



Significance of putrescine conversion in filling grain embryos of wheat plants subjected to drought stress

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Abstract

Background and Aims Polyamines (PAs) play important roles in drought tolerance, but the physiological significance of putrescine (Put) conversion into other forms of PAs is not clear in filling grain embryos of wheat (*Triticum aestivum* L.) plants under drought stress.

Methods The changes in the levels of three main free PAs, Put, spermidine (Spd), and spermine (Spm), covalently conjugated PAs (perchloric acid-soluble), and covalently bound PAs (perchloric acid-insoluble), were investigated in embryos of filling grains, in two

wheat cultivars, Longmai No. 079 (drought-tolerant) and Wanmai No. 52 (drought-sensitive). Furthermore, the activities of arginine decarboxylase, S-adenosyl-methionine decarboxylase, and transglutaminase, which affect free Put biosynthesis, Spd and Spm biosynthesis from Put, and conversion of free PAs into bound PAs, respectively, were also determined. Exogenous PAs and PA biosynthesis inhibitors were also applied in the experiment.

Results Under drought stress, the levels of conjugated PAs did not significantly differ between the two cultivars. However, the levels of free Spd, free Spm and bound Put increased more markedly ($p < 0.05$) in drought-tolerant Longmai No. 079 than in drought-sensitive Wanmai No. 52, suggesting that free Spd and Spm, and bound Put, which were converted from free Put, were possibly involved in drought tolerance. Exogenous Spd treatment enhanced the drought-induced increases in endogenous Spd and Spm contents in drought-sensitive Wanmai No. 52, and increased drought tolerance, as judged by the decrease in flag leaf relative plasma membrane permeability and increases in flag leaf relative water content, 1000-grain weight and grain number per spike. Methylglyoxal-bis guanyldiazide and *o*-phenanthroline inhibited drought-induced increases in free Spd, Spm, and bound-Put contents in drought-tolerant Longmai No. 079, and decreased in drought tolerance.

Conclusions Collectively, the conversions of free Put into free Spd, Spm, and bound Put in filling grain

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embryos enhanced the tolerance of wheat plants to drought stress.

Keywords Bound polyamine · Conjugated polyamine · Drought stress · Free polyamine · Wheat (*Triticum aestivum* L.) filling grain

Introduction

Environmental factors, such as drought, salt, high temperature, and heavy metal stress, greatly affect plant growth and development (Beshamgan et al. 2019; Ouyang et al. 2020; Ebmeyer et al. 2021; Zhong et al. 2020; Rakić et al. 2021) and thereby pose severe threats to agricultural productivity and sustainability (Lechowska et al. 2022).

Globally, arid and semi-arid areas are spreading rapidly and crop tolerance to drought stress is of particular importance. Therefore, it is increasingly interesting to analyse the mechanisms underlying crop drought tolerance. Wheat is one of the main food crops and often experiences drought and dry-hot wind stresses during grain filling and embryo maturation. Plant water deficits alter physiological metabolism, reduce photosynthetic performance, inhibit normal development of wheat, and ultimately decrease yield. Therefore, it is important to explore mechanisms of wheat drought tolerance at the grain filling stage.

Polyamines (PAs) are plant growth regulatory substances with positive charges and strong biological activity and are found in all living organisms (Martin-Tanguy 2001; Sobieszczuk-Nowicka et al. 2019). Three PAs, putrescine (Put), spermidine (Spd), and spermidine (Spm), are the most common members of the PA family. Diamine Put is mainly produced from arginine by the action of arginine decarboxylase (ADC; EC 4.1.1.19), which is one of the key enzymes in Put biosynthesis (Nam et al. 1997), characterized by absolute specificity for L-arginine, and can be inhibited by D-arginine (D-Arg) potently and exclusively (Lutts et al. 2013). Another key enzyme involved in Put synthesis is ornithine decarboxylase (ODC; EC 4.1.1.17), which catalyzes the transform from substrate ornithine to Put through decarboxylation (Martin-Tanguy 2001; Gardiner et al. 2010). Put can be converted to Spd and Spm by being linked with one and two aminopropyls by Spd synthase and Spm synthase, respectively. The aminopropyl group

is from decarboxylated *S*-adenosylmethionine, which is converted from *S*-adenosylmethionine by *S*-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) (Martin-Tanguy 2001). Many processes and factors are involved in Put conversions into Spd and Spm. Among these, SAMDC is the most important enzyme and can be inhibited by methylglyoxal-bis guanylhydrazide (MGBG) potently and exclusively (Tiburcio et al. 1993; Lutts et al. 2013).

Free PAs can be converted into conjugated and bound forms. The former are perchloric acid-soluble and formed via covalent binding of PAs with small molecules, such as hydroxyl cinnamic acid and cinnamon, by the action of acyl-transferase. Conjugated PAs are regarded as temporary storage pools of free PAs (Bais et al. 2000). Most studies indicate that conjugated PAs are mainly involved in plant disease resistance with external exposure triggering hypersensitivity reactions (Rabiti et al. 1998; Musetti et al. 1999). However, conjugated PAs have been associated with the development of flowers (Scaramagli et al. 2000), fruits (Shinozaki et al. 2000) and seeds (Puga-Hermida et al. 2003) and PEG-osmotic stress tolerance (Kong et al. 1998). Bound PAs are perchloric acid-insoluble and formed via the linking of PAs to endo-glutamine residues of proteins by the action of transglutaminase (TGase; EC 2.3.2.13), which can be inhibited by *o*-phenanthroline (Zhong et al. 2020; Du et al. 2022). The conversion of PAs can play important roles in the process of post-translational modification of proteins (Del Duca et al. 1995, 2014). Our previous studies showed that this PA form in the plasma membrane could enhance the chilling tolerance of postharvest plum fruit (Du et al. 2021) and maize drought tolerance by maintaining the membrane stability of maturing grain embryos (Du et al. 2022).

In summary, PAs, as plant growth regulators, widely exist in plants and play important regulatory roles in plant growth, development, morphogenesis and response to environmental stress (Tomosugi et al. 2006; Kasukabe et al. 2004; Paschalidis and Roubelakis-Angelakis 2005; Alcázar et al. 2006; Chen et al. 2019; Gondor et al. 2021; Hashem et al. 2021; Pál et al. 2021). Therefore, the homeostatic balance of free, conjugated and bound PAs is vital in growth and development processes (Lutts et al. 2013). For example, Yang et al. (2007) reported that at a later stage of drought, the increases in free Spd/free Spm and

bound Put in the flag leaves of rice plants were significantly positive correlated with rice grain yield. However, the mechanism underlying PA function in wheat (*Triticum aestivum* L.) drought tolerance during grain filling remains to be explored. Our recent research showed free Spd and Spm in filling grain embryos enhanced maize drought tolerance. Furthermore, non-covalently conjugated Spd and Spm, and conjugated covalently Put and Spd in embryo cell plasma membrane functioned in increasing H^+ -ATPase activity, mitigating cell plasmolysis and maintaining the plasma membrane conformation under drought stress (Du et al. 2022). However, due to sampling on the 7th day after treatment, dynamic changes in levels of free PAs were not elucidated. In particular, the significance of converting Put into other forms was not clear. Therefore, this research aimed to uncovering the significance of Put conversion by estimating the dynamic changes in levels of endogenous free, conjugated and bound PA accumulation in embryos during wheat grain filling under drought, with two wheat cultivars differing in drought tolerance. The hypothesis of the research was that the conversions of free Put into free Spd and Spm, and bound Put, rather than free Put itself, functioned in enhancing wheat drought tolerance. Furthermore, the finding could address the issue of whether free Put accumulation at an earlier stage of drought could bring about or alleviate injury.

Materials and methods

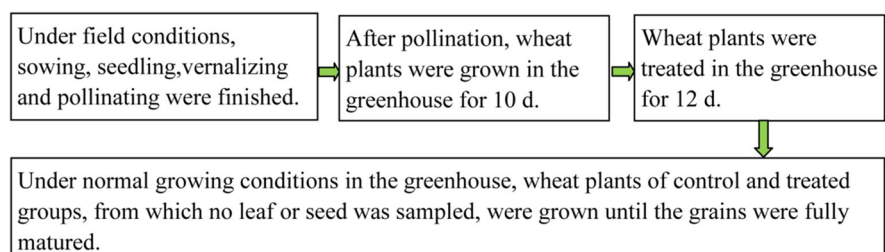
Plant materials and growth conditions

The typical winter wheat (*Triticum aestivum* L.) cultivars (Longmai No. 079 and Wanmai No. 52) were applied in the research. Longmai No. 079 (F_1 generation of Loumai No. 1 and TW98-829-1) and Wanmai No. 52 (F_1 generation of Zhengmai No. 8329 and

Wanmai 19) are all hybrids and bred by Pingliang Academy of Agricultural Sciences, Gansu Province, China, and Suzhou Seed Company, Anhui Province, respectively. The former was approved by Gansu Provincial Variety Certification Committee in 2016 (No. 2016007) and the latter was examined by the second session of the National Crop Variety Approval Committee of China in 2007 (No. 2007009). The wheat seeds were obtained from Prof. Guozhang Kang of Henan Agricultural University and were permitted to be used in this experiment according to the legislation and guidelines of China. Longmai No. 079 is distributed in Northwest China, a drought ecotope, while Wanmai No. 52 is mainly planted in Central China, a rainy ecotope. From the geographical distribution of the two cultivars, it could be prospectively inferred that Longmai No. 079 is drought-tolerant, and Wanmai No. 52 is drought-sensitive. In our preliminary experiment, under the normal water supplying condition, the 1000-grain weight of Longmai No. 079 and Wanmai No. 52 were about 44 and 41 g, respectively, while under D.S., the parameter of both cultivars were about 40 and 30 g, respectively. Furthermore, the tolerance difference in D.S. between the two cultivars was verified in this research. This experiment was carried out at Zhoukou Normal University in Henan Province, North China, from 2017 to 2020 by the scheme (Fig. 1).

On October 25, 2017, 2018 and 2019, wheat seeds were planted in plastic basins (33 seeds/basin) after the seed surface was sterilized for 5 min with 0.1% $HgCl_2$ (w/v) and rinsed with distilled water three times. Each basin (height: 50 cm, bottom diameter: 35 cm, rim diameter: 40 cm) was filled with 15 kg of screened soil, which was taken from the surface in the wheat experimental field and contained 13.5 g organic matter kg^{-1} , 120 mg available nitrogen kg^{-1} , 30 mg available phosphorus kg^{-1} , 105 mg available potassium kg^{-1} and the other macroelements and

Fig. 1 The experimental scheme throughout whole wheat growing period



microelements, meeting wheat's demand for nutrients during the growth period. The basins with seeds were placed in the field and the seedlings were vernalized under natural conditions in winter. Then they were thinned to 10 seedlings/basin and cultivated under natural conditions. After pollination, wheat plants were transferred to a greenhouse with a 25 °C/15 °C (day/night) temperature, 75% relative air humidity, 16 h/8 h light/dark photoperiod, and 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic quantum flux density and grown for 10 d.

Material treatments and sampling

As soon as the basins with wheat plants had been placed in the greenhouse, the soil water potentials of all basins were detected with a potential instrument (Zhejiang Top Yunong Technology Co., LTD, Hangzhou, China, Model: TRS-II), of which the probe sensor was buried at a depth of approximately 15 cm in the soil of the basin. The soil water potentials of the control and treated groups were monitored every 3 h. When the water potential readings of the control and treated groups dropped to -0.15 and -1.0 MPa, respectively, the basins were replenished manually in a timely manner with tap water to ensure that the water potential of the control and treated groups were -0.15 and -1.0 MPa, respectively. On the 10th day after pollination, the wheat plants were treated as follows. The greenhouse was under the aforementioned conditions except for the 35% relative air humidity.

Control Flag leaves and spikes were sprinkled with deionized water, and the roots were in a soil water potential of -0.15 MPa.

Drought treatment Flag leaves and spikes were sprinkled with water, and the roots were in a soil water potential of -1.0 MPa.

Drought + D-Arg treatment Flag leaves and spikes were sprinkled with 1 mM D-Arg, and the roots were in a soil water potential of -1.0 MPa.

Drought + D-Arg + Put treatment Flag leaves and spikes were sprinkled with 1 mM D-Arg + 1 mM

Put, and the roots were in a soil water potential of -1.0 MPa.

Drought + Spd treatment Flag leaves and spikes were sprinkled with 1 mM Spd, and roots were in a soil water potential of -1.0 MPa.

Drought + MGBG treatment Flag leaves and spikes were sprinkled with 0.5 mM MGBG, and the roots were in a soil water potential of -1.0 MPa.

Drought + *o*-phenanthrolin treatment Flag leaves and spikes were sprinkled with 0.2 mM *o*-phenanthrolin, and the roots were in a soil water potential of -1.0 MPa.

The PAs and inhibitors mentioned-above were from Sigma Chemical Co. (USA). The dose determination of the abovementioned reagents was based on our preliminary experiments (Supplementary Data, Fig. 1). Wheat flag leaves and spikes of treated groups were sprayed with 25 mL/basin of a solution containing the abovementioned reagents, 0.1% ethanol and 0.01% (V/V) Tween 20 at 6:00 and 18:00 every day. The control group was sprayed with water containing 0.1% ethanol and 0.01% (V/V) Tween-20. The flag leaves and seeds in the middle of the spikes were collected at 6:00 on the 0, 2nd, 4th, 6th, 8th, 10th and 12th days after treatment. The embryos were carefully separated from the seeds with a scalpel under a dissection microscope and washed with deionized water.

Assessment of flag leaf relative water content (FLRWC)

After the fresh flag leaves were weighed for fresh weight (LFW), they were immediately immersed in deionized water for approximately 5 h to fully absorb water until the weight of the flag leaves was constant, which was regarded as the saturation weight (LSW). Then, the sample was put in an oven and dried at 75 °C for approximately 10 h until its weight was constant. The dried constant flag leaf weight was named the dry weight (LDW). FLRWC was assessed by the formula:

$$\text{FLRWC}(\%) = (\text{LFW} - \text{LDW}) / (\text{LSW} - \text{LDW}) \times 100.$$

Assessment of flag leaf relative plasma membrane permeability (FLRPMP)

Wheat FLRPMP was assessed according to the method described by Jahan et al. (2019) with minor adjustments. Wheat flag leaves (1 g) were immersed in 10 mL of deionized water. Then, under dark conditions, the samples were incubated in a water bath at 25 °C for 2 h. The original electrical conductivity (OEC) of the water medium containing flag leaves was determined with a portable conductivity metre (Guangzhou Ruibin Technology Co., Ltd, Guangzhou, China, Model: DDB-11A). Afterwards, the flag leaves were boiled for 20 min at 100 °C, cooled to 25 °C, and left to stand for 30 min. The terminal electrical conductivity (TEC) was determined. DEC represents the electrical conductivity of deionized water. FLRPMP was assessed by the formula:

$$\text{FLRPMP}(\%) = (\text{OEC} - \text{DEC}) / (\text{TEC} - \text{DEC}) \times 100.$$

Determination of 1000-grain weight and grain number per spike

The wheat plants, from which leaves and grains were not sampled, were left growing in a greenhouse under normal growing conditions with a soil water potential of -0.15 MPa, 25 °C/15 °C (day/night) temperature, 75% relative air humidity, 16 h/8 h light/dark photoperiod, and 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic quantum flux density until the grains were fully matured. Then, 1000 grains and spikes, which were randomly selected from the control and treatment groups, were sampled for the determination of 1000-grain weight and grain number per spike, respectively. At the tillering stage, the tillering number was controlled to ensure 20 wheat plants per basin, so the spike number per basin was 20, and the grain yield per basin was approximately 20 to 50 g.

Assay of arginine decarboxylase (ADC) in filling grain embryos

ADC was extracted and assayed by the method of Nam et al. (1997) with modifications. Wheat embryos (0.2 g) were homogenized with 4 mL of precooled extract solution, which was composed of 50 mM

phosphate buffer (pH 6.3), 5 mM ethylene diamine tetraacetic acid (EDTA), 0.1 μM phenyl methyl sulfonyl fluoride (PMSF), 40 μM phosphopyridoxal, 5 mM dithiothreitol (DTT), and 20 mM vitamin E, in an ice bath and filtered with 4 layers of gauze. The solution was precipitated by 20%-50% $(\text{NH}_4)_2\text{SO}_4$. The precipitate was redissolved in 10 mM phosphate buffer (pH 6.3), dialyzed overnight at 4 °C, and then added to a double volume of -15 °C acetone. After centrifugation at 5,000 $\times g$ for 5 min at 4 °C, the precipitate was redissolved in 10 mM phosphate buffer, dialyzed overnight at 4 °C and centrifuged at 15,000 $\times g$ for 15 min. The supernatant was used to measure enzyme activity. The ADC reaction system, including Tris HCl buffer (100 mM, pH 7.5), 5 mM EDTA, 40 μM phosphopyridoxal and 5 mM DTT and ADC extraction above, was placed in a water bath at 37 °C for 2 min. The reaction lasted for 60 min at 37 °C and was terminated with perchloric acid (PCA, 5% final concentration). The sample was centrifuged at 3,000 $\times g$ for 10 min. Then, 0.5 mL of supernatant was added to 1 mL of 2 M NaOH and 10 μL of benzoyl chloride, vortexed vigorously for 20 s, left to stand for 60 min at 25 °C and added to 2 mL of saturated NaCl. After mixing, 2 mL of diethyl ether was added, vortexed vigorously for 20 s, and centrifuged at 1,500 $\times g$ for 5 min. One millilitre of diethyl ether was collected and evaporated at 50 °C. The precipitate was redissolved in 3 mL of methanol and measured by an ultraviolet-spectrophotometer at 254 nm. A total of 1 nmol agmatine g^{-1} fresh weight (FW) h^{-1} was regarded as one enzyme activity unit.

Assay of S-adenosylmethionine decarboxylase (SAMDC) and transglutaminase (TGase) activities in filling grain embryos

SAMDC activity was assayed by examining the release of $^{14}\text{CO}_2$ using substrates labelled with the isotope ^{14}C according to the method of Kaur-Sawhney and Shin (1982) with a few modifications. Wheat embryos (0.2 g) were homogenized with 0.5 mL of phosphate buffer (100 mM, pH 7.6) and then centrifuged at 25,000 $\times g$ for 20 min at 4 °C. The supernatant was used to assess SAMDC activity. The assay solution consisted of the aforementioned supernatant, EDTA (0.2 mM), Tris-HCl buffer (0.1 M, pH 8.3) and 2-mercaptoethanol (2.0 mM). S-adenosyl-L-[carboxyl- ^{14}C] methionine (0.5 nM) was added to

the assay solution, and at 30 °C, the reaction went on for 30 min. Then, 0.4 ml of KH_2PO_4 (1 M) was used to quench the reaction, and $^{14}\text{CO}_2$ was collected in a glass vial. One microlitre of $^{14}\text{CO}_2 \text{ min}^{-1}$ was defined as one unit.

TGase activity was determined by detecting the rate of incorporation of Put labelled with $^3\text{[H]}$ into proteins according to the method of Ickson and Apelbaum (1987) with proper adjustments. Embryo samples of wheat grains (0.2 g) were homogenized with 1 mL of 100 mM Tris–HCl (pH 8.8) and centrifuged for 30 min at $1200\times g$ at 4 °C. The supernatant was used to assess TGase activity. Put labelled with $^3\text{[H]}$ was added to the enzyme solution, and the determination of TGase activity was performed by assessing the conjugation rate of $^3\text{[H]}$ Put into proteins. One TGase activity unit was expressed with 1 nmol of $^3\text{[H]}$ Put mg^{-1} protein h^{-1} . Protein content was detected by the Bradford method of protein–dye binding (1976), with bovine serum albumin used as a standard.

Extraction and quantification of free PAs

The Extraction and quantification of free PAs were conducted by the method of Quinet et al. (2010) with minor adjustments. Wheat embryos (0.2 g) were homogenized in 3 mL of perchloric acid (5%, v/v), and the homogenate was left to stand for 1 h in a refrigerator at 4 °C. Then it was centrifuged at $21,500\times g$ for 30 min. The supernatant was collected for the free PA assay. By the method of Di Tomaso et al. (1989), benzoyl chloride was used for the derivatization of the free PAs in the supernatant. Then, 0.5 mL of supernatant was added to 1 mL of 2 M NaOH and 7 μL of benzoyl chloride, vortexed vigorously for 5 s three times, left to stand for 60 min at 25 °C and added to 2 mL of saturated NaCl. Then the mix was added to 2 mL of diethyl ether, vortexed vigorously for 5 s three times, and centrifuged at $1,500\times g$ for 5 min. One millilitre of diethyl ether was collected and evaporated with warm air. The precipitate was redissolved into 1 mL of methanol. Free Put, Spd and Spm were quantified by HPLC (Waters 2695, USA). A C-18 reverse-phase separation column was used, with 1, 6-Hexanediamine as the internal standard and 254 nm as the detecting wavelength. At 25 °C, the PA sample was eluted from

the separation column by a Perkin-Elmer Series 410 pump at 0.6 mL/min.

Extraction and quantification of conjugated PAs

The Extraction and quantification of conjugated PAs were conducted by the method of Quinet et al. (2010) with some adjustments. One millilitre of the supernatant of the free PA extraction mentioned above was mixed with 1 mL of 12 N HCl, sealed immediately in ampere glass bottles, and acid-hydrolysed at 110 °C for 15 h. The glass bottles were carefully crevassed to evaporate HCl at 80 °C. Finally, the residue was added with 10% PCA, benzoylated with benzoyl chloride and quantified by HPLC for quantification of conjugated PAs as described for the free PA assay.

Extraction and quantification of bound PAs

The extraction and quantification of bound PAs were conducted by the method of Quinet et al. (2010) with some modifications. The sediment of the free PA extraction mentioned above was added to 0.5 mL of 5% PCA, vortexed vigorously, and centrifuged at $21,500\times g$ for 30 min at 4 °C. The precipitate was resuspended in 0.5 mL of 1 N NaOH, vortexed, kept in an ice bath for 30 min, and centrifuged at $21,500\times g$ for 30 min at 4 °C. The sample was collected, acid-hydrolysed and quantified as described for the extraction and quantification of conjugated polyamines.

Data and statistical analysis

The experiment was performed three times. Three biological replicates and three technical replicates were carried out for every biological replicate. Therefore, the data shown in the paper were averages of 9 values \pm S.E. The data were analysed by SPSS 16.0 and Microsoft Excel (SPSS Inc., Armonk, NY, USA). The deviation of the averages was statistically evaluated by two-way analysis of variance (ANOVA) and Duncan's method was used to compare means at the $P < 0.05$ level. The significant differences among multiple groups are indicated by different letters above the columns in the figures and in the tables, which are shown as Supplementary Data 1 and 2.

Results

Dynamic changes in FLRWC and FLRPMP during wheat grain filling under drought, exogenous PAs and inhibitors

Drought resulted in decreased water content in wheat plants, especially in leaves. In the experiment, the FLRWC of the control group with a soil water potential of -0.15 MPa was regarded as 100%. As displayed in Fig. 2A, the FLRWC of wheat plants decreased with decreasing soil water potential. Furthermore, as the soil drought treatment lasted longer, the FLRWC decreased more severely. The FLRWC of the two wheat cultivars decreased over the whole drought period, and it decreased more significantly ($P < 0.05$) in drought-sensitive Wanmai No. 52 (Fig. 2B) than in drought-tolerant Longmai No. 079 (Fig. 2A). Especially at the end drought stage, it decreased to 71% in Wanmai No. 52, whereas in Longmai No. 079, it decreased only to 88%. Treatment with D-Arg, an ADC inhibitor of Put biosynthesis, aggravated the drought-induced decreases in FLRWC of both cultivars, and in Wanmai No. 52 and Longmai No. 079, the FLRWCs decreased to 65% and 67%, respectively. Applying exogenous Put reversed the effect of D-Arg on FLRWC. Exogenous Spd treatment markedly ($P < 0.05$) alleviated the drought-induced decreases in FLRWC of both cultivars, especially the drought-sensitive Wanmai No. 52, while treatment with the inhibitor MGBG or *o*-phenanthroline markedly ($P < 0.05$) aggravated the drought-induced decreases in FLRWC of both cultivars, especially the drought-tolerant Longmai No. 079 (Fig. 2A, B).

The FLRPMPs of the two wheat cultivars increased throughout the whole drought period, and the parameter increased more significantly ($P < 0.05$) in drought-sensitive Wanmai No. 52 (Fig. 2D) than in drought-tolerant Longmai No. 079 (Fig. 2C). The FLRPMPs increased to 38% and 22% at the end stage of treatment in Wanmai No. 52 and Longmai No. 079, respectively. D-Arg treatment aggravated the drought-induced increases in FLRPMPs of both cultivars, especially Longmai No. 079. The FLRPMPs of Wanmai No. 52 and Longmai No. 079 increased to 42% and 40%, respectively. Applying exogenous Put markedly ($P < 0.05$) reversed the effect of D-Arg treatment on FLRPMP. Exogenous Spd treatment markedly ($P < 0.05$) alleviated the drought-induced

increases in FLRPMPs of the two cultivars, especially the drought-sensitive Wanmai No. 52, while the treatment with inhibitor, MGBG or *o*-phenanthroline, markedly ($P < 0.05$) aggravated the drought-induced increases in FLRPMPs of both cultivars, especially the drought-tolerant Longmai No. 079 (Fig. 2C, D).

Effects of drought, exogenous PAs and inhibitors on wheat 1000-grain weight and grain number per spike

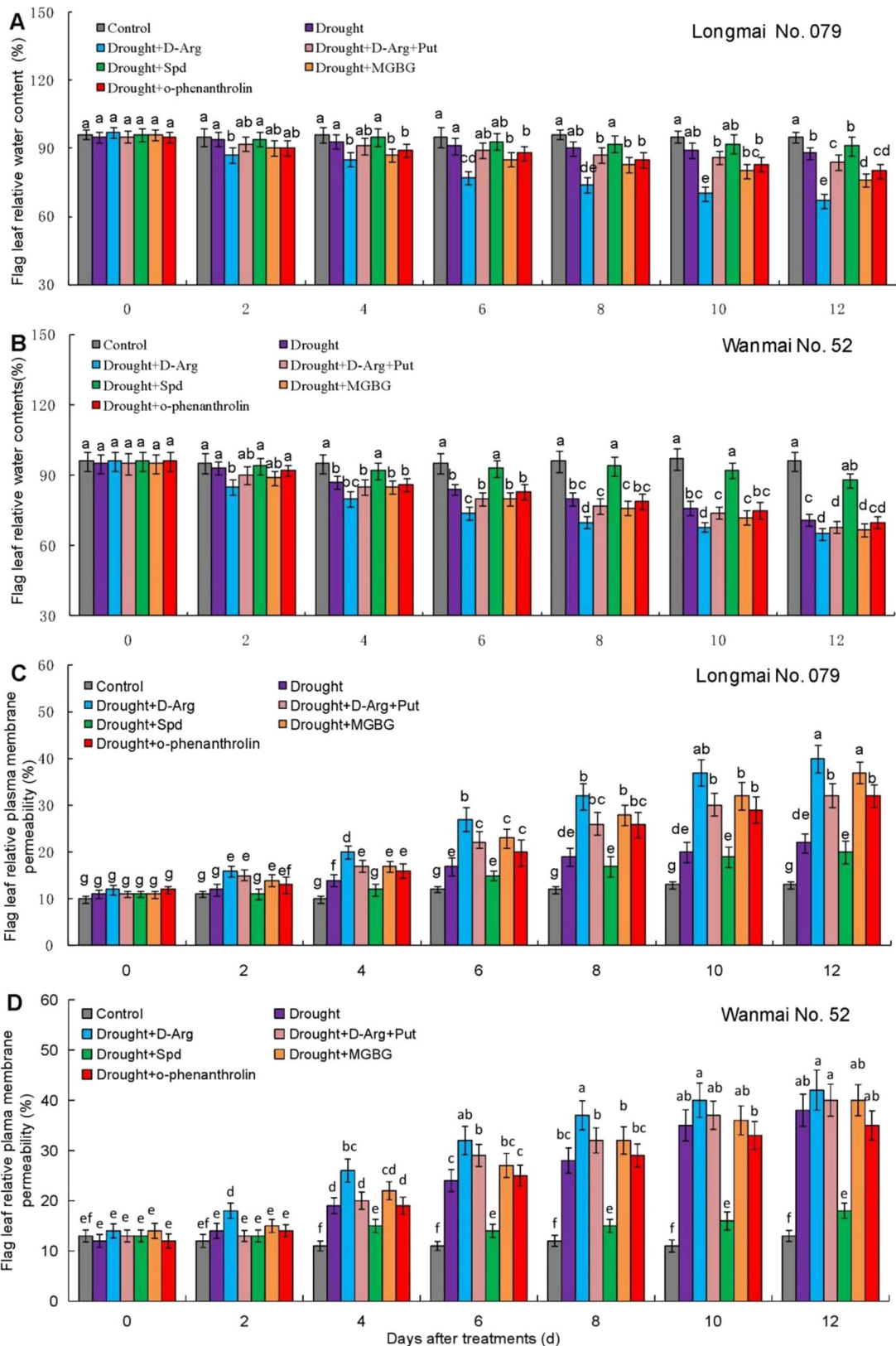
Drought treatment distinctly decreased the 1000-grain weight (Fig. 3A) and grain number per spike (Fig. 3B) of the two wheat cultivars compared with the control, and the two parameters of Wanmai No. 52 decreased by 25.9% and 32.3%, respectively, whereas they decreased by 6.9% and 8.8% in Longmai No. 079, respectively, indicating that they decreased more significantly in Wanmai No. 52 than in Longmai No. 079. Applying D-Arg, MGBG or *o*-phenanthroline aggravated the drought-induced decreases in the two parameters of the two wheat cultivars, and the effects of the inhibitors were more marked ($P < 0.05$) on Longmai No. 079 than on Wanmai No. 52. Exogenous Put treatment obviously reversed the D-Arg effect. Exogenous Spd or Spm alleviated the effects of drought on the two parameters of both cultivars, especially the drought-sensitive Wanmai No. 52.

Dynamic changes in ADC activity in grain embryos under drought and inhibitor D-Arg

The ADC activities in grain embryos of both cultivars rose continuously with the prolonged stress time (Fig. 4). At the end drought stage, the ADC activities were 329 and 320 nmol Agm g^{-1} FW h^{-1} in Longmai No. 079 (Fig. 4A) and Wanmai No. 52 (Fig. 4B), respectively. The results indicated that there was no marked difference in ADC activity between the two cultivars. D-Arg partially inhibited ADC activity over the whole drought period.

Dynamic changes in the levels of free PAs in grain embryos under drought, exogenous PAs, and inhibitors

The free Put contents in grain embryos of both cultivars increased drastically at the earlier drought stage, while on the 4th day, the contents began to decrease (Fig. 5). However, the decrease in free Put at the later



◀**Fig. 2** Dynamic changes in FLRWC (A, B) and FLRPMP (C, D) during wheat grain filling under drought, exogenous PAs and inhibitors. The data shown here were averages of 9 values \pm S.E. The deviation of the averages was statistically evaluated by two-way analysis of variance (ANOVA) and the multiple means of the groups were compared at $P < 0.05$ level by Duncan's method. The significant differences among multiple groups were indicated by different letters above the columns in the figures

drought stage was more significant in drought-tolerant Longmai No. 079 (Fig. 5A) than in Wanmai No. 52 (Fig. 5B). For example, at the end drought stage, the free Put contents reached 137 and 365 nmol g⁻¹ FW in Longmai No. 079 (Fig. 5A) and Wanmai No. 52 (Fig. 5B), respectively. D-Arg inhibited the drought-induced increase in free Put in the two cultivars. Applying exogenous Put, Spd, Spm or the inhibitor MGBG enhanced the drought-induced increase in free Put and the MGBG effect was more significant than the others. Due to the similar effects to exogenous Spd, the data on the effects of exogenous Spm on endogenous free PAs are not shown here.

The free Spd contents in the embryos of both cultivars increased continuously throughout the whole drought period (Fig. 6). However, the increase was more significant ($P < 0.05$) in drought-tolerant Longmai No. 079 (Fig. 6A) than in Wanmai No. 52 (Fig. 6B) during the later period (from the 4th to the 12th day). Applying exogenous Put and Spd enhanced the increase induced by drought in endogenous free Spd, while inhibitor D-Arg, MGBG and *o*-phenanthroline treatment dramatically inhibited the increase. The trends of change in free Spm were similar to those of free Spd in grain embryos of both wheat cultivars under drought, exogenous PAs and inhibitors (Fig. 7).

Dynamic changes in the levels of conjugated PAs in grain embryos under drought

Three conjugated PAs, Put (Fig. 8A, B), Spd (Fig. 8C, D) and Spm (Fig. 8E, F), were detected in this study. On the 6th day of drought treatment, the conjugated Put levels of both cultivars began to increase and reached the highest level at the end stage. However, there was no marked difference in the conjugated Put level between the two cultivars. Furthermore, the levels of conjugated Spd and conjugated Spm increased from the 8th to the 12th day of drought treatment, but

it seemed that there was no regularity in dynamic changes in the two conjugated PA levels.

Dynamic changes in the levels of bound PAs in grain embryos under drought and the inhibitor *o*-phenanthroline

Among the three bound PAs, the Put content was the highest (Fig. 9A, B), followed by Spd (Fig. 9C, D), and the Spm content was the lowest (Fig. 9E, F). The three bound PAs began to increase on the 4th day of drought treatment, and the increases in bound PAs were more significant ($P < 0.05$) in drought-tolerant Longmai No. 079 than in drought-sensitive Wanmai No. 52. In particular, the bound Put level in Longmai No. 079 rose from 75 to 235 nmol g⁻¹ FW, while in Wanmai No. 52, it only rose from 62 to 83 nmol g⁻¹ FW. Applying the inhibitor *o*-phenanthroline markedly inhibited ($P < 0.05$) the drought-induced increases in bound PAs.

Dynamic changes in SAMDC activity in grain embryos under drought and the inhibitor MGBG

SAMDC activity in grain embryos of the control group was approximately invariable and began to increase slightly at the end stage of grain filling (Fig. 10). Nevertheless, drought obviously elevated SAMDC activity over the whole treatment period. More interestingly, the drought-induced increase in SAMDC activity was much more significant ($P < 0.05$) in drought-tolerant Longmai No. 079 (Fig. 10A) than in drought-sensitive Wanmai No. 52 (Fig. 10B), especially from the 4th to the 12th day of the drought treatment period. On the 12th day, it increased by 57.1% and 37.8% in Longmai No. 079 and Wanmai No. 52, respectively. MGBG treatment significantly inhibited ($P < 0.05$) the drought-induced increase in SAMDC activity.

Dynamic changes in TGase activity in grain embryos under drought and the inhibitor *o*-phenanthroline

The change in TGase activity in grain embryos was indistinctive at the earlier drought stage (Fig. 11). However, on the 4th day, the activity began to increase in both Longmai No. 079 (Fig. 11A) and Wanmai No. 52 (Fig. 11B), and the increase in the former was more significant ($P < 0.05$) than that in the latter.

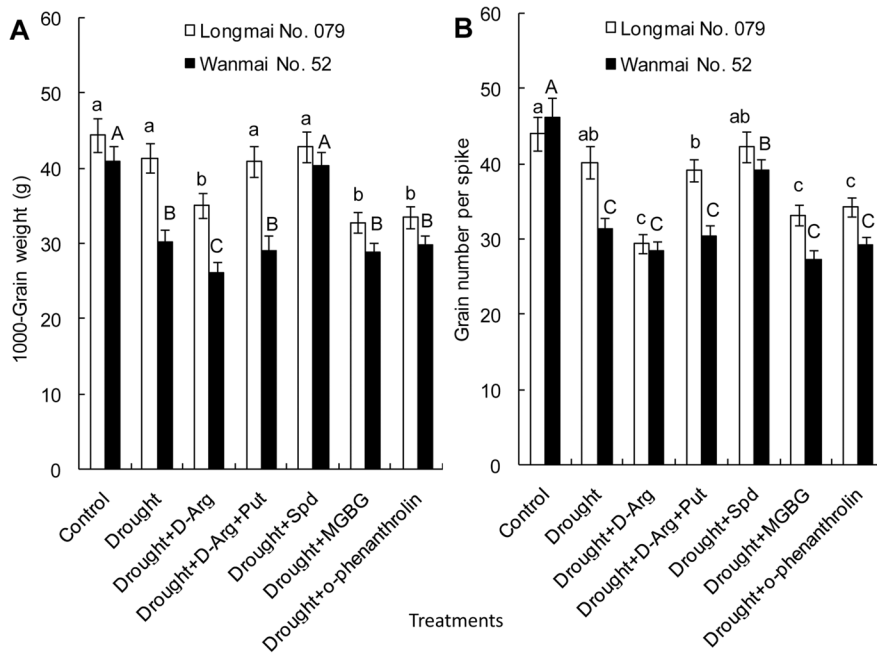


Fig. 3 Effects of drought, exogenous PAs, and inhibitors on wheat 1000-grain weight (A) and grain number per spike (B). The data shown here were averages of 9 values \pm S.E. The deviation of the averages was statistically evaluated by two-way analysis of variance (ANOVA) and the multiple means of the groups were compared at $P < 0.05$ level by Duncan's

method. The significant differences among multiple groups were indicated by different letters above the columns in the figures. Lower case letters discriminated treatment differences in Longmai No. 079, and capital letters discriminated treatment differences in Wanmai No. 52

On the 12th day, in Longmai No. 079, the activity was over 3 times as high as the value of the control group, while in Wanmai No. 52, it rose by merely 90%. Applying the inhibitor *o*-phenanthrolin markedly inhibited ($P < 0.05$) the drought-induced increase in TGase activity.

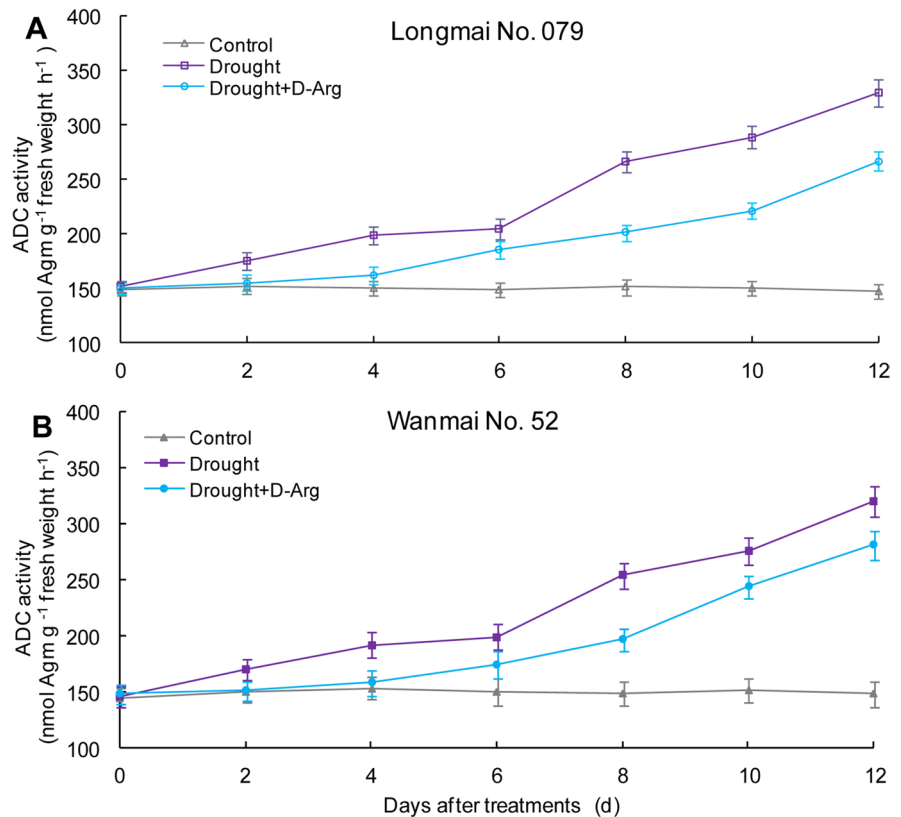
Discussion

Selecting wheat cultivars that differ in drought tolerance

Selecting wheat cultivars that differ in drought tolerance was useful to analyse cultivar differences in dynamic changes in different PA types and forms in grain embryos during the drought period. Two wheat cultivars, Longmai No. 079 and Wanmai No. 52, were selected as experimental materials since Longmai No. 079 is planted in dry ecotopes of Northwest China, such as the Gansu and

Ningxia Provinces, while Wanmai No. 52 is distributed in rain-rich ecotopes of Central China, such as the Anhui, Jiangsu and Hubei Provinces. Secondly, drought inhibits growth, which is usually quantified by biomass accumulation (Bányai et al. 2017; Kaur and Asthir 2017); therefore, by analysing cultivar differences in important agronomic traits, such as 1000-grain weight (Fig. 3A) and grain number per spike (Fig. 3B), it could be inferred that Longmai No. 079 and Wanmai No. 52 were drought tolerant and drought sensitive, respectively. To further verify the two cultivar differences in drought tolerance, FLRPMP and FLRWC, which were closely associated with membrane injury and drought stress respectively, were assessed. Drought stress increased FLRPMP (Fig. 2C, D) and decreased FLRWC (Fig. 2A, B) much more markedly in Wanmai No. 52 than in Longmai No. 079. Therefore, we concluded that Wanmai No. 52 was drought sensitive and Longmai No. 079 was drought tolerant.

Fig. 4 Dynamic changes in ADC activity in grain embryos under drought and D-Arg. The data shown here were averages of 9 values \pm S.E



Significance of free Put accumulation in filling grain embryos at the earlier drought stage

NO unanimous conclusion can be drawn regarding the significance of free Put accumulation under abiotic stresses. Free Put build-up can cause injury and decrease plant tolerance to environmental stresses because oxidation and degradation products of Put, such as H₂O₂, amido aldehyde and propylene aldehyde, can be cross-linked with proteins and nucleic acids, leading to cell senescence and apoptosis (Di Tomaso et al. 1989; Pedrol et al. 2000; Rodriguez et al. 2001). For example, Jing et al. (2020) reported that the free Put content in grains was negatively correlated with the grain weight of wheat plants under heat stress. Furthermore, exogenous Put enhanced the inhibitory effect of drought on wheat grain filling (Liu et al. 2016). However, some recent studies have suggested that Put can alleviate the injury symptoms induced by stresses and improve plant tolerance to abiotic stresses (Ebeed et al. 2019; Ahmad and Ali 2019; Hassan et al. 2020; Upadhyay et al. 2021). These differences might be

attributed to different treatment periods and experimental materials, especially cultivar variation in tolerance. To address this issue, dynamic changes in free Put were examined throughout the whole stress period, in two wheat cultivars differing in drought tolerance. Drought substantially increased free Put not only in the drought-sensitive wheat cultivar (Fig. 5B), but also in the drought-tolerant cultivar at the earlier stress stage (Fig. 5A). Furthermore, coupled with the significant decreases in the free Put level and ADC activity, which was inhibited by D-Arg (Fig. 4), drought tolerance of both cultivars decreased markedly, as judged by changes in FLRW (Fig. 2A, B), FLRPMP (Fig. 2C, D), 1000-grain weight (Fig. 3A) and grain number per spike (Fig. 3B). Thus free Put accumulation during early drought might be essential for plant stress tolerance, as free Put could play a crucial role in signal transduction under abiotic stresses (Alcázar et al. 2020; Gondor et al. 2021; Gonzalez et al. 2021). Whether drought-induced increases in free Put levels could enhance stress tolerance would depend on whether

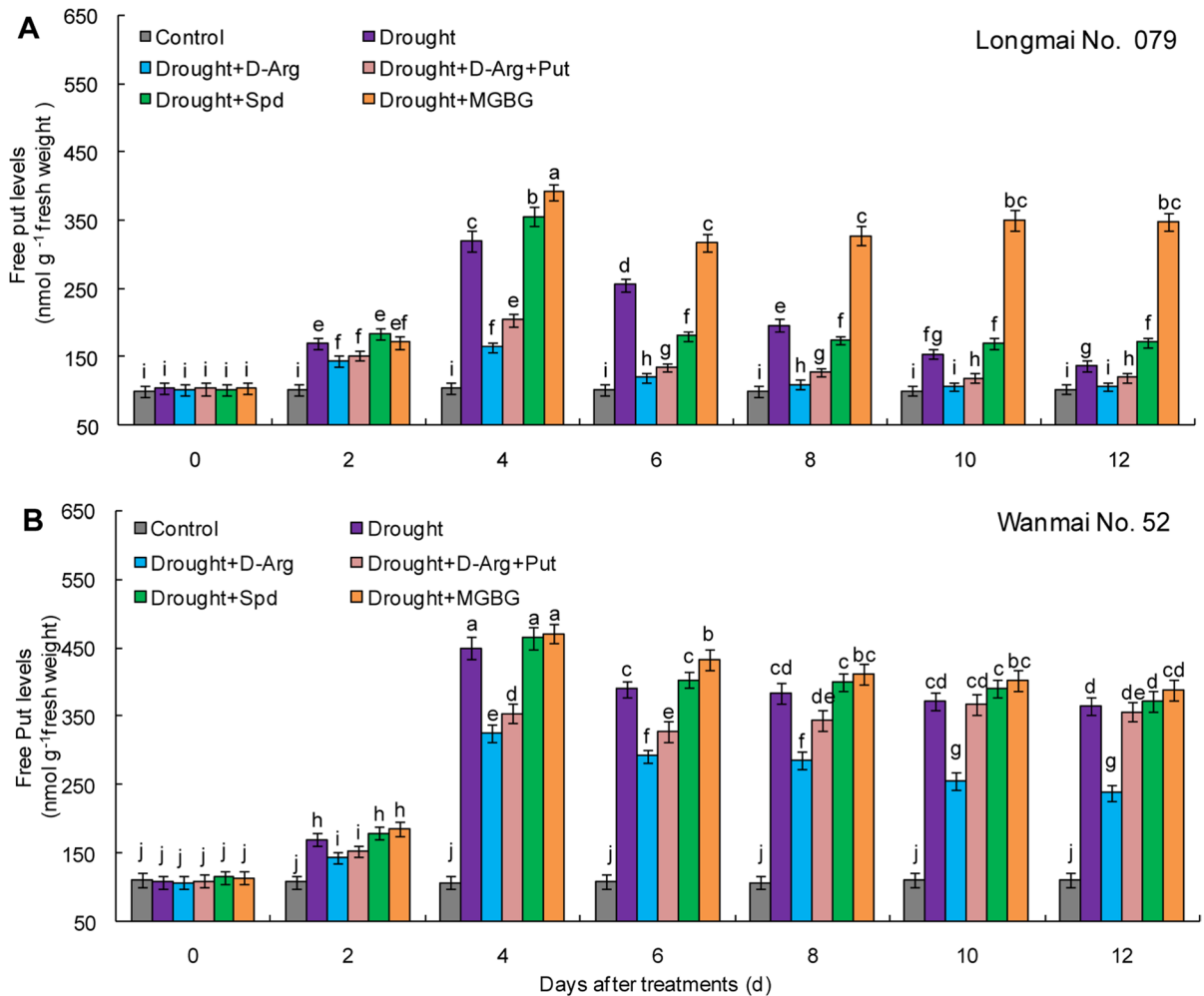


Fig. 5 Dynamic change in free Put level in grain embryos under drought, exogenous PAs, and inhibitors. The data shown here were averages of 9 values \pm S.E. The deviation of the averages was statistically evaluated by two-way analysis of

variance (ANOVA) and the multiple means of the groups were compared at $P < 0.05$ level by Duncan's method. The significant differences among multiple groups were indicated by different letters above the columns in the figures

free Put could be converted into free Spd and Spm, and other forms of PAs at the later drought stage.

Significance of free Put conversions into free Spd and Spm in filling grain embryos at the later drought stage

Since drought-induced increases in free Spd (Fig. 6) and Spm (Fig. 7) were more significant ($P < 0.05$) in the drought-tolerant cultivar than in the drought-sensitive cultivar during the later period, we concluded that free Spd and Spm might enhance drought tolerance. Furthermore, by analysing the dynamic changes

in the three free PAs, increases in free Spd and Spm in the drought-tolerant cultivar agreed well with the decrease in free Put (Fig. 5A) and all the changes in free PAs began simultaneously after 4 days of drought. It was inferred that the drought-induced increases in free Spd and Spm were mainly from free Put conversion, which was confirmed by applying the inhibitors D-Arg and MGBG. D-Arg inhibited free Put biosynthesis, coupled with the decreases in free Spd and Spm; while MGBG inhibited the conversion of free Put into free Spd and Spm by inhibiting the drought-induced increase in SAMDC activity (Fig. 10), coupled with the decreased drought

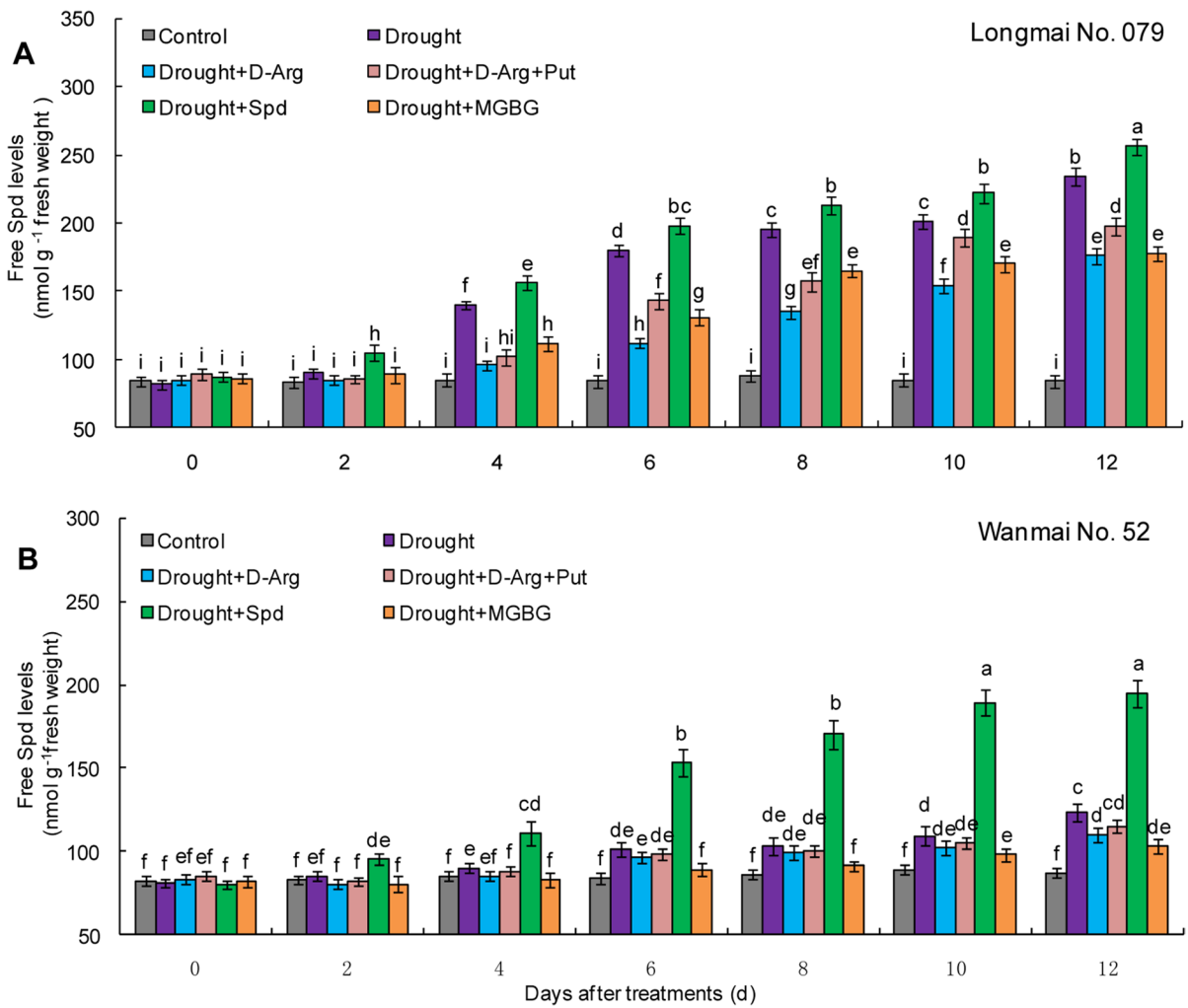


Fig. 6 Dynamic change in free Spd level in grain embryos under drought, exogenous PAs, and inhibitors. The data shown here were averages of 9 values \pm S.E. The deviation of the averages was statistically evaluated by two-way variance

(ANOVA) and the multiple means of the groups were compared at $P < 0.05$ level by Duncan’s method. The significant differences among multiple groups were indicated by different letters above the columns in the figures

tolerance evidenced by the decreases in FLRW (Fig. 2A, B), 1000-grain weight (Fig. 3A) and grain number per spike (Fig. 3B), and increased FLRPMP (Fig. 2C, D). Additionally, applying exogenous Spd increased endogenous free Spd and Spm in grain embryos, coupled with an increase in drought tolerance (Figs. 2 and 3), especially in the drought-sensitive cultivar due to the inherently high background levels of Spd and Spm in the tolerant cultivar. Correlation analysis further supported these findings. After 12 days of drought, the total content of free Spd+free Spm in grain embryos was positively

significantly correlated with 1000-grain weight ($r_{0.05} = 0.95$, $n = 10$) (Fig. 12A) and grain number per spike ($r_{0.05} = 0.90$, $n = 10$) (Fig. 12B). Additionally, the ratio of free Spd+free Spm/free Put was also positively correlated with 1000-grain weight ($r_{0.05} = 0.76$, $n = 10$) (Fig. 12C) and grain number per spike ($r_{0.05} = 0.59$, $n = 10$) (Fig. 12D).

Our findings agreed with previous studies (Yang et al. 2007; Gupta et al. 2013; Luo et al. 2019; Jing et al. 2020; Zarza et al. 2020). Since Spd and Spm carry more positive charges, they could be linked to biomacromolecules, such as acidic proteins and

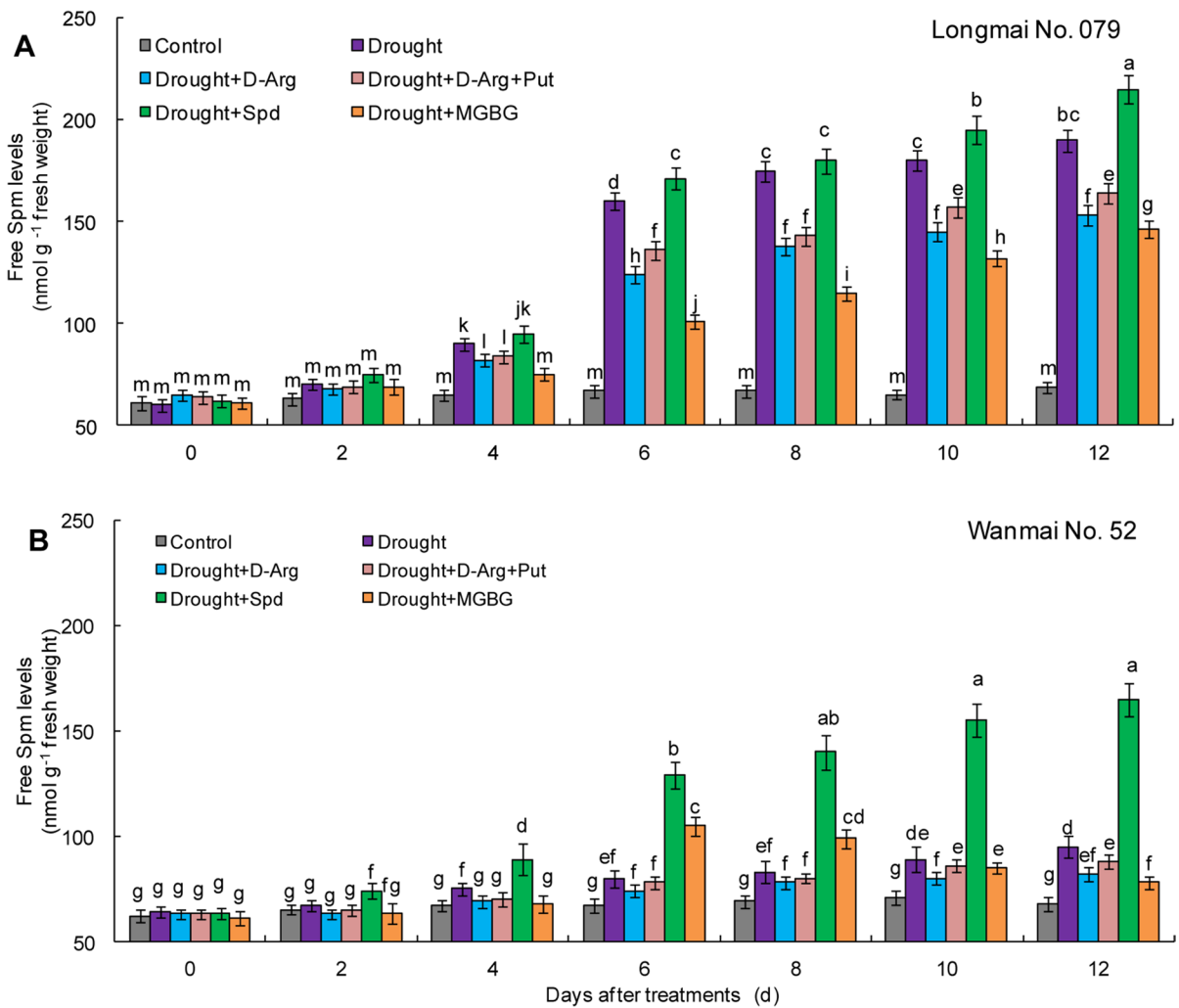


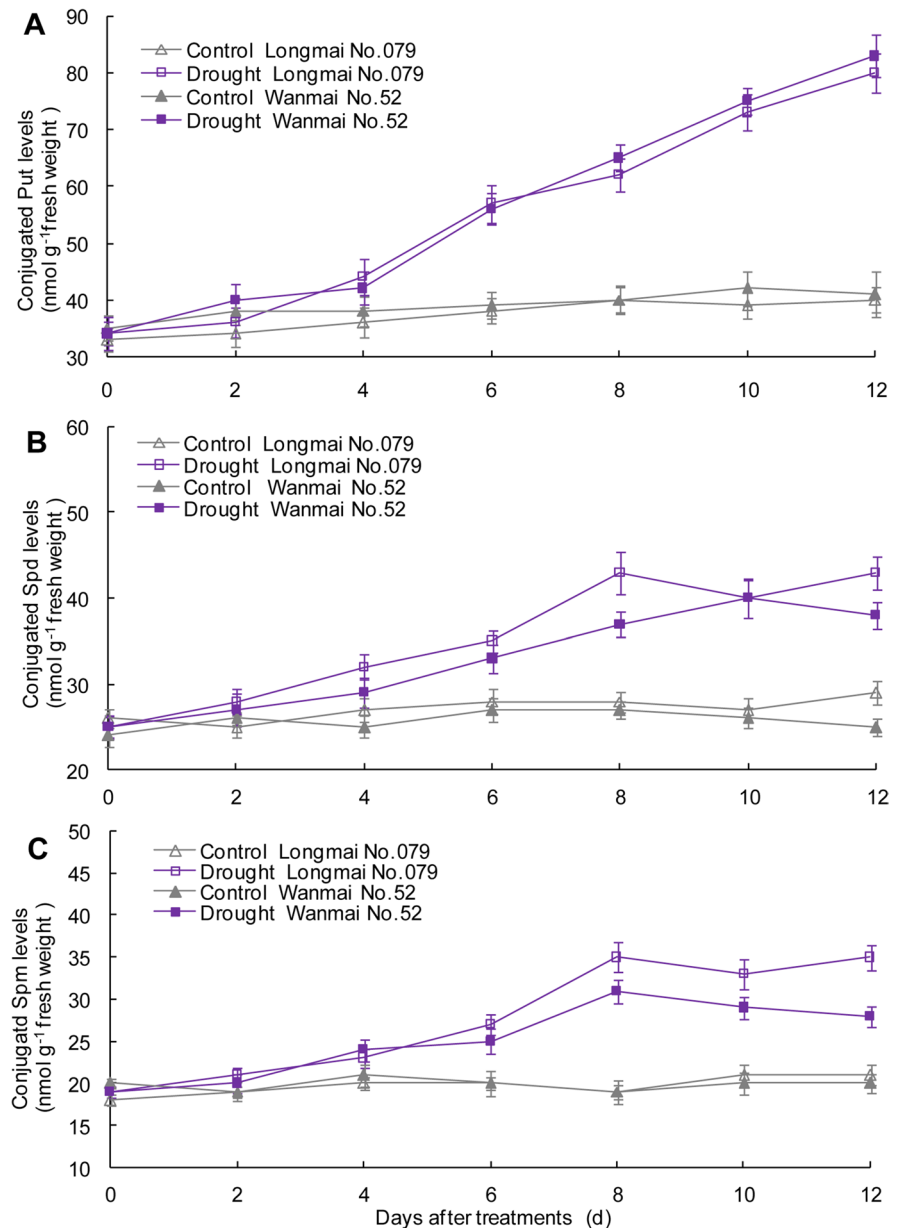
Fig. 7 Dynamic change in free Spm level in grain embryos under drought, exogenous PAs, and inhibitors. The data shown here were averages of 9 values \pm S.E. The deviation of the averages was statistically evaluated by two-way variance

(ANOVA) and the multiple means of the groups were compared at $P < 0.05$ level by Duncan's method. The significant differences among multiple groups were indicated by different letters above the columns in the figures

membrane phospholipids, more easily than Put, and could play important roles in drought responses (Yamaguchi et al. 2007; Kubis 2008; Dutra et al. 2013). Farooq et al. (2009) reported that Spm was the most effective in enhancing drought tolerance among PAs. Additionally, polyamines seem to regulate cell membrane proteins such as two major vacuolar cation channels and plasma membrane H^+ -ATPase (Janicka-Russak et al. 2010; Pottosin and Shabala 2014; Pottosin et al. 2021; Du et al. 2022). Thus Jing et al. (2020) found that the free Spd level in grains was positively correlated

with wheat grain weight and that exogenous Spm/Spd enhanced heat tolerance of wheat plants. Furthermore, Jing et al. (2020) reported that exogenous Spm better alleviated heat stress injury in wheat plants than Spd, unlike our experiment where exogenous Spm and exogenous Spd had similar effects on enhancing wheat drought tolerance. Different abiotic stresses and wheat cultivars might account for this discrepancy. Additionally, our findings agreed with observations that the grain weight and filling rate were positively and significantly correlated with endogenous free Spd and Spm levels, and

Fig. 8 Dynamic changes in the levels of conjugated Put (A, B), conjugated Spd (C, D) and conjugated Spm (E, F) in grain embryos under drought. The data shown here were averages of 9 values \pm S.E



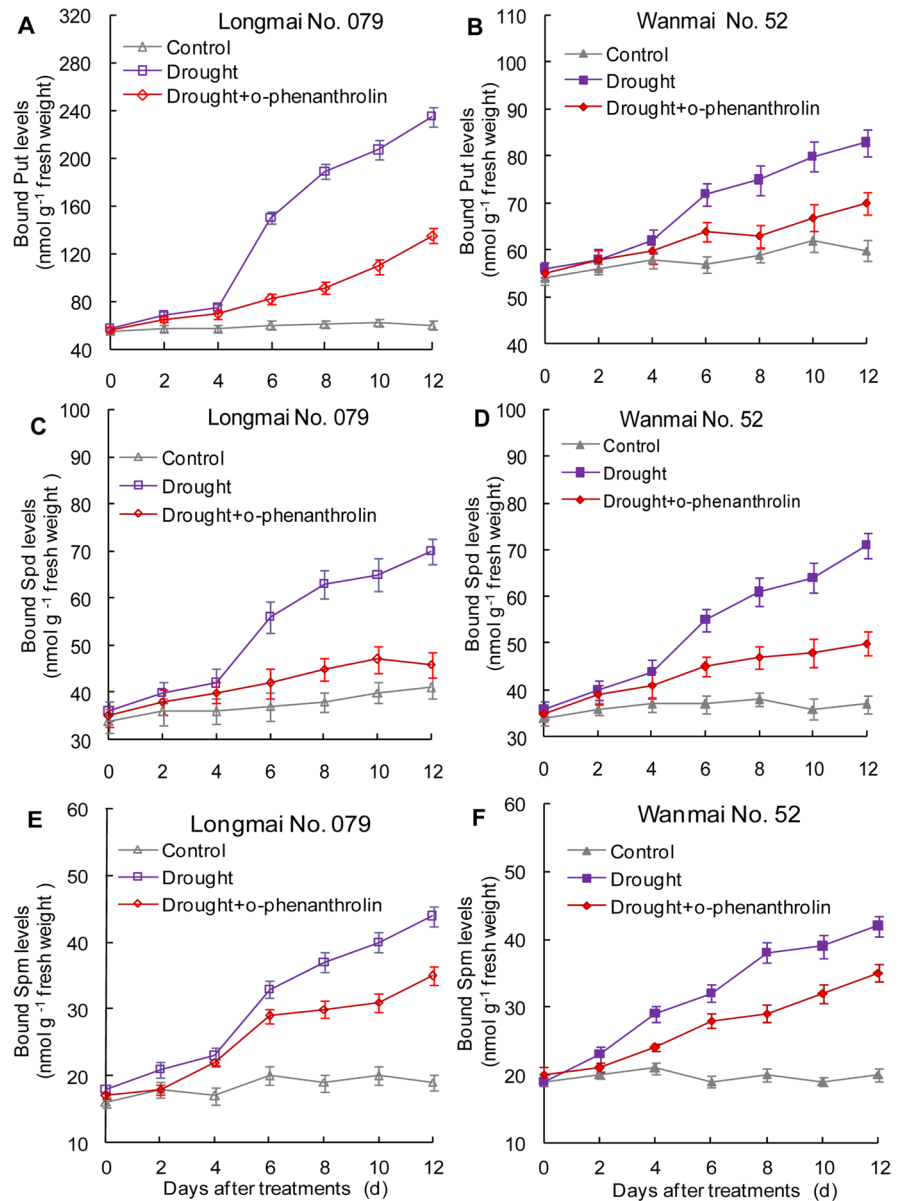
higher SAMDC activity in wheat grains enhanced the conversions of free Put into free Spd and Spm and increased the levels of free Spd and Spm (Liu et al. 2013a, b; 2016). Although Luo et al. (2019) used well-watered plants, their discovery also agreed with our findings in droughted wheat. The precise function of free Put in enhancing drought tolerance is still not clear, but our research revealed that under drought, the conversions of free Put into free Spd and Spm were the main pathway by which

the Spd and Spm levels were elevated and therefore drought tolerance was enhanced.

Free Put conversion to conjugated Put in filling grain embryos at the later drought stage

Free PAs can be converted into acid-soluble conjugated PAs. Drought increased three conjugated PAs, especially the conjugated Put level. However, the two cultivars did not markedly differ in conjugated

Fig. 9 Dynamic changes in the levels of bound Put (A, B), bound Spd (C, D) and bound Spm (E, F) in grain embryos under drought and *o*-phenanthrolin. The data shown here were averages of 9 values \pm S.E



PA contents (Fig. 8), suggesting that this PA form in filling grain embryos might not be directly involved in drought tolerance, as in the study of Yang et al. (2007) in rice. In the present study, since conjugated PA biosynthesis inhibitors were not applied, more convincing data could not be supplied for further verification. However, Bais et al. (2000) showed that conjugated PAs could be regarded as temporary storage pools of free PAs and that there was no direct relationship between conjugated PAs and

abiotic stress tolerance. While conjugated PA was important in callus proliferation under PEG osmotic stress (Kong et al. 1998), most previous studies suggested that conjugated PAs were correlated with organ development (Scaramagli et al. 2000; Shinozaki et al. 2000; Puga-Hermida et al. 2003) and disease resistance (Rabiti et al. 1998; Musetti et al. 1999). In any case, whether conjugated PAs might function in enhancing drought tolerance deserves further exploration.

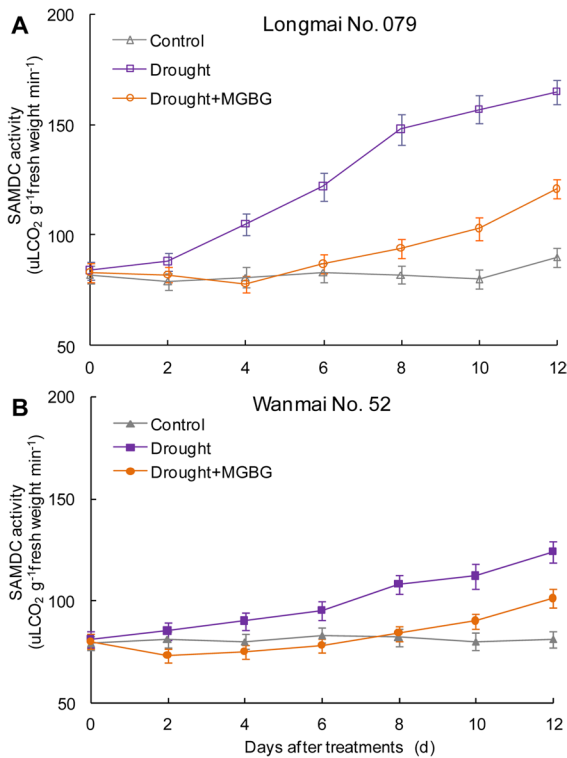


Fig. 10 Dynamic changes in SAMDC activity in grain embryos under drought and MGBG. The data shown here were averages of 9 values \pm S.E

Significance of free Put conversion to bound Put in filling grain embryos at the later drought stage

Bound PAs, especially bound Put, might be involved in drought tolerance because this PA form increased more significantly in the drought-tolerant cultivar than in the sensitive cultivar (Fig. 9). Furthermore, the bound Put content in grain embryos was positively significantly correlated with the 1000-grain weight ($r_{0.05}=0.99$, $n=4$) (Fig. 12E) and grain number per spike ($r_{0.05}=0.99$, $n=4$) (Fig. 12F). Additionally, the total content of bound Put + Spd + Spm was also positively and significantly correlated with the 1000-grain weight ($r_{0.05}=0.98$, $n=4$) (Fig. 12G) and grain number per spike ($r_{0.05}=0.98$, $n=4$) (Fig. 12H). Furthermore, analysing dynamic changes in bound Put determined that the increases in bound Put agreed with the decrease in free Put (Fig. 5), and both changes in bound and free Put occurred simultaneously on the 4th day of drought. Therefore, the

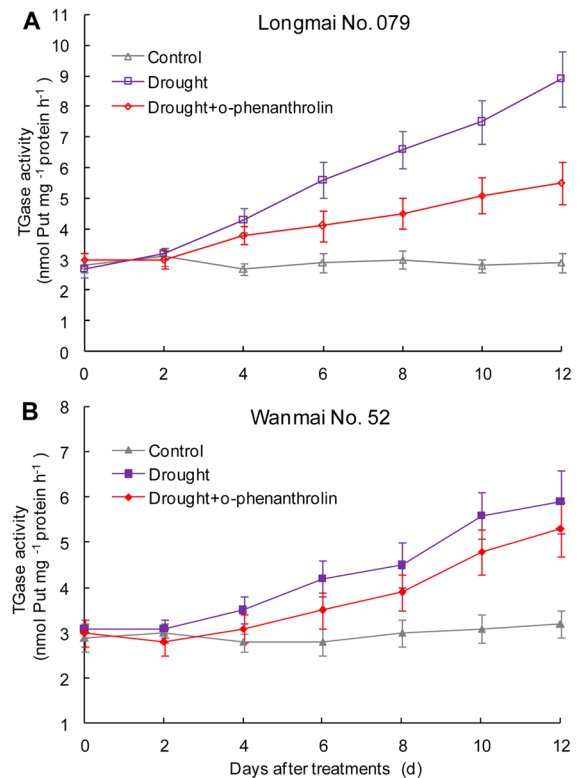
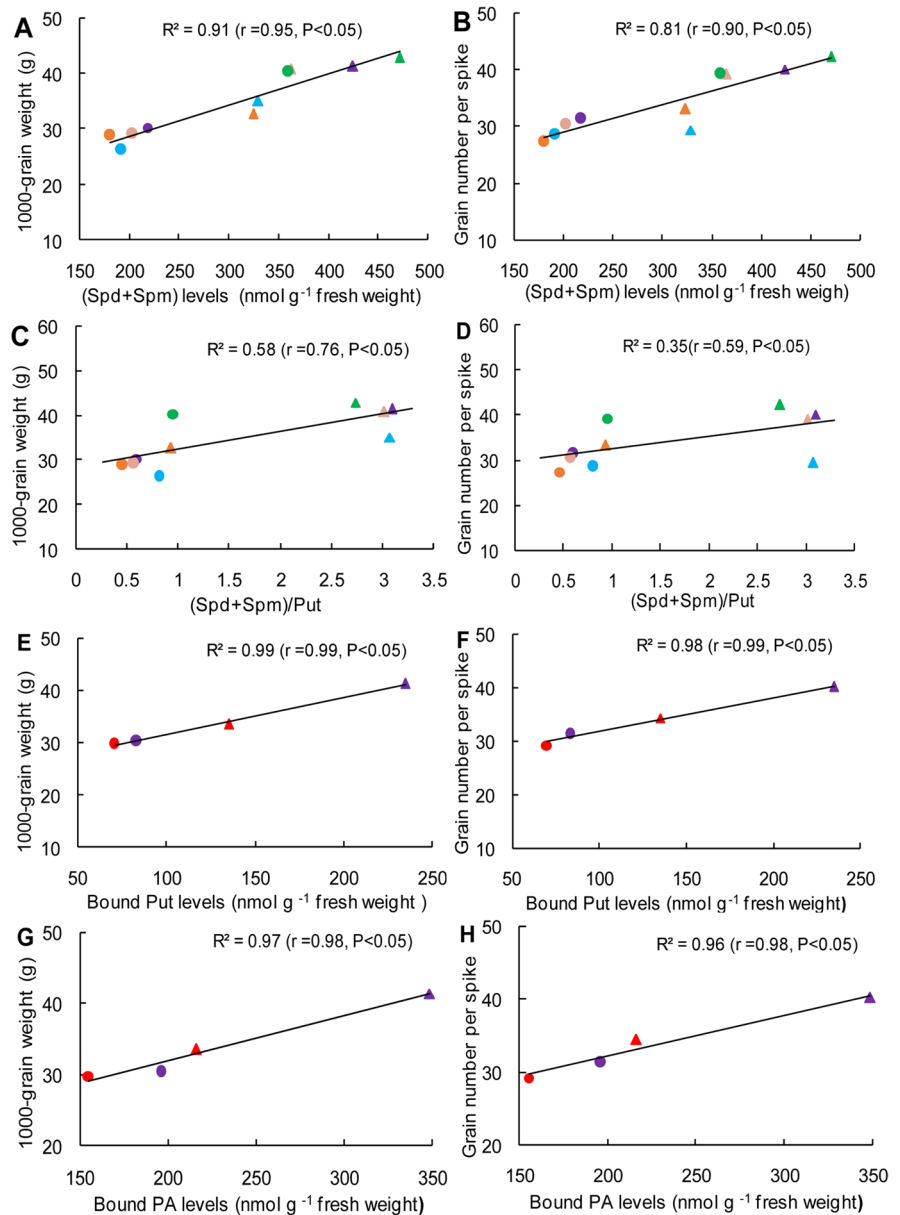


Fig. 11 Dynamic changes in TGase activity in grain embryos under drought and *o*-phenanthroline. The data shown here were averages of 9 values \pm S.E

drought-induced increase in bound Put was inferred to originate mainly from free Put conversion, as verified by applying the inhibitor *o*-phenanthroline, that attenuated drought-induced increases in TGase activity (Fig. 11) and bound PA levels (Fig. 9) and decreased drought tolerance (Figs. 2 and 3). This notion was also supported by previous studies, with the conversion of free PAs into bound PAs functioning in posttranslational modification of chlorophyll proteins and stabilising protein conformation by preventing protein denaturation associated with senescence and programmed cell death (Del Duca et al. 1995, 2014; Serafini-Fracassini et al. 1995). In rice flag leaves, the drought-induced increase in insoluble conjugated Put, which was the same as bound Put in our experiment, was correlated with rice grain yield formation (Yang et al. 2007). Recently, it was reported that bound PAs might enhance salt stress tolerance in tomato and tobacco (Zhong et al. 2019, 2020) and chilling tolerance in

Fig. 12 The results of statistical analysis for correlation ($P < 0.05$). **A**: between total content of free Spd + free Spm and 1000-grain weight; **B**: between total content of free Spd + free Spm grain number per spike; **C**: between ratio of free Spd + free Spm/free Put and 1000-grain weight; **D**: between ratio of free Spd + free Spm/free Put and grain number per spike; **E**: between bound Put content and 1000-grain weight; **F**: between bound Put content and grain number per spike; **G**: between total content of bound Put + Spd + Spm and 1000-grain weight; **H**: between total content of bound Put + Spd + Spm and grain number per spike). Triangular symbol and circular symbol represented Longmai No. 079 and Wanmai No. 52 cultivars, respectively. Furthermore, purple, blue, pink, green, orange and red were used to show drought, drought + D-Arg, drought + D-Arg + Put, drought + Spd, drought + MGBG and drought + o-phenanthrolin, respectively



plum fruit (Du et al. 2021). In particular, our previous research suggested that bound PAs in the plasma membrane could mitigate cell plasmolysis and thereby maintain the membrane conformation of maize grain embryos under drought (Du et al. 2022). By estimating the dynamic changes in PA levels, the present study revealed that the conversions of free Put into free Spd and Spm, and bound Put, rather than free Put itself, enhanced wheat drought tolerance.

Free Put conversion under well-watered and droughted conditions

In well-watered plants, PAs also play a significant role in grain developing and ripening (Hussain et al. 2011). Luo et al. (2019) have proposed that the direct synthetic pathway from free Put to free Spd in the grain is a key factor in promoting grain filling and thousand grain weight in well-watered wheat plants, and the promotive effect of free Spd on the grain

filling is notably related to carbohydrate metabolism in that grain. Under well-watered condition, exogenous Spd, Spm and inhibitor MGBG can affect wheat grain filling by modulating endogenous PA biosynthesis (Liu et al. 2013a, b; Luo et al. 2019). However, whether or how PA levels are modulated during grain filling under drought is yet to be ascertained. All plant tissues and organs contain PAs, especially in the metabolically active embryos, indicating their indispensable role under adverse environmental conditions (Gupta et al. 2013). Free Put accumulation occurred during the earlier drought period and the conversions of free Put into free Spd and Spm, and bound Put occurred at the later drought stage. By analyzing the relationship between the conversions and the tolerance of the two cultivars with different drought tolerance, we conclude that the free Put conversions happen only under drought condition, but not under well-watered condition, thereby conferring drought tolerance to wheat plants. Furthermore, treating drought-stressed plants with exogenous PAs increased the levels of free Spd and Spm, and bound Put in embryos and enhanced the drought tolerance, while treating with inhibitors had contrary effects. Further research should analyze the effects of exogenous PAs and inhibitors in well-watered wheat plants.

Conclusions

In filling grain embryos, the conversions of free Put into free Spd and Spm, and bound Put, rather than free Put itself, function in enhancing the tolerance of wheat plants to drought. This finding might explain why exogenous Put application has different effects on different plant cultivars. In stress-sensitive cultivars, the excessive accumulation of free Put would be oxidized by polyamine oxidase, and the degradation products might have adverse effects on plant cells. Conversely, in stress-tolerant cultivars, exogenous Put could be converted into other forms of PAs, which function in enhancing tolerance. Future work should explore the application of conjugated PA biosynthesis inhibitors and/or use gene (related to ADC, SAMDC and TGase) knockout and recombination technology. Coupling ^{14}C -isotope-labelled exogenous Put with electron microscope autoradiography techniques could examine where Put conversion occurs in the embryo. Additional data would help understand the

mechanisms by which PA enhances plant drought tolerance.

Author contributions HPL and HYD conceived the project; HYD and GTL performed the experiment (except enzyme activity detection); DXL detected enzyme activity; HYD and GTL wrote original draft; HPL and RK reviewed and edited the MS. Authors have already read the MS and agreed to being published in the final version.

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Code availability Not applicable.

Data Availability The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval Not applicable.

Conflict of interest Authors declare no conflict of interest.

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