharmacological treatments that reduce AGP activity enhanced the abortion of fertilized ovaries. Drought also reduced the expression of *AthFLA9* in the siliques of *Arabidopsis thaliana*. Gain- and loss-of-function mutants of Arabidopsis showed a negative correlation between *AthFLA9* and seed abortion. On the basis of gene expression patterns, pharmacological, and genetic evidence, we propose that stress-induced reductions in the expression of selected *FLA* genes enhance abortion of fertilized ovaries in maize and Arabidopsis.

KEYWORDS

corn, drought stress, FLA, seed abortion

1 | INTRODUCTION

In maize (*Zea mays*), grain yield increases have been mostly related to an improved number of harvestable kernels per unit land area, indicative of a predominant sink limitation to crop yield (Borrás, Slafer, & Otegui, 2004). Kernel abortion at the tip of the maize ear can be severe, particularly under stress conditions such as drought (Hall, Lemcoff, & Trápani, 1981; Westgate & Boyer, 1986) or high stand densities (Otegui, 1997; Vega, Andrade, Sadras, Uhart, & Valentinuz, 2001). One of the critical components of yield is the number of fertilized ovaries, which can be affected by pollen availability. However, abortion of fertilized ovaries positioned at the tip of the ear can occur even when fresh pollen is added to their late-appearing silks (Otegui, Andrade, & Suero, 1995; Oury, Tardieu, & Turc, 2016), indicating that factors other than pollen availability are responsible for setting kernel number in the field (Boyer & Westgate, 2004; Westgate & Boyer, 1986). The development of the kernels positioned at the tip of the ear is dominated by ovules positioned at the base of the ear, which normally become fertilized first (Cárcova & Otegui, 2007). Thus, the degree of synchronization of the pollination of ovaries with different position along the ear also affects kernel abortion (Freier, Vilella, & Hall, 1984; Cárcova & Otegui, 2001; Oury, Tardieu, et al., 2016).

The comparative analysis of the timing of either water stress and abortion (Boyer & Westgate, 2004) or pollination and abortion (Cárcova & Otegui, 2007) indicates that the fate of the kernel can be defined by signals perceived by the ovary, before the embryo or endosperm are present. Traditionally, reduced availability of carbohydrates at the ovaries has been considered a major trigger of kernel abortion (Boyer & Westgate, 2004; Boyle, Boyer, & Morgan, 1991; Ruan, Patrick, Bouzayen, Osorio, & Fernie, 2012). Sucrose is transported from the source leaves to the maize ear and unloaded from phloem at the ovary pedicel, where it becomes hydrolysed to glucose by wall-bound invertases (McLaughlin & Boyer, 2004a). Glucose accumulates in the upper section of the pedicel below the nucellus and is present at low concentrations in the nucellus, whereas starch accumulates around vascular pedicel tissues (McLaughlin & Boyer, 2004a). Low water potentials can reduce glucose and starch accumulation as well as cell wall invertase activity in the upper pedicel. It can also lead to a reduced expression of the cell wall invertase 1 and 2, soluble invertase 2, sucrose synthase 1 and 2 genes and also an increased expression of senescence-associated genes and ovary abortion (Zinselmeier, Jeong, & Boyer, 1999; Andersen et al., 2002; McLaughlin & Boyer, 2004b; Qin et al., 2004). Some of these effects are prevented by feeding sucrose to the stem (Boyle et al., 1991; McLaughlin & Boyer, 2004a; Zinselmeier et al., 1999). Based on these findings, abortion has been proposed to result from low leaf photosynthesis under water stress, which reduces sugar concentrations at the ovary and creates a starvation signal causing reduced activity of sugar metabolism enzymes and programmed cell death (Boyer & McLaughlin, 2007; Ruan et al., 2012).

However, several observations suggest the occurrence of additional control points. First, only some of the effects of low water potential are reversed by feeding sucrose to the stem (McLaughlin & Boyer, 2004a). Second, limiting leaf photosynthesis by neutral shading to the levels observed when water stress causes ovary abortion reduced ovary starch and glucose while failing to induce abortion (Hiyane, Hiyane, Tang, & Boyer, 2010). Third, kernel or cob reducing sugars, sucrose, and starch concentrations are not necessarily lowered within the time frame when shade or low water potentials cause kernel abortion (Reed & Singletary, 1989; Schussler & Westgate, 1991a, 1995; Cheng & Lur, 1996; Setter, Flannigan, & Melkonian, 2001; Andersen et al., 2002; Qin et al., 2004). Actually, during the first days of silking, drought may increase sucrose, glucose, and fructose levels while maintaining starch levels at the tip of the ear (Oury, Caldeira, et al., 2016). Fourth, hormones such as ethylene and abscisic acid have also been implicated in shade-induced (Cheng & Lur, 1996) and drought-induced (Reed & Singletary, 1989; Asch, Andersen, Jensen, & Mogensen, 2001; Andersen et al., 2002; Yu & Setter, 2003) abortion. Fifth, on the basis of the detailed kinetics analysis, Oury, Tardieu, et al. (2016) have proposed an integral model where ovary growth interruption by water stress is linked to the arrest of silk growth, which might impede pollen tube progression along the silk.

Several studies have addressed the effects of water stress on the transcriptome of maize kernels, 1 (Kakumanu et al., 2012), 5–9 (Yu & Setter, 2003), 10 (Marino et al., 2009), or 25–45 days after pollination (Luo, Liu, Lee, Scully, & Guo, 2010). Changes in the expression of genes involved in carbohydrate metabolism are consistent with a down-regulation of starch and sucrose biosynthesis and an up-regulation of starch and sucrose degradation in the ovaries of drought stressed plants 1 day after pollination (Kakumanu et al., 2012). The early ovary transcriptome is consistent with increased abscisic acid signalling, enhanced programmed cell death, senescence, and arrest of the cell cycle in response to drought (Kakumanu et al., 2012). Nine days after pollination, genes encoding heat shock proteins, chaperonins, and major intrinsic proteins are up-regulated in placenta, whereas genes involved in the cell division, growth, and cell wall degradation are down-regulated in endosperm in response to drought (Yu & Setter, 2003). At late stages (25–45 days after pollination), drought enhanced the expression of stress-related genes (e.g., abscisic acid response genes) and down-regulated defence-related genes (Luo et al., 2010). However, Oury, Caldeira, et al., (2016) harvested ovaries and silks at husk emergence and 5 days later and observed that the earliest changes occurred in silks rather than in ovaries, and involved genes affecting extension growth rather than sugar metabolism, which is consistent with a model where abortion is caused by premature silk arrest under stress (Oury, Tardieu, et al., 2016).

In order to gain insight into the mechanisms involved in the control of kernel abortion in maize, we examined transcriptome patterns and sugar levels of the tip of the ear, where fertilized ovules might be aborted, at early stages (2–6 days after pollination). The assay included all the possible combinations of three irrigation protocols, two maize genotypes, and two stand densities. The results provided correlative evidence for a role of selected *FASCICLIN-LIKE ARABINOGALACTAN PROTEIN* (*FLA*) genes in seed abortion. This hypothesis was tested by a pharmacological approach in maize and genetic approaches in *Arabidopsis thaliana*.

2 | MATERIALS AND METHODS

2.1 | Plant material, growth conditions, and design of Experiment 1

Single cross maize hybrids NK880 and NK900 from Syngenta (both carrying the Bt transgene and of almost identical cycle duration up to anthesis) were sown at two stand densities (6 and 10 plants m^{-2}) at the experimental field of the Faculty of Agronomy of the University of Buenos Aires (34°25′S, 58°25′W), Argentina, in December 2005. The experiment was conducted in plots fitted with a separately controlled set of drip irrigation lines. The plots contained a 180-cm-deep layer of a silty clay loam soil (Aeric Argiudol, USDA taxonomy) resting on native loess (C). Each plot was isolated from contiguous plots and the surrounding area by 2-m-deep 500-mm polyethylene film barriers to impede lateral flow of water. The plots were protected from rain by an automatic rain shelter. The clear polycarbonate roof of the shelter rolled over the plots as soon as the rainfall sensor was wetted and opened again when the sensor dried. Plots were over sown manually to ensure the desired stand densities. Plants were arranged in rows placed 50 cm apart. Each plot covered an area of approximately 25 m^2, divided in four equal subplots. The crop was maintained free of weeds and sanitary problems, fertilized with 10 g N m^{-2} at sowing and at V6 (Ritchie, Hanway, Benson, & Herman, 1993), and watered as needed.

The experiment was arranged in a split-split plot design where main plots were assigned to drought treatments (three plots per condition), which included a well-irrigated control condition, an early drought treatment (i.e., drought during presilking ear growth; Otegui & Bonhomme, 1998), and a late drought treatment (i.e., drought during anthesis and silk exposure). Each plot contained four subplots, which resulted from the combination of genotype and stand density. The sub-sub plots corresponded to sampling time. The tip of the ear was harvested 2, 4, and 6 days after pollination. These sampling times were chosen because 8 days after pollination, the signals of abortion are already evident in terms of grain size and morphology (Westgate & Boyer, 1986).

The experiment included 3 irrigation conditions × 3 replicates × 2 stand densities × 2 cultivars × 3 sampling times; that is, 108 samples processed independently to quantify biological variation. For each sample, the tips of at least three ears were pooled to obtain representative data. All samples were collected at midday to avoid interference with changes in expression due to diurnal rhythms. Each tip was divided in three sections, one used for the analysis of the transcriptome, another used for the carbohydrate determinations, and the third reserved and used for RNA extraction if needed due to low yield of the first subsample. To determine the number of aborted kernels in the upper third of the ear, six plants (each one surrounded by intact plants spaced at the specified plant population density) were harvested from the central rows of each subplot (i.e., for each one of the three replicates of each watering × stand density × genotype combination) at the stage of physiological maturity (black layer maturity).

In the early drought treatment, watering was interrupted 26 days after sowing, and the first signs of wilting were detected 18 days later, when the spikelets corresponding to the upper 40% of representative apical ears were at early stages of development. The treatment ended 5 days after the beginning of silking. At this time, the stigmas (silks) of the upper third of the ear in the watered controls were already emerged. By the time the stigmas of the upper third of the ear were emerging in the early drought treatment, the plants were already well watered. The rationale behind this treatment is that drought periods during the formation of the ovules can leave no obvious morphological indication of the stress event and yet can affect the fate of the ovaries after pollination (Chimenti, Marcantonio, & Hall, 2006; Westgate & Boyer, 1986). Late drought started 44 days after sowing, and the first signs of wilting were detected 17 days later, before the stigmas of the upper third of the ear were visible and ended at the start of active grain filling (Otegui & Bonhomme, 1998). In both case,s the duration of water shortage was 37 days. The leaf water potential at sunset was recorded in six plants (three plants per plot from two pots) per condition with a pressure bomb.

In order to avoid shortage of pollen due to a delay in ear development in response to drought treatments, an irrigated plot with plants sown 10 days after the date of sowing of the actual experiment was included. Pollination was made by hand between 10 in the morning and 12 (noon) with fresh pollen collected from neighbour plants immediately before pollination. Ears were covered before silking with white paper bags. All silks exposed from these ears were hand pollinated synchronously on Day 5 after the beginning of silking (i.e,. ovules positioned either at the top or at the base received pollen simultaneously). This procedure was chosen to minimize the inhibitory effect of early-fertilized ovaries from the base on the development of late-fertilized ovaries from the tip, with the concomitant reduction in kernel abortion among the latter (Cárcova, Uribelarrea, Borrás, Otegui, & Westgate, 2000). Silk receptivity lasts 6–7 days after being exposed from the husks (Bassetti & Westgate, 1993).

Only the ovaries with at least minimal signals of postpollination growth were included in the count of aborted kernels. The number of unfertilized ovaries was minimized by the application of simultaneous pollination because delaying the pollination of basal grains favours the growth and emergence of silks of the upper ovaries (Bassetti & Westgate, 1993; Cárcova & Otegui, 2007). Because the growth of the silks is arrested simultaneously (Oury, Tardieu, et al., 2016) and pollination was done simultaneously, it is clear that all silks had the minimum time required after pollination to complete fecundation (Oury, Tardieu, et al., 2016). In the early harvests used for microarray analysis, no silks attached to the ovaries were evident, indicating successful pollination (Nielsen, 2016).

2.2 | Plant material, growth conditions, and design of Experiment 2

The hybrid AX886 was sown in 70-L pots containing 70% sand and 30% peat in December 2011. Seedlings were thinned to one per plot and fertilized with 10 g N m^{-2} at V3. The drought treatment started 20 days before anthesis and continued until harvest. Drought-treated plants were irrigated with 15% of the demand (potential evapotranspiration) whereas the controls received 100%. Pollination was as described for Experiment 2. We harvested the kernels at the upper (tip), central, and lower portions of the ear on 2, 4, and 6 days after

pollination to measure the expression of *FLA* genes by real-time PCR and the abundance of AGP in protein blots.

2.3 | Plant material, growth conditions and design of Experiment 3

Single cross maize hybrids AX852 and AX886 from Nidera (both carrying the Bt transgene and of similar cycle duration up to anthesis) were sown at 5 plants m^{-2} at the experimental field of Faculty of Agronomy of the University of Buenos Aires, on a silty clay loam soil (Vertic Argiudol, USDA taxonomy), in December 2008. The experiment was kept free of weeds and pests, fertilized with 10 g N m^{-2} at V3 and drip irrigated. For synchronized pollination, the ears were bagged before silking and unbagged 4 days after silking to receive pollen naturally. These plants were used to localize the presence of AGP in maize kernels and to test pharmacologically the involvement of AGP in the control of kernel abortion.

2.4 | Plant material and growth conditions of experiments with *A. thaliana*

We used plants of *A. thaliana* accession Columbia and of the *fla9-1* (SALK_051688C) and *fla9-2* (SALK_088265) mutants (mutations confirmed by sequencing Figure S1). Seeds were sown on agar in plastic boxes, stratified, and transferred to continuous white light 160 µmol m^{-2} s^{-1} (400–700 nm, Li-188B sensor; LI-COR) provided by fluorescent tubes (Philips) at 22 °C. Five-day-old seedlings were transplanted to 70-cm^3 pots containing equal amounts of perlite (Perlome), peat moss (Finca Don Calvino), and vermiculite (Intersum) and watered as needed with a solution containing 1 g L^{-1} Hakaphos R (Compos). We quantified the number of aborted, unfertilized, and viable seeds under magnifying glass in mature siliques (siliques that had reached maximum length and changed from green to yellow colour at the extremes) sampled from the bottom, medium, and top sections of the inflorescence (five per section). The rate of abortion was calculated as the ratio between aborted seeds and the sum of aborted ovules and viable seeds.

In the experiments with drought treatments, the pots were watered at field capacity until bolting. Then, the substrate was maintained either at 0.3 or at 1.0 of field capacity for the drought and control conditions, respectively. Plants were watered every 3–5 d, that is, the time required for the evaporation of 0.3 of the water volume contained in a beaker.

2.5 | Analysis of carbohydrates

The determination of carbohydrates is based on published protocols (Quaggiotti, Trentin, Dalla Vecchia, & Ghisi, 2004). The frozen tissues of the upper third of the ears were ground in 5-ml liquid nitrogen and extracted with methanol/chlorophorm/20-nM Hepes-KOH (3/1.5/0.5 *v*/v/v) pH 8.5 for 30 min at 4 °C. After adding 3 ml of distilled water and centrifuging 5 min at 3000 *g*, the upper (methanol-aqueous) phase was dried in a rotary evaporator at 40 °C and re-suspended in 2 ml distilled water for the determination of soluble sugars by high-performance liquid chromatography (HPLC). The insoluble pellet was re-suspended in 0.8-ml KOH 0.2 M, boiled for 30 min, allowed to cool down and then incubated 3 hr at 55 °C in 0.5-ml sodium acetate (pH 4.8) containing α-amiloglucosidase from *Aspergillus niger* (Sigma). The reaction was stopped by heating at 95 °C for 1 min and used for HPLC determinations of glucose produced by the hydrolysis of starch. HPLC was performed on an Agilent 1100 with an Agilent Zorbax Carbohydrate Analysis column, detector of refraction index, and a flow of 1.4 ml/min of Acetonitrile: water (75:25) at 30 °C.

2.6 | Analysis of the transcriptome

RNA was extracted from the upper third of the ears using an RNeasy Plant Mini Kit (Quiagen). RNA quality was assessed by gel electrophoresis. The hybridization images were obtained and processed by Affymetrix' MAS 5.0. Hybridization signals of perfect matched probes of a set were condensed into a single index based on 72 percentile using a custom script. The condensed data were globally normalized/scaled so that the average probe set signal intensity of all arrays equals to 100.

The statistical analysis was restricted to 76,678 genes showing the presence call of the Affymetrix software in the three replicate samples of at least one treatment condition (5,322 genes were eliminated from further analysis). Data were log-transformed and analysed by analysis of variance (ANOVA) following a split-split plot design. The first level of this analysis corresponded to the different watering treatments (control, early drought, and late drought); the second level corresponded to the factorial combination of genotype (NK880 or NK900) and plant density (high or low); and the third level to the time after pollination (2, 4, or 6 days) and all the interactions with time after pollination. For each gene, the analysis yielded 9 *p* values that were taken into account for subsequent analysis: water condition, stand density, genotype, genotype × stand density interaction, water condition × time interaction, water condition × stand density-genotype interaction, water condition × time interaction, stand density-genotype × time interaction, and water condition × stand density-genotype × time interaction. We transformed these *p* values of the ANOVA into *q* values, which are a measure of significance in terms of the false discovery rate (Storey & Tibshirani, 2003). We selected 1,322 genes with a *q* value <0.05 for at least one of the nine tests. The analysis also yielded genes showing significant effects only for time after pollination. These genes are not relevant to the analysis of the impact of water conditions, genotype, and/or stand density and are therefore excluded from the subsequent analysis. To analyse the correlation between gene expression and abortion, we used the multiple linear regression analysis tool of InfoStat. The average number of aborted kernels per ear for each treatment (12 treatments) was used as independent variable, and the expression data of each gene on 2, 4, and 6 days after pollination were used as independent variables for multiple regression. We identified 218 genes with *p* value <.05, which corresponds to a *q* value <0.18.

2.7 | Real-time RT-PCR

Maize kernels were harvested in liquid nitrogen, total RNA was extracted with TRIzol (Invitrogen). Arabidopsis siliques were harvested 3, 6, 9, and 12 days after hand pollination. cDNA derived from this RNA

was synthesized using Invitrogen SuperScript III and an oligo-dT primer. We used a 7500 Real Time PCR System (Applied Biosystem). The primers used for *ZmFLA7* were ACAGCCTCGTCGTCTACCAG (forward) and GCGTCTTGCCCTTCTTCTTGG (reverse). For *ZmFLA8* (used as a negative control), the primers were CTTCCCGTTCGCCGTCTACTC (forward) and ACATCGTCGCCGCTCTTTGG (reverse). For *ZmACTIN*, used to standardize expression, the primers were CGGTTTCGCTGGTGATGATGC (forward) and GCTCGTTGTAGAAGGTGTGATGC (reverse). The primers used for *AthFLA9* (*AT1G03870*) were TGCGAACACCGTCATACC (reverse) and GGCTACCACTCGTCTAACTC (forward). For *AthUBC* (*AT5G25760*) were TTGTGCCATTGAATTGAACCC (reverse) and CTGCGACTCAG^GGAATCTTCTAA (foward). For *AthATPXG2* (*AT5G55240*) were TACCAGCAGCCAAGATAAGC (reverse) and GCCGAGAGCACYACAAGC (forward). For *AthCAB2* (*AT1G29920*) were TGGCTTGGCAACAGTCTTCC (reverse) and CCTCAACAATGGCTCTCTCCTC (forward).

2.8 | Protein blot analysis

A total of 100 mg of dry milled maize kernels were extracted for 10 min with 200 µl of 50-mM Tris-HCl, pH 7.4, containing 1-mM ethylenediaminetetraacetic acid, 1 mM DTT, and protease inhibitor cocktail (Sigma). After extraction, the mixture was centrifuged, a volume of the supernatant containing 30 µg of protein was mixed with 3X running electrophoresis buffer (60-mM Tris-Cl, pH 6.8, containing 2% SDS, 30% Glycerol, 15% β-mercaptoethanol, 3-mM DTT, 0.2% bromophenol blue) and loaded on 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Gels were transferred to nitrocellulose membranes and stained with Ponceau Red. Ponceau Red stain was washed away, and the same membranes were probed with specific anti-AGP antibodies (JIM13 and JIM16, Yates et al., 1996; Knox, Linstead, Cooper, & Roberts, 2001). Primary antibodies were used at 1:150 dilution and secondary anti-rat HRP at 1:1,000, and bands were visualized with chemiluminescence reaction (Super-Signal; Pierce Chemical, Rockford, IL, USA). Intensity signal was quantified in the nitrocellulose membranes using GelPro32 software, and the images were processed with Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA, USA).

2.9 | Treatments with Yariv reagents

β-Glucosyl Yariv reagent [1,3,5-tris (4-β-D-glycopyranosyloxyphenylazo)-2,4,6-trihydroxybenzene] is a red dye that specifically binds AGPs, whereas α-Mannosyl Yariv [1,3,5-tris (4-α-D-mannopyranosyloxyphenylazo)-2,4,6-trihydroxybenzene] does not recognize AGPs and can be used as a negative control (Yariv, Lis, & Katchalski, 1967). α-Mannosyl or β-Glucosyl Yariv reagents were synthesized as described (Yariv, Rapport, & Graf, 1962). Binding of α-Mannosyl or β-Glucosyl Yariv reagents were tested against gum Arabic AGPs (Sigma) as dot blots in nitrocellulose membranes. Lower detection limit was around 100 ng of AGPs incubated with 500 µg/ml of β-D-glucosyl Yariv in 1% NaCl.

To access the fertilized ovaries for Yariv reagent treatments, on 4 days after pollination, we cut two windows on the husks, one at each side of the ear. A gauze bandage soaked in 100 µM of either α-Mannosyl or β-Glucosyl Yariv was placed on the fertilized ovules. The windows were closed, and the number of aborted kernels counted 20 days later. To evaluate the impact of the disturbance, other plants were exposed to a mock treatment (distilled water rather than Yariv reagents) and compared to control plants where no window was cut.

2.10 | AGP localization with Yariv

β-Glucosyl Yariv phenyl glycoside was used to localize total AGPs, and α-Mannosyl Yariv phenyl glycoside was used as a control (Yariv et al., 1962). Sections were incubated overnight at 4 °C in 0.5% in NaCl (*w*/*v*, two times) 500-µg/ml Yariv solutions. Sections (5–7 µm) were mounted on glass slides and then observed with a Carl Zeiss Axiolab light microscope (Carl Zeiss, Jena, Germany).

2.11 | Microscopy

Flowers of Arabidopsis were identified by means of colour threads at pollination. After harvest, the siliques were incubated 24 hours in Chloride Hydrate and then ethanol, where they were cut to expose the fertilized ovaries. The ovaries were mounted on slides and photographed in a Zeiss Axiostrar Plus microscope (Carl Zeiss, Oberkochen, Germany).

3 | RESULTS

3.1 | Effects of drought and stand density stress on kernel abortion in two maize hybrids

The aim of the first experiment was to characterize the transcriptome and sugar levels at the tip of the ear of maize plants on 2, 4, and 6 days after pollination, and the number of aborted kernels at maturity. Two hybrids (NK900 and NK880) from the same maturation zone, but with different rates of tip abortion under normal field growth conditions, were grown in the field at two stand densities (6 and 10 plants m^{-2}) under an automatic rain shelter and three irrigation regimes; a well-irrigated control condition, an early drought treatment (i.e., drought during presilking ear growth; Otegui & Bonhomme, 1998), and a late drought treatment (i.e., drought during anthesis and silk exposure). All possible combinations of genotype, stand density, and water availability were included in a split-split plot design. Reduced watering caused a gradual decay in leaf water potential reaching a minimum of −1.3 and −2.1 MPa in late and early drought, respectively (Figure S2), which is typical of field-grown plants. The ears were hand pollinated synchronously on Day 5 after the beginning of silking to minimize the inhibitory effect of early-fertilized ovaries on the development of late-fertilized ovaries.

At harvest, kernel abortion was observed at the tip of the ear (Figure 1a). The number of aborted kernels per ear increased with drought treatments and with the high stand density and was higher in hybrid NK900 compared to hybrid NK880 (Figure 1b). There was a significant interaction between genotype and water availability ($p = .01$), as hybrids NK880 and NK900 responded differently to either early or late drought, respectively. There was significant interaction between stand density and water availability ($p < .0001$), as late

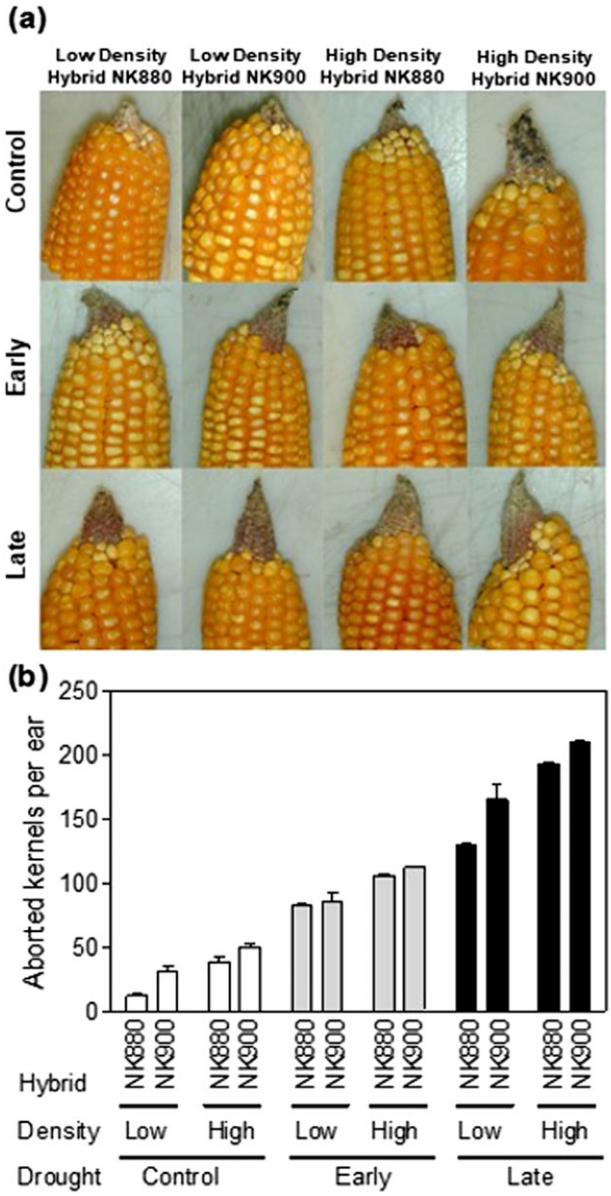

FIGURE 1 Effects of water deficit, stand density, and genotype on the abortion of fertilized ovaries at the tip of the ear in maize crops. Plants of the maize Syngenta hybrids NK880 and NK900 were grown at high (10 plants m^{-2}) or low (6 plants m^{-2}) stand densities under well-irrigated conditions or exposed to drought either before pollination (early drought) or during pollination (late drought). (a) Representative ear tips. (b) Number of aborted kernels (mean ± SE). Data from Experiment 1

drought was particularly effective to induce abortion at high stand densities. The treatments produced a wide range of abortion intensities (Figure 1b).

3.2 | Kernels with higher risk of abortion tend to accumulate sugars early after pollination

On 2, 4, and 6 days after pollination, we harvested ears of randomly chosen plants within the same plots used to score abortion and subsequently measured their levels of glucose, fructose, and starch (Table S1). The levels of starch and those of glucose and fructose showed no significant effects of the treatments 2 and 4 days after pollination. Beyond a threshold of approximately 150 aborted kernels per spike, kernels with higher risk of abortion showed increased glucose and fructose 6 days after pollination (Figure 2a,b). This observation supports the idea that abortion is probably not caused by a general decay of carbohydrate availability and could be caused by regulatory processes.

3.3 | Correlation between gene expression and kernel abortion

Factorial ANOVA revealed that gene expression at the tip of the ear was also significantly affected by the different genotype, stand density, and irrigation treatment combinations. We selected 1,322 genes with a q value <0.05 for at least one of the main effects (except the main effect of time after pollination) or one of the interactions (including the interactions with time after pollination). For these 1,322 genes, we analysed the correlation with the subsequent abortion. We identified 218 genes with expression levels significantly correlated with the number of

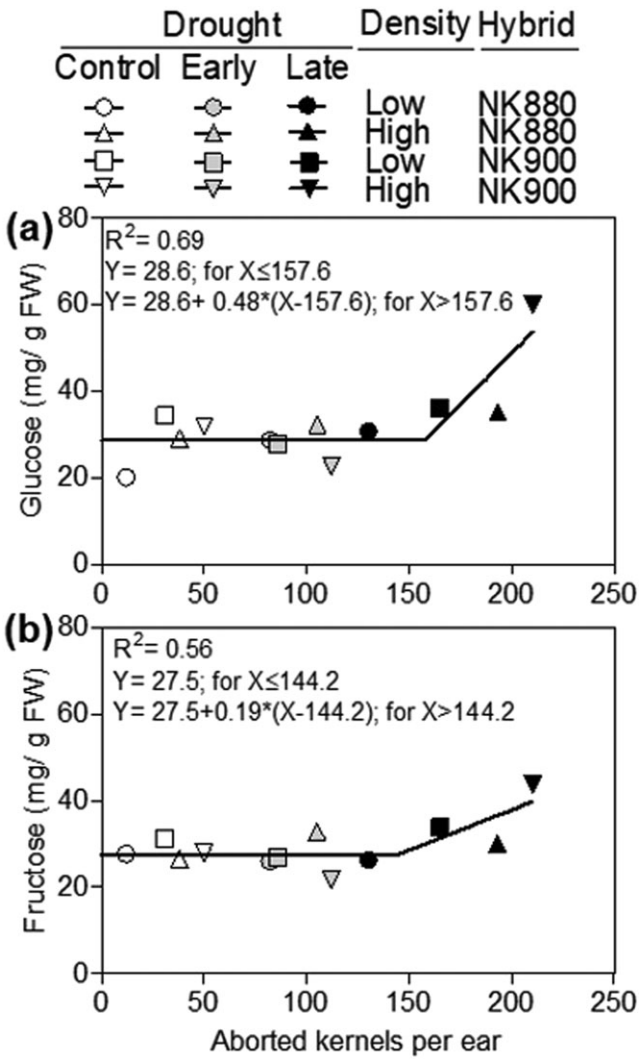

FIGURE 2 Glucose and fructose levels tend to increase with aborted kernels at high stress conditions. Correlation between glucose and fructose levels at the tip of the ear 6 days after pollination and the number of aborted kernels at the tip of the ear at harvest. Data from Experiment 1 (Table S1)

aborted kernels (p value <.05, q value <0.18, Table S2). Representative genes are shown in Figure 3, and several can be linked to cell wall processes, embryogenesis, and/or carbohydrate metabolism.

Several cell wall-related genes showed expression correlation with subsequent abortion. The expression of a β-1,3-glucanase, a β-Expansin (*EXPB15*), a kinesin-like protein (which could be involved in deposition of cellulose microfibrils; Zhong, Burk, Morrison, & Ye, 2002), and of UDP-glucose 4-epimerase (which is involved in cell wall biosynthesis, including Arabinogalactan Proteins (AGP)-glycans; Seifert, Barber, Wells, Dolan, & Roberts, 2002) was positively related to abortion. The expression levels of an endo-xyloglucan transferase, an extensin, a second β-1,3-glucanase, and four clones, representing

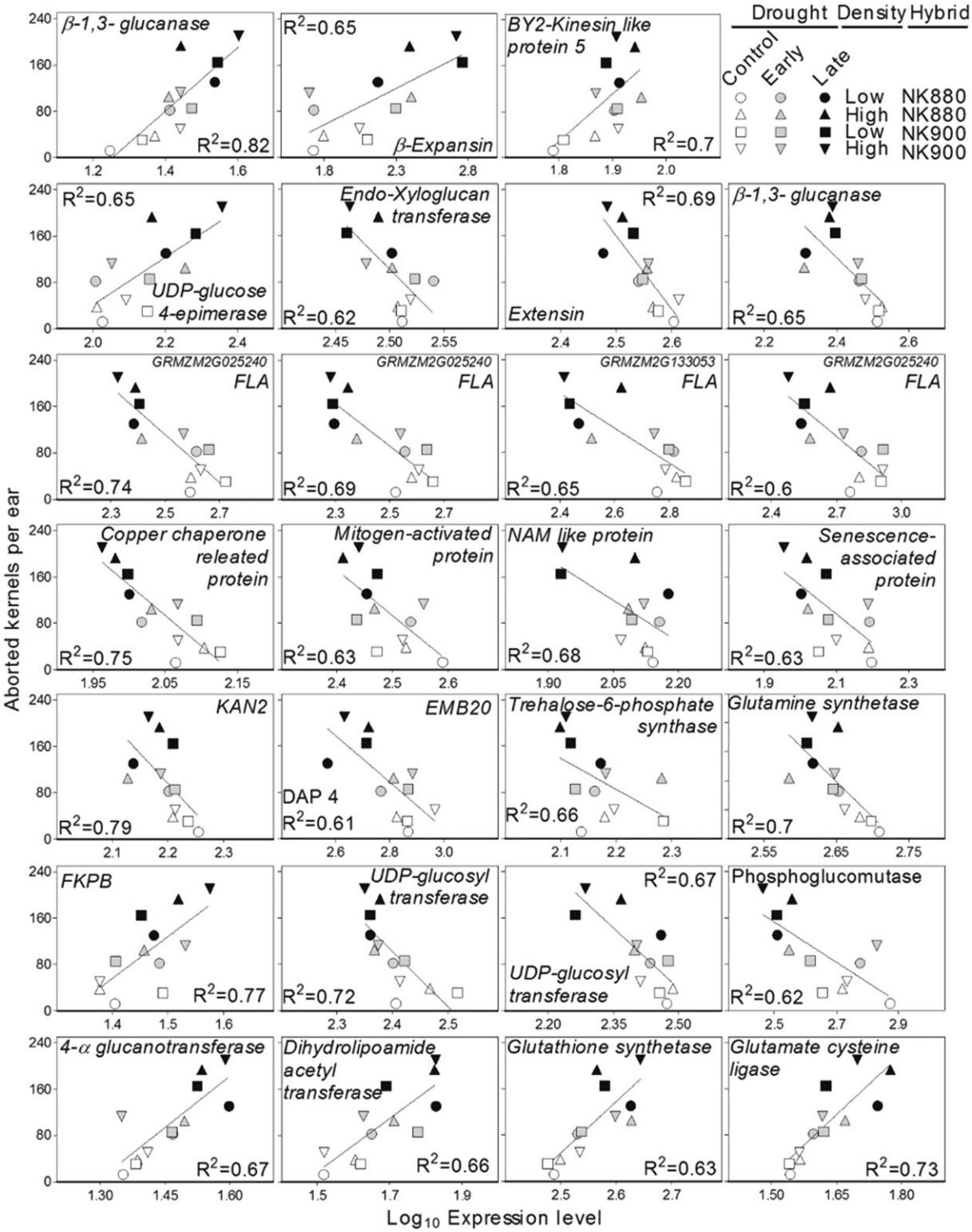

FIGURE 3 Correlation between gene expression at the tip of the ear early after pollination and the number of aborted kernels at the tip of the ear at harvest. Data from Experiment 1

genes encoding AGPs of the subclass of fasciclin AGPs (FLAs) were negatively related to abortion. Three of these four FLA-related probes (Zm010066_at, Zm010066_x_at, and Zm014734_x_at, Table S2) correspond to the GRMZM2G025240 maize gene (http://www.maizegdb.org; identity: 100, 99.43, and 92.82; alignment length: 114, 349, and 376, respectively). The remaining probe (Zm013703_x_at, Table S2) corresponds to the GRMZM2G133053 maize gene (% identity: 99.76; alignment length: 411). These genes are expressed in maize kernels early after pollination (Maize eFP Browser http://bar.utoronto.ca; Sekhon et al., 2011; Winter et al., 2007).

Several genes that can be related to embryo development showed expression correlation with subsequent abortion (Figure 3). These genes include those encoding the myb family transcription factor KANADI 2 (KAN2; Eshed, Izhaki, Baum, Floyd, & Bowman, 2004), the embryonic abundant protein 20 (EMB20; Patton et al., 1998), the trehalose-6-phosphate synthase-like protein (Gómez, Baud, Gilday, Li, & Graham, 2006; Paul, Primavesi, Jhurreea, & Zhang, 2008); and glutamine synthetase (Martin et al., 2006), which are positively involved in embryo development in Arabidopsis or kernel development in maize and negatively correlated with abortion. AGPs have also been linked to somatic embryogenesis (Ellis, Egelund, Schultz, & Bacic, 2010; Poon, Heath, & Clarke, 2012; Seifert & Roberts, 2007; Tang, He, Wang, & Sun, 2006) and negatively correlated with abortion. The expression of the *FKBP* (FK506-binding protein) gene positively correlated with abortion, and the immunophilin protein FKBP12 interacts with AtFIP37 (FKBP12 interacting protein 37 kD), which is required for normal embryogenesis in Arabidopsis (Vespa et al., 2004). Genes associated to cell death and reactive oxygen species scavenging are significantly up-regulated in abortive embryos of chrysanthemum (Zhang et al., 2014). In addition, proper regulation of cell death is required for normal embryo development (Zhao et al., 2013) and stress (drought) can delay the normal advance to programmed cell death in the central endosperm close to the embryo (Yu & Setter, 2003). The expression of four genes potentially involved in senescence/programmed cell death was negatively correlated with abortion. The list includes a copper chaperone-related protein gene (Miller, Arteca, & Pell, 1999), a mitogen-activated protein kinase 4 homolog gene (Li, Smaj, & Franklin-Tong, 2007), a NAM-like protein gene (Uauy, Distelfeld, Fahima, Blechl, & Dubcovsky, 2006), and a senescence-associated protein gene (Figure 3). The expression of two genes encoding enzymes involved in glutathione synthesis (glutathione synthetase and glutamate cysteine ligase) was positively correlated with abortion (Figure 3).

In addition to trehalose, other carbohydrate-metabolism genes (two UDP glucosyltranferases, a phosphoglucomutase, a 4 α-glucanotransferase, and a dihidrolipoamide acetyltransferase) showed negative or positive correlation with abortion (Figure 3).

The analysis of correlation between expression and abortion provides many hypotheses to be tested. In the rest of this work, we will focus on FLAs for a more detailed analysis of its functional relationship with fruit setting. The reason for this choice is that we observed four FLA-related probes with expression (inversely) correlated with abortion (indicating consistency) and AGPs are linked to both cell wall and embryo development (i.e., the two major subjects emerging from the transcriptome analysis). FLAs are hydroxyproline-rich glycoproteins with one or more arabinogalactan side chains O-linked to the protein backbone and cell-adhesion domains found on the plasma membrane and apoplast (Ellis et al., 2010; Johnson, Jones, Bacic, & Schultz, 2003; Seifert & Roberts, 2007; Showalter, Keppler, Lichtenberg, Gu, & Welch, 2010; Figure S3).

3.4 | Water stress reduces the expression of *ZmFLA7* in the upper section of the ear

The aim of the second experiment was to test the effects of drought on the expression of selected *FLA* genes by real-time PCR and evaluate the effects of drought on the abundance of AGP in protein blots. Despite the use of different pairs of primers, we were unable to measure GRMZM2G025240 or GRMZM2G133053 (identified in microarray experiments) by real-time RT-PCR, likely because the design of the primers was based on the B73 sequence and the experiments were conducted with hybrids that did not include B73 between their parental lines. Therefore, to evaluate whether there is a general effect on the expression of *FLA* genes, we measured GRMZM2G021794 (*ZmFLA7*; which is close to GRMZM2G025240, Figure S4) and GRMZM2G177142 (*ZmFLA8*; which is relatively distant from both genes identified in microarray experiments Figure S4). In control plants, mRNA abundance of both genes increased from the base to the tip of the ear (Figure 4). Drought reduced the expression of *ZmFLA7* at the tip of the ear whereas *ZmFLA8* showed no significant

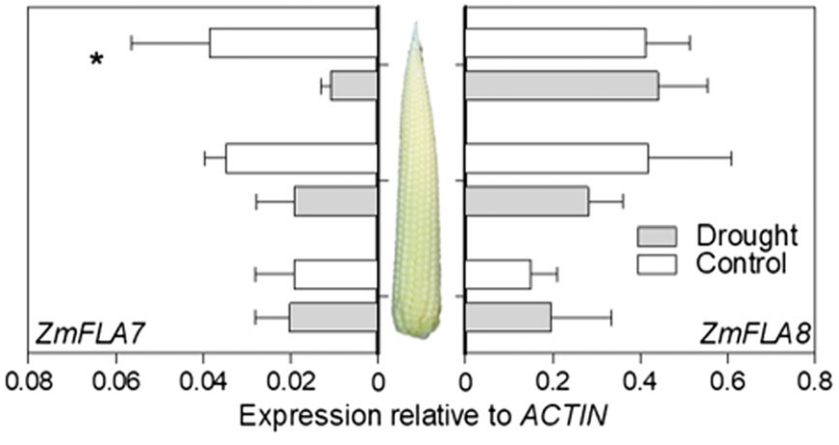

FIGURE 4 Drought reduces the expression of *ZmFLA7* at the tip of the ear but not at lower ear positions. The ears were harvested 2–6 days after pollination and data for different times were pooled because no significant differences were observed. The lack of response of *ZmFLA8* expression demonstrates that the effects are specific. Data from Experiment 2. * indicates $p < .05$ in *t* tests [Colour figure can be viewed at wileyonlinelibrary.com]

effects of drought (Figure 4). The response of *ZmFLA7* was not observed in samples taken from middle or lower portions of the ear (Figure 4), that is, regions where no kernel abortion was observed under our experimental conditions. These results are consistent with microarray analysis where reduced expression for selected *FLA genes* correlated with abortion whereas other *FLA* genes did not.

3.5 | Drought reduces the abundance of extractable glycosyl moieties specific to AGPs/FLAs

Because drought reduces the expression of selected *FLA (AGP)* genes, and FLAs are member of the AGP family, we investigated the response of extractable AGP abundance at the tip of the ear by using monoclonal antibodies JIM16 and JIM13 directed against glycosyl moieties specific to AGPs (Knox et al., 2001; Yates et al., 1996). Two groups of bands with different response patterns were recognized by these antibodies. The bands with a molecular mass <135 kDa showed no significant effects of drought. The bands with molecular mass >135 kDa increased with time after pollination to higher levels in well-irrigated controls than in drought-treated plants (Figure 5a,b). These

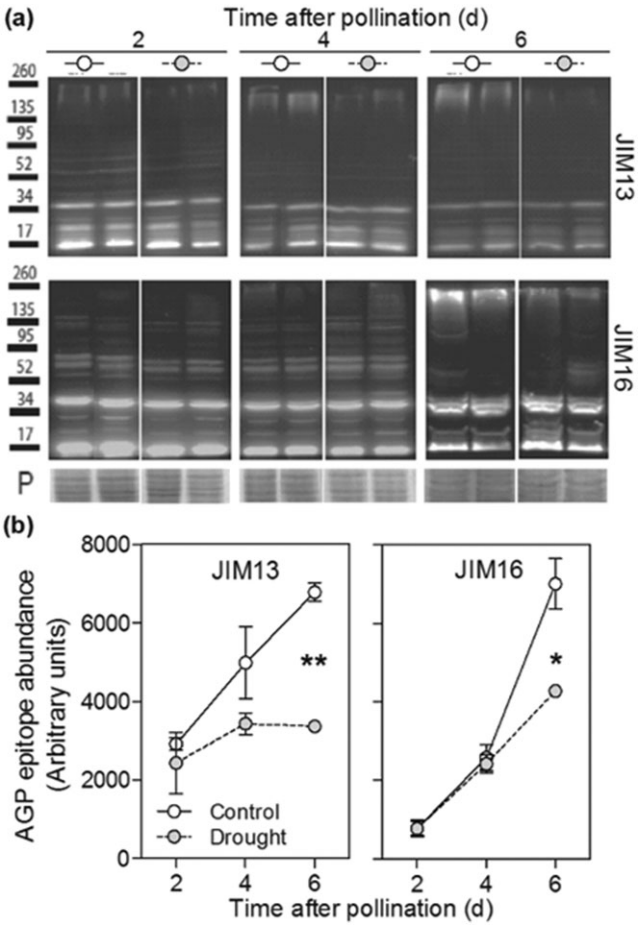

FIGURE 5 Drought reduces the abundance of arabinogalactan protein (AGP) epitopes detected with either JIM13 or JIM16 antibodies at the tip of the ear early after pollination. (a) Protein blots for control and drought conditions at the indicated times after pollination. P = Ponceau staining as loading control. (b) Quantification of the signal performed with GelPro32 and normalized to Ponceau staining. Data from Experiment 2. * and ** indicate $p < .05$ and $p < 0.01$, respectively, in *t* tests

observations are consistent with the higher expression of selected *FLA* genes in control than drought-treated ear tips.

3.6 | Pharmacological evidence for AGP-mediated prevention of kernel abortion in maize

The aim of the third experiment was to localize the presence of AGP in maize kernels and to test pharmacologically their involvement in the control of kernel abortion in plants grown in well-irrigated crops at low density. Yariv reagent stained the vascular structures and phloem termini of the pedicel (Figure 6a). For the pharmacological treatment, 4 days after pollination, a small window was opened to reach the kernels by gently cutting the husks (Figure 6b). A similar window was opened at the opposite side of the ear. A mesh soaked in β-Glucosyl Yariv reagent, which specifically binds beta-(1 → 3)-galactan chains in AGPs (Kitazawa et al., 2013; Yariv et al., 1967), was placed on the kernels visible through one of the windows; the negative control α-Mannosyl Yariv (Yariv et al., 1967) was placed at the other side, and both windows were closed. Mock ears with two windows treated with distilled water rather than Yariv reagents caused no significant effects on kernel abortion when compared with controls not exposed to surgical operation (Figure 6c, inset). In both tested hybrids, kernel abortion was significantly higher at the side treated with β-Glucosyl Yariv compared to the side treated with α-Mannosyl Yariv (Figure 6c).

3.7 | Drought reduces the expression of *AthFLA9* in Arabidopsis

To investigate whether the proposed role of FLAs in maize is conserved in Arabidopsis, we analysed the expression of the *AthFLA9* gene, which shows high similarity to *ZmFLA7* (Figure S4) and decreased expression in whole seedlings exposed to drought (Huang, Wu, Abrams, & Cutler, 2008). In developing siliques, maximum levels were observed 3 days after pollination (Figure 7a). Drought severely reduced the expression of *AthFLA9* in Arabidopsis siliques harvested at that time point (Figure 7b).

3.8 | *AthFLA9* reduces seed abortion in Arabidopsis

We did not observe flower or silique abortion at the inflorescence position where we measured *AthFLA9* gene expression; we therefore calculated abortion at the silique scale. We compared the proportion of abortions in wild-type plants with normal *FLA9* expression, *fla9-1* mutant with reduced *FLA9* expression, and *fla9-2 mutant* with 10-fold higher enhanced *AthFLA9* expression (Figure 7a) caused by a mutation affecting the promoter (Figure S2). The plants were either well-watered (control) or exposed to restricted irrigation (drought). Compared to the wild type, *fla9-1 mutant* showed increased abortion rates in siliques of the middle and upper third of the main shoot inflorescence under well-watered conditions (Figure 7c). Under restricted irrigation, the abortion rates were similar in wild-type and *fla9-1mutant* plants (which is consistent with the reduced expression of *AthFLA9* in the wild type). The *fla9-2* mutant, with enhanced *AthFLA9* expression, showed reduced abortion rate under drought conditions and increased

FIGURE 6 Enhanced kernel abortion in areas of the ear treated with β-Glucosyl Yariv reagent to precipitate AGPs. (a) Localization of AGP with Yariv staining 2 days after pollination. (b) Four days after pollination, small windows were opened at opposite sides of the ear by cutting husk leaves. A mesh soaked in the active β-Glucosyl Yariv reagent or the α-Mannosyl Yariv control was placed on the kernels exposed by the window, which was then closed. (c) Number of aborted kernels in response to the treatments with Yariv reagents in maize hybrids AX886 and AX852. The inset shows abortion in windows treated with distilled water (letter W) compared to controls not exposed to manipulations (letter C) in AX852. Data are means and SE of 10 plants from Experiment 3. * indicates $p < .05$ in t tests [Colour figure can be viewed at wileyonlinelibrary.com]

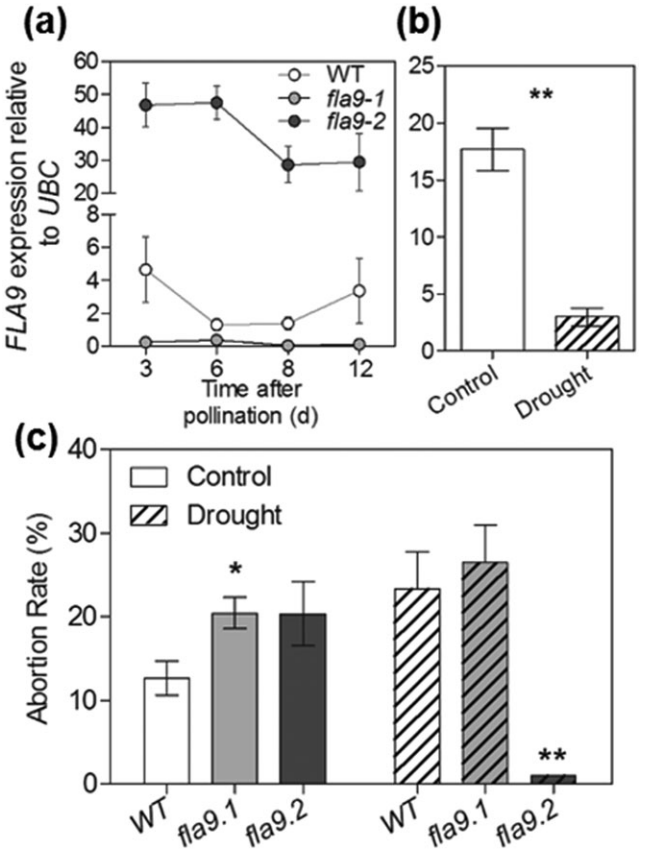

FIGURE 7 Enhanced seed abortion in *fla9 mutants* of *Arabidopsis thaliana*. (a) Expression of *FLA9* in hand-pollinated siliques of the wild type and of the *fla9-1* and *fla9-2* mutants measured by real-time RT-PCR. (b) Expression of *FLA9* in siliques of the wild type at 3 days after pollination under control and drought conditions. (c) Abortion rate of siliques of the middle and upper thirds of the main shoot inflorescence under control and drought conditions. Data are means and SE of 3 biological replicates. * and ** indicate significant differences with the wild type at $p < .05$ and $p < .01$ in two-way analysis of variance followed by Bonferroni tests

abortion under control conditions (Figure 7c). The high *AthFLA9* expression levels of the *fla9-2* mutant would be supra-optimal under control conditions and compensate the putative decrease of other FLAs under drought conditions.

Under control conditions, the *fla9-1 mutant* increased abortion without affecting the proportion of unfertilized ovaries (%, ±SE, wild type: 3 ± 0.6, *fla9-1* 3 ± 1.5) but the *fla9-2 mutant* increased the proportion of unfertilized ovaries (20% ± 5.1, $p < .001$). The *fla9-1 mutant* showed reduced expression of the *CHLOROPHYLL A/B-BINDING PROTEIN 2* (*CAB2*) and *ARABIDOPSIS THALIANA PEROXYGENASE 2* (*ATPXG2*; Figure 8a), which are linked to seed development (Andriotis, Pike, Bunnewell, Hills, & Smith, 2010; Becerra, Puigdomenech, & Vicient, 2006). The timing of expression of these genes was also altered in the *fla9-2 mutant* (Figure 8a). Analysis by microscopy revealed abnormal embryo development of the fertilized ovules in *fla9-1* and *fla9-2* in a proportion compatible with the rate of subsequent abortion (Figure 8b). Thus, molecular markers and morphological observations indicate that both reduced and enhanced levels of *AthFL9* expression can distort normal embryo development leading to abortion under control conditions.

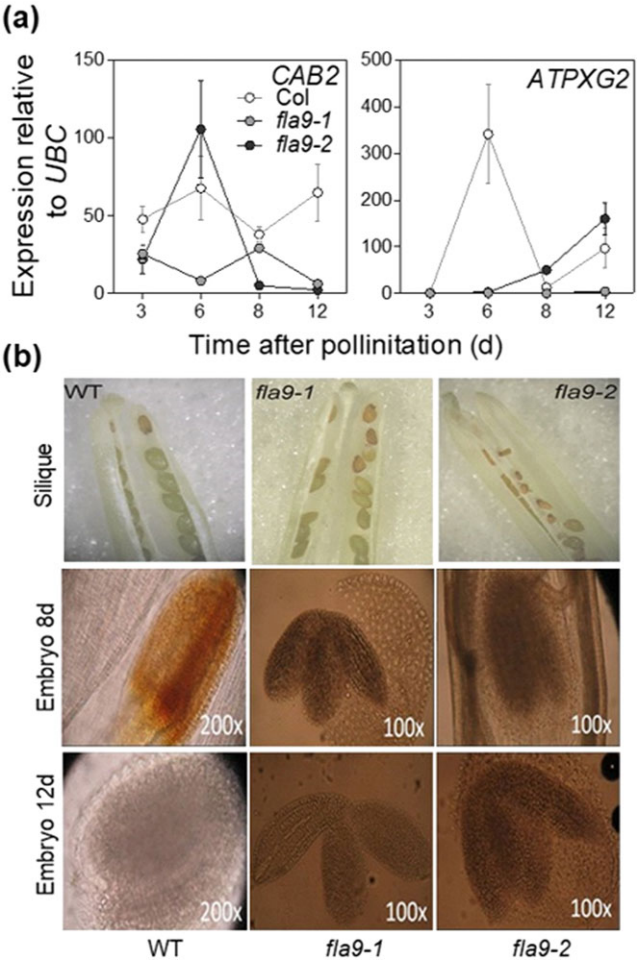

FIGURE 8 Impaired seed and embryo development in *fla9 mutants* of *Arabidopsis thaliana*. (a) Distorted expression of *CAB2 and ATPXG2* in hand-pollinated siliques of the wild type and of the *fla9-1* and *fla9-2* mutants indicates abnormal developmental progression. Data are means and SE of 3 biological replicates. (b) Siliques and embryos (8 and 12 days after hand pollination) images of the wild type and of the *fla9-1* and *fla9-2* mutants [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

To obtain insight into the mechanisms that control the abortion of fertilized ovaries, we cultivated two hybrids of maize at two sowing densities and under three irrigation regimes, that is, well-watered, early drought (before pollination), and late drought (at pollination) in the field and searched for the genes that showed correlation of their expression early after pollination with the rate of abortion at the tip of the ear observed later on (Figure 3, Table S2). Not unexpectedly, many of the identified genes can be related to the control of embryo development (which would be affected during abortion). Another function emerging from this analysis is related to cell wall processes, which is consistent with previous observations showing that genes related to cell wall processes are over-represented among the genes responding to drought 9–10 days after pollination of maize ovaries (Marino et al., 2009; Yu & Setter, 2003). At the intersection between these two functional groups, we identified selected *FLA* genes (which belong to the AGP family). Several pieces of evidence presented here support a role of specific FLAs in the prevention of the abortion of fertilized ovaries.

First, the expression of selected *FLA* genes (GRMZM2G025240, GRMZM2G133053, GRMZM2G021794 [*ZmFLA7*]) at the tip of the ear early after pollination decreased in response to treatments that increased the rate of maize kernel abortion at harvest (Figure 3), and (at least for *ZmFLA7*) this response occurs at the tip of the ear (where kernel abortion was subsequently observed) but not at lower ear positions (Figure 4). A *FLA* gene is also included in a recently published list of genes down-regulated by drought stress in the silk and ovaries of maize plants grown under controlled conditions (Oury, Caldeira, et al., 2016). Similarly, in Arabidopsis, drought reduced the expression of *AthFLA9* in the siliques 3 days after pollination and increased the rate of abortion (Figure 7b,c). Furthermore, drought reduced the abundance of extractable AGP epitopes (which are present in FLA proteins, Figure S3) at the tip of the ear early after pollination (Figure 5a,b).

Second, in field experiments with nonstressed maize plants, treatment of a small area of the ear with β-Glucosyl Yariv reagent, which specifically binds beta-(1 → 3)-galactan chains of at least seven residues present in AGPs (Kitazawa et al., 2013; Yariv et al., 1967) and hence in FLAs (Figure S3), increased subsequent kernel abortion compared to the negative control α-Mannosyl Yariv (Figure 6c). This observation provides pharmacological evidence in favour of a role of AGP in the prevention of kernel abortion in maize (Yariv et al., 1962).

Third, plants of the *fla9-1 mutant* of *A. thaliana*, with severely reduced expression of *AthFLA9*, showed enhanced seed abortion compared to the wild type when grown under nonstressful conditions indoors (Figure 7c). Drought conditions increased the rate of abortion in the wild type, which reached the largely constitutive level of abortion observed in the *fla9-1 mutant*. The gain-of-function *fla9-2 mutant* reduced abortion under stress but not under control conditions (Figure 7c). This negative correlation between *AthFLA9* expression and abortion provides genetic evidence in favour of a role of *AthFLA9* in seed setting in Arabidopsis.

There is abundant literature concerning the role of AGPs in somatic embryogenesis (Ellis et al., 2010; Seifert & Roberts, 2007), an in vitro process that would share similarities to the development of the embryo *in planta* during fruit setting. For instance, the in vitro development of pollen grains of *Brassica napus* into haploid embryos in vitro is impaired by the addition of β-Glucosyl Yariv reagent to the culture medium (Tang et al., 2006). Conversely, in several species, AGPs produced by calli undergoing in vitro somatic embryogenesis promote this process when added back into tissue culture (Poon et al., 2012, and references therein). In maize pollen embryogenesis, many proteins secreted during the course of the culture are glycosylated, including AGPs; adding tunicamycin to inhibit glycosylation arrests embryo development, but adding "Arabic gum" (which contains AGPs) partially restores embryo development (Borderies et al., 2004). Both molecular and morphological markers indicate distorted development of the siliques and the embryo in the *fla9* mutants of Arabidopsis (Figure 8).

We did not observe changes in carbohydrate levels consistent with a role of restricted photo-assimilates in the induction of abortion in maize. Glucose and fructose levels at the tip of the ear were unaffected early (2–4 days) after pollination and actually increased 6 days

after pollination in the tip of ears with high risk of kernel abortion due to stress (Figure 2a,b). The latter confirms and extends previous observations that moderate stress can cause kernel abortion without reducing or even increasing sugar levels (Reed & Singletary, 1989; Schussler & Westgate, 1991b, 1995; Cheng & Lur, 1996; Setter et al., 2001; Andersen et al., 2002; Qin et al., 2004; Oury, Caldeira, et al., 2016), and reducing ovary sugar levels not always leads to kernel abortion (Hiyane et al., 2010). Like many other processes in plants (e.g., flowering, cell expansion), kernel setting could be under the control of multiple signalling pathways. Sugar availability and signalling could be limiting under certain conditions, and kernel growth restriction by additional signalling pathways would result in the accumulation of sugars in others.

AGPs belong to the superfamily of plant hydroxyproline-rich glycoproteins (Showalter et al., 2010) that contain a core protein, one or more arabinogalactan side chains, and often a glycosylphosphatidylinositol lipid anchor (Seifert & Roberts, 2007). AGPs are found on the plasma membrane transiently linked by the glycosylphosphatidylinositol anchor, in the wall, in the apoplastic space, and in secretions (Ellis et al., 2010). FLAs are a subclass of AGPs that have putative cell-adhesion domains known as fasciclin domains, which are 110–150 amino acids long, have low overall sequence similarity and two highly conserved regions of approximately 10 amino acids each (Johnson et al., 2003; Figure S3). Proteins containing a fasciclin domain have been shown to function as adhesion molecules (Elkins, Zinn, McAllister, Hoffmann, & Goodman, 1990). There are 21 *A. thaliana FLA* loci grouped based on domain organization and sequence similarity (Johnson et al., 2003; Schultz et al., 2002; Schultz, Johnson, Currie, & Bacic, 2000). Only few FLAs have been assigned a role showing highly diverse arrays of functions linked to cell wall assembly in cell division and cell expansion processes. *AtFLA3* was linked to microscopore development (Li, Yu, Geng, & Zhao, 2010), mutants at the *AtFLA4* gene showed abnormal cell expansion and increased sensitivity to salt (Griffiths et al., 2014; Harpaz-Saad et al., 2011; Seifert, Xue, & Acet, 2014; Shi, Kim, Guo, Stevenson, & Zhu, 2003; Xue et al., 2017), *AtFLA11/12* contribute to stem strength by regulating cellulose deposition and cell wall matrix integrity (Huang et al., 2013; MacMillan, Mansfield, Stachurski, Evans, & Southerton, 2010; Wang et al., 2015), *AtFLA1* played a role in shoot regeneration (Johnson, Kibble, Bacic, Schultz, & Pu, 2011), and *GhFLA1* is involved in modulating the biosynthesis of cell wall polysaccharides during fibre development of cotton (Huang et al., 2013). Yariv reagent stained the vascular structures and phloem termini of the pedicel (Figure 4a) suggesting a possible interaction with sugar signalling. Both AGPs (Gao & Showalter, 2000) and FLA (Roach & Deyholos, 2007) have been previously observed in phloem tissues in other systems. Noteworthy, both sugar signalling (Ruan et al., 2012; Ruan, Jin, Yang, Li, & Boyer, 2010) and AGPs (Coimbra, Almeida, Junqueira, Costa, & Pereira, 2007; Levitin, Richter, Markovich, & Zik, 2008; Li et al., 2010) are also important for pollen development, indicating that these signals converge to control different processes.

Stress-induced abortion of fertilized ovaries at the tip of the ear can generate economically significant yield losses in maize crops. Based on experiments where the stresses caused by drought and canopy shade developed with a timing and intensity typical of field conditions, this paper identifies the reductions in the expression of *FLA* genes and the associated decay in the abundance of AGPs, as early events after pollination that anticipate the occurrence of abortion at the tip of the ear in maize. A similar function is proposed for *AthFLA9* in Arabidopsis plants exposed to drought, and this is consistent with the current view of conserved fruit-setting mechanisms between monocot and eudicot herbaceous species (Ruan et al., 2012).

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