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Abscisic acid and jasmonic acid are involved in drought priming-induced tolerance to drought in wheat

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ABSTRACT

Drought stress is a limiting factor for wheat production and food security. Drought priming has been shown to increase drought tolerance in wheat. However, the underlying mechanisms are unknown. In the present study, the genes encoding the biosynthesis and metabolism of abscisic acid (ABA) and jasmonic acid (JA), as well as genes involved in the ABA and JA signaling pathways were up-regulated by drought priming. Endogenous concentrations of JA and ABA increased following drought priming. The interplay between JA and ABA in plant responses to drought priming was further investigated using inhibitors of ABA and JA biosynthesis. Application of fluridone (FLU) or nordihydroguaiaretic acid (NDGA) to primed plants resulted in lower chlorophyll-fluorescence parameters and activities of superoxide dismutase and glutathione reductase, and higher cell membrane damage, compared to primed plants (PD) under drought stress. NDGA + ABA, but not FLU + JA, restored priming-induced tolerance, as indicated by a finding of no significant difference from PD under drought stress. Under drought priming, NDGA induced the suppression of ABA accumulation, while FLU did not affect JA accumulation. These results were consistent with the expression of genes involved in the biosynthesis of ABA and JA. They suggest that ABA and JA are required for priming-induced drought tolerance in wheat, with JA acting upstream of ABA.

1. Introduction

Wheat (*Triticum aestivum* L.) is one of the main staple food crops in the world, and it has been predicted that global wheat production should increase by 60% from the current yield level of 3.3 t ha^{-1} to 5 t ha^{-1} by 2050, to feed a population of more than 9 billion [1]. Wheat is susceptible to drought stress, resulting in reduced grain yield and quality [2,3]. The frequency and intensity of drought stress events are expected to increase [4,5]. Improving wheat tolerance to drought stress is thus required for food security under future climate scenarios. Drought priming, defined as pre-exposure of plants to a moderate drought episode, increasing stress tolerance to subsequent stress events [6], has been used as a promising strategy for improving plant tolerance [7,8]. Drought-

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primed wheat plants can achieve higher yield and higher capacity of photo-protection as well as of antioxidant enzyme activities under subsequent drought events than non-primed plants [2,9]. However, the mechanisms of priming-induced tolerance to drought stress are unknown.

Abscisic acid (ABA) has been shown to play a crucial role in the regulation of many processes of plant development and of stress responses, especially under drought conditions [10–12]. ABA can stimulate short-term responses such as closure of stomata and maintenance of water balance [13], and stimulates the transcription and activities of antioxidant enzymes [14,15]. The *9-cis-epoxycarotenoid dioxygenase 3* (*NCED 3*) gene, which encodes the limiting enzyme for ABA biosynthesis in *Arabidopsis* leaves, can be induced by drought stress [16]. The diverse effects of ABA on regulating abiotic stress tolerance are due mainly to the multiple downstream substrates modulated by Snf1-related kinase 2 s (SnRK2s) [17]. In our previous study, primed wheat plants accumulated higher ABA concentrations than non-primed plants under drought stress [2]. ABA mediated increased heat tolerance in tall fescue by stimulating the transcription of stress signaling, ABA responses, and heat protection [18]. However, the role of ABA biosynthesis and signaling involved in drought priming-induced tolerance in wheat remains unknown.

In addition to its involvement in biotic stress, JA participates in abiotic stress responses including drought stress [19,20]. JA pretreatment alleviated the negative effects of drought-induced membrane damage in barley [21]. Genes involved in JA biosynthesis, signaling, and JA-mediated stress responses are involved in drought priming induced stress tolerance [22]. Endogenous JA content transiently increased in *Arabidopsis* after drought stress [23]. JA increased the drought resistance of cauliflower by activating the enzymatic antioxidative system [24]. In transgenic potato overexpressing *allene oxide synthase* (*AOS*), which encodes the enzyme involved in JA biosynthesis, stress damage is alleviated by activation of the expression of drought stress-responsive genes [25]. By contrast, in the absence of *allene oxide cyclase* (*AOC*) gene, rice showed increased tolerance to drought stress in the form of higher stomatal conductance, water use efficiency, and shoot ABA content compared to the wild type [26]. The role of JA in priming-induced drought tolerance in wheat awaits further investigation.

Both ABA- and JA-regulated pathways are critical for maintaining drought priming induced stress tolerance in *Arabidopsis* [27,28]. In rice, both ABA and JA-associated genes were regulated during the first drought exposure, but showed different patterns under the next several drought-stress cycles [29], suggesting a role of ABA and JA in stress memory. Studies of the interaction between ABA and JA are not conclusive, depending partly on the applied stress type and plant species under investigation. For example, JA accumulation is required for ABA accumulation after drought treatment in *Arabidopsis* [30] and increased JA content stimulates the production of ABA in rice under drought stress [31]. These results suggest that JA plays an upstream role relative to ABA. However, other studies show that ABA activates herbivore-induced resistance in plants primed for JA-dependent defenses [32], suggesting that ABA plays an upstream role relative to JA. It is thus still far from clear how JA and ABA interact under drought priming in wheat.

We hypothesized that 1) endogenous ABA and JA are the major signals required for drought priming-induced stress tolerance, and 2) JA is involved in the upstream regulation of ABA in priming-induced tolerance to drought stress. To test these hypotheses, the transcriptome and physiological analyses of plants in response to drought priming were linked to those of endogenous ABA and JA levels and their signaling genes. Inhibitors of ABA and JA biosynthesis were used to elucidate the roles of ABA and JA in resistance mechanisms of drought priming against drought stress.

2. Materials and methods

2.1. Experiment I: effects of drought priming on plant tolerance to a subsequent drought stress event

Uniform seeds of winter wheat cultivar Yangmai 16 were selected and surface-sterilized with 2.5% sodium hypochlorite for 10 min and then rinsed several times with sterile distilled water. The seeds were placed in quartz sand for germination and uniform seedlings were transplanted to plastic containers (45 cm in length, 35 cm in width and 18 cm in height) for hydroponic cultivation at the one-leaf stage. The temperature was set at 22 °C/18 °C (day/night), with a 14 h photoperiod at 300 μmol m^{-2} s^{-1}. At the three-leaf stage, plants were separated into two groups, one group labeled as control (C) with normal hydroponic solution and the other as drought priming (P), with 10% PEG 6000 for 48 h followed by washing and cultivation in normal hydroponic solution. After recovery for 10 days, drought stress was applied with 20% PEG 6000 for 5 days. Three treatments were formed: no drought priming + no drought stress (CC), no drought priming + drought stress (CD), drought priming + drought stress (PD).

2.2. Experiment II: interaction between ABA and JA in drought priming-induced tolerance to drought stress

The treatments were the same as experiment I with four extra treatments under drought stress. In FLU + PD, 1 μmol L^{-1} fluridone (FLU, an ABA synthesis inhibitor, CAS number 59756-60-4) was sprayed before drought priming; in NDGA + PD, 0.2 mmol L^{-1} nordihydroguaiaretic acid (NDGA, a JA synthesis inhibitor, CAS number 500-38-9) was sprayed before drought priming; in FLU + JA + PD, 1 μmol L^{-1} FLU and 0.1 mmol L^{-1} JA were sprayed before drought priming; in NDGA + ABA + PD, 0.2 mmol L^{-1} NDGA and 0.01 mmol L^{-1} ABA were sprayed before drought priming. The concentrations of the inhibitors ABA and JA were chosen following a previous study [33]. The spray volume per square meter was approximately 70 mL, and all growth conditions were the same as in experiment I.

2.3. Leaf relative water content, leaf water potential, photosynthesis, and chlorophyll fluorescence

The latest fully expanded leaves were used for measuring leaf relative water content and leaf water potential following [34]. Leaf photosynthesis was measured with a LI-6400 portable photosynthesis system (LI-COR Biosciences, Lincoln, NE, USA) [35]. Chlorophyll fluorescence was measured with a chlorophyll

fluorescence imager (CF Imager, Technologica, Essex, UK). The latest fully expanded leaf was secured with a clip, the measurement procedure was performed following the user manual, and plants were dark adapted for 30 min. Maximum efficiency of PSII photochemistry (F_v/F_m) and actual PSII photochemical efficiency (Φ_{PSII}) were calculated [36].

2.4. Leaf relative electricity conductivity, H_2O_2 content, malondialdehyde (MDA) content, and antioxidant enzyme activities

Leaf relative electricity conductivity (REC) was measured with a Cole-Parmer 1481-55 conductivity meter (Cole Parmer Instrument Co., Niles, IL, US) [37]. The extraction method for measuring H_2O_2 content, MDA content and antioxidant enzyme activities followed Tan et al. [38], with minor modifications. A leaf sample (0.1 g) was finely homogenized in 3 mL of extraction solution (50 mmol L^{-1} phosphate buffer saline (PBS, pH 7.0), 0.4% (w/v) polyvinylpyrrolidone) and the extraction was used for the assay of H_2O_2 content, MDA content, superoxide dismutase (SOD) and catalase (CAT), and with the addition of 1 mmol L^{-1} ascorbic acid for the ascorbate peroxidase (APX) and glutathione reductase (GR) assays. The content of MDA was measured by the thiobarbituric acid reaction method [39]. The reaction was conducted at 95 °C for 30 min and then cooled on ice immediately. The mixture was centrifuged at 10,000 ×*g* for 10 min and the supernatant was collected for absorbance measurement at 532 and 600 nm. The content of H_2O_2 was measured according to the user's manual of the Hydrogen Peroxide Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China). The activity of SOD (EC 1.15.1.1) was assayed by the photochemical reaction of nitro blue tetrazolium (NBT) [38]. A 3 mL reaction mixture was prepared with 50 mmol L^{-1} PBS containing 130 mmol L^{-1} methionine, 750 μmol L^{-1} NBT, 100 μmol L^{-1} EDTA, and 50 μL of enzyme extract (pH 7.8). The reaction was started with 20 μmol L^{-1} riboflavin by exposure of the tube to light for 10 min and terminated by removal from light and covering of the tubes with black cloth. Absorbance at 560 nm was recorded. The activity of CAT (EC 1.11.1.6) was determined by titration [38], using 100 μL of enzyme extract mixed with 4 mL of 50 mmol L^{-1} PBS (pH 7.0) and incubated at 30 °C for 10 min. The reaction was started by addition of 1 mL of 50 μmol L^{-1} H_2O_2 and stopped after 1 min by addition of 2 mL of 10% H_2SO_4.

Activity of APX (EC 1.11.1.11) was measured by absorbance at 290 nm for 1 min [40]. To 1 mL of reaction solution containing 50 mmol L^{-1} PBS (pH 7.0), 0.5 mmol L^{-1} ascorbic acid, and 0.1 mmol L^{-1} H_2O_2 was added 100 μL of extract. APX activity was determined by monitoring the absorbance at 290 nm. GR (EC 1.6.4.2) activity was determined by absorbance monitored at 340 nm for 3.0 min [41]. The assay mixture contained 500 μL extract mixed with 50 mmol L^{-1} PBS (pH 7.8), 2 mmol L^{-1} Na_2EDTA, 0.15 mmol L^{-1} NADPH.

2.5. ABA and JA assays

Last fully expanded leaves (0.5 g) were ground in liquid nitrogen mixed with 1 mL Milli-Q water and shaken overnight at 4 °C to extract ABA. ABA content was assayed by indirect enzyme-linked immunosorbent assay (ELISA) [42]. JA content was determined with a Plant Jasmonic Acid kit from Dongge Institute of Biotechnology (Beijing, China). The same sample as used for ABA assay was ground in liquid nitrogen and mixed with 5 mL PBS buffer (pH 7.4), and then centrifuged at 14,000 ×*g* for 20 min, the measurement procedure was performed, and JA content was determined following the user manual. Three biological replicates for each treatment were measured.

2.6. RNA-Seq analysis

Last expanded leaves with or without drought priming were frozen in liquid nitrogen and stored at –80 °C. Three biological replicates were used for each treatment. Total RNA was isolated with an OminiPlant RNA Kit (DNase I) (CoWin Biosciences) following the manufacturer's protocol. The ligation products were size-selected by agarose gel electrophoresis, PCR amplified, and sequenced on an Illumina HiSeq 4000 system. The clean reads were matched to the *Triticum aestivum* cDNA database (ftp://ftp.ensemblgenomes.org/pub/release-28/plants/fasta/triticum_aestivum/cdna/Triticum_aestivum.IWGSC1.0) using TopHat v2.0.12/Bowtie v2.2.3 software (open source software available from http://tophat.cbcb.umd.edu).

Gene abundances were quantified by software RSEM (RNA-Seq by Expectation Maximization) [43]. To identify differentially expressed genes (DEGs) across samples or groups, the edgeR package (http://www.r-project.org/) was used. Genes showing an absolute Fold change (FC) > 2 with an adjusted *P*-value (false discovery rate, FDR) < 0.05 were considered as differentially expressed between control and drought-primed plants. These DEGs were then subjected to enrichment analysis of Gene Ontology (GO) functions and KEGG pathways. The primers of genes used for verifying the RNA sequencing data were shown in Table S1.

2.7. Genes expression by quantitative real-time PCR

Total RNA was extracted from 100 mg fresh leaves using RNA TRIzol reagent (Invitrogen, San Diego, CA). The purity and concentration of the RNA extract solution were measured with the NanoDrop 2000 (Thermo Scientific, USA). First-strand cDNA synthesis was performed using Super Smart cDNA Synthesis Kit (Takara, Japan). The primers for genes associated with physiological parameters are listed in Table S2. Quantitative real-time PCR was conducted on an ABI PRISM 7300 Fast Real-Time PCR system (Applied Biosystems, USA) using a SYBR Green I Master mix Kit (Takara) according to the manufacturer's instructions.

2.8. Statistical analysis

Data were subjected to One-way ANOVA using SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Differences were identified at the 0.05 probability level by least significant difference test.

3. Results

3.1. Effects of drought priming on drought stress in wheat seedlings

Leaf relative water content decreased under drought stress, with primed plants (PD) showing higher leaf relative water

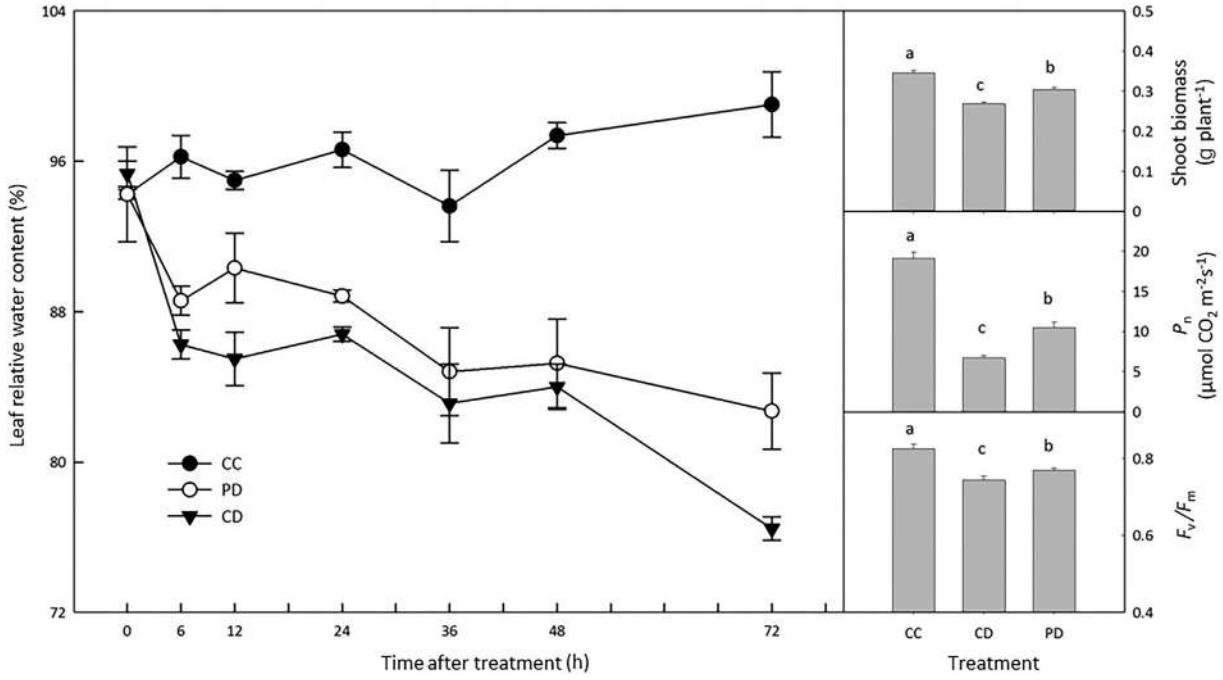

Fig. 1 – Effects of drought priming on shoot biomass and photosynthetic parameters in wheat under drought stress. P_n, *photosynthesis rate*; F_v/F_m, maximum efficiency of PSII photochemistry. CC, no drought priming + no drought stress; CD, no drought priming + drought stress; PD, drought priming + drought stress. Three biological replicates were measured. Different letters indicate statistical significance at $P < 0.05$ within treatments.

contents than non-primed plants (CD) starting 6 h after the start of drought stress treatment. Shoot biomass, leaf photosynthesis, and F_v/F_m decreased with drought stress, with the decrease in these parameters much higher in PD than in CD after three days of treatment (Fig. 1). Root biomass was reduced after three days of drought stress, and PD showed much higher root biomass after five days of drought stress. Root length and mean diameter decreased with drought stress, and the reductions were much lower in PD than in CD, though no significant differences between PD and CD in root surface area and volume were found (Table 1).

3.2. RNA-Seq and physiological analyses of plants after drought priming

In total, 3724 genes were differently regulated by drought priming, with 1245 up-regulated and 2479 down-regulated relative to control (Fig. 2). Many genes associated with the GO terms “response to cellular process”, “metabolic process”, “single-organism process”, and “response to stimulus” were found (Fig. S1). The significantly enriched KEGG pathways were plant pathogen interaction, phenylalanine metabolism, plant hormone signal transduction, photosynthesis, and alpha-linolenic acid metabolism (Fig. 2). Genes involved in

Table 1 – Effects of drought priming on root morphology and root biomass of wheat under drought stress.

Time	Treatment	Root length (m)	Root surface area (cm^2)	Root mean diameter (mm)	Root volume (cm^3)	Root dry biomass (mg)
1 d after stress	CC	17.8 ± 0.5 a	160 ± 3.1 a	0.27 ± 0.01 a	1.06 ± 0.10 a	97.1 ± 5.1 a
	CD	15.4 ± 0.9 b	125 ± 6.7 b	0.26 ± 0.02 a	0.85 ± 0.07 b	96.1 ± 3.9 a
	PD	15.5 ± 0.6 b	126 ± 4.3 b	0.27 ± 0.02 a	0.85 ± 0.08 b	97.2 ± 6.27 a
3 d after stress	CC	19.2 ± 0.9 a	174 ± 56.0 a	0.28 ± 0.02 a	1.29 ± 0.09 a	124.3 ± 13.1 a
	CD	15.6 ± 0.7 b	131 ± 4.5 b	0.26 ± 0.01 b	0.87 ± 0.08 b	98.2 ± 3.4 b
	PD	15.8 ± 0.5 b	135 ± 4.1 b	0.28 ± 0.01 a	0.87 ± 0.08 b	106.0 ± 7.7 b
5 d after stress	CC	20.8 ± 1.3 a	191 ± 6.8 a	0.29 ± 0.02 a	1.26 ± 0.05 a	157.6 ± 6.5 a
	CD	16.0 ± 0.3c	134 ± 4.5 b	0.27 ± 0.01 b	0.87 ± 0.07 b	102.6 ± 7.2c
	PD	18.2 ± 0.8 b	148 ± 4.7 b	0.29 ± 0.01 a	1.00 ± 0.09 b	114.6 ± 9.3 b

CC, no drought priming + no drought stress; CD, no drought priming + drought stress; PD, drought priming + drought stress. Three biological replicates were measured. Different letters within the same column indicate statistical significance at $P < 0.05$.

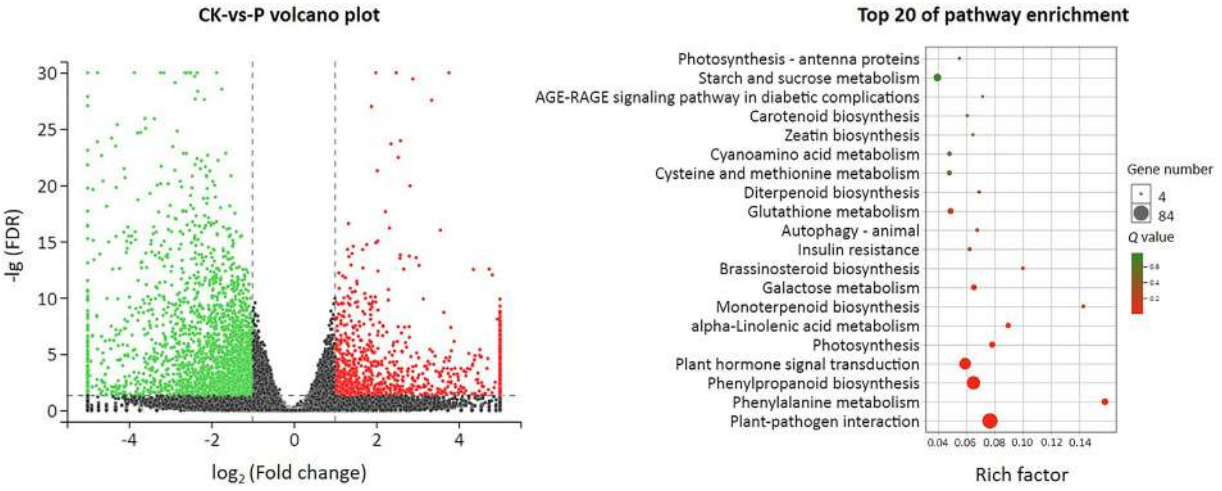

Fig. 2 – RNA-Seq analysis of wheat plants under drought priming. C, no drought priming; P, drought priming. The KEGG pathway annotated genes differently expressed between primed plants and non-primed plants during drought priming.

biosynthesis of ABA and JA, stress response, and sugar metabolism were up-regulated by drought priming relative to the control treatment. Seven differentially expressed transcripts from RNA-Seq were used to validate the RNA-Seq expression data and its reliability using qPCR analysis. The qPCR measurements were highly correlated with the transcript abundance from RNA-Seq analysis ($y = 1.1334x - 8.124$, $r = 0.8567$) (Fig. S2).

Time-course analysis showed that drought priming increased leaf ABA content after 3 h, with leaf JA showing a similar tendency (Fig. 3). Under drought stress, the contents of ABA and JA were increased, compared with CC. ABA content was higher in PD than in CD after 6 h, whereas JA content was higher from six to 12 h and lower from 24 h to 48 h in PD than in CD. The expression of *NCED1*, which encodes the key biosynthetic enzyme in ABA biosynthesis, was up-regulated

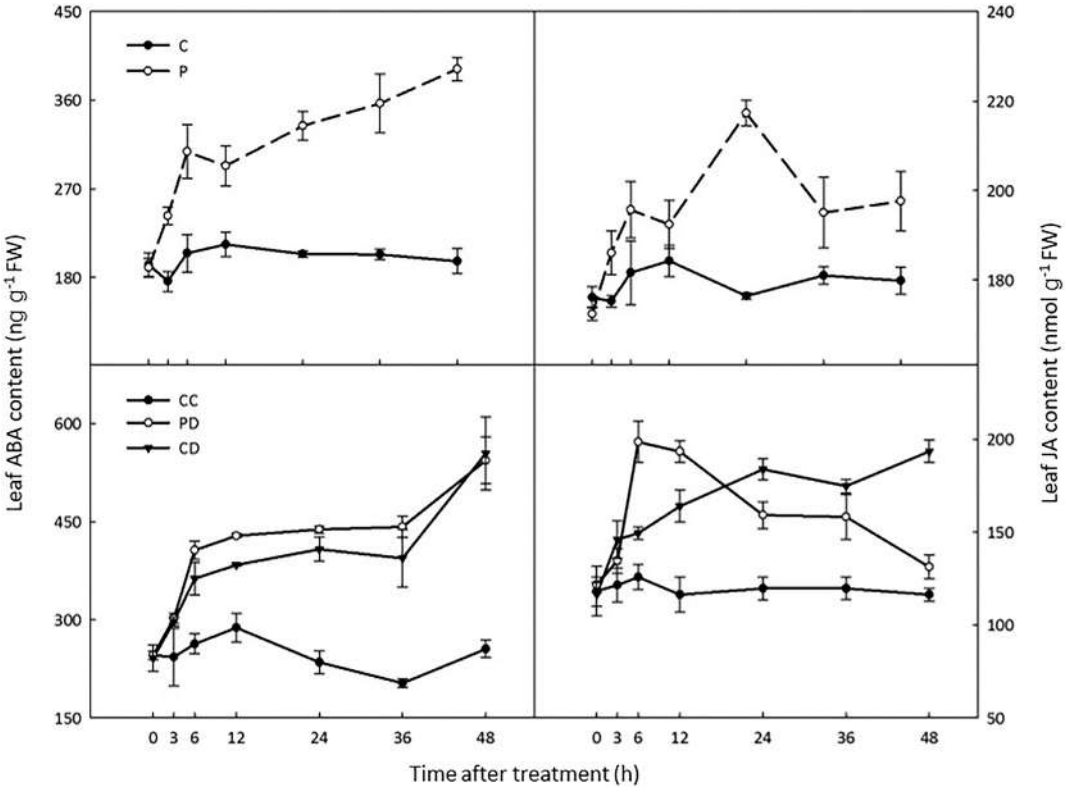

Fig. 3 – Time courses of ABA and JA concentration in wheat leaves under drought priming and drought stress. C, no drought priming; P, drought priming; CC, no drought priming + no drought stress; CD, no drought priming + drought stress; PD, drought priming + drought stress. Three biological replicates were measured.

by drought priming, especially at 0.5 h and 1 h. Several genes are involved in the JA biosynthetic pathway, including the lipoxygenase enzyme (LOX), which is required for 12-oxo-phytodienoic acid (OPDA), the precursor of JA biosynthesis; AOS and AOC constituting a branch point leading to OPDA; and 12-OPDA reductase (OPR), which catalyzes the reduction of 10, 11-double bonds of OPDA to yield 3-oxo-2-(2′-pentenyl)-cyclopentane-1-octanoic acid. The expressions of *LOX1*, *AOS1*, and *AOC1* genes were more highly induced at later stages (at 12 h or 24 h), while *OPR3* was more highly expressed in the early stages (0.5 h and 1 h). The expression of *MYC2* gene, encoding a bHLH-type transcription factor, was more highly induced at the early stage at 0.5 h, showed an expression pattern similar to that of *NCED* (Fig. 4).

3.3. Roles of ABA and JA in drought priming-induced drought tolerance

Drought stress lowered leaf water potential, with PD showed (non-significantly) greater differences than CD. NDGA + PD showed lower leaf water potential, FLU + PD showed no significant differences compared with PD. FLU + JA + PD showed no differences compared with FLU + PD, while NDGA + ABA + PD showed higher leaf water potential than NDGA + PD (Fig. 5).

Φ_{PSII} was reduced by drought stress, and PD showed higher ΦPSII than CD. The FLU + PD and NDGA + PD showed lower Φ_{PSII} than PD, while no significant differences were found with CD. FLU + JA + PD showed no differences in Φ_{PSII} compared to

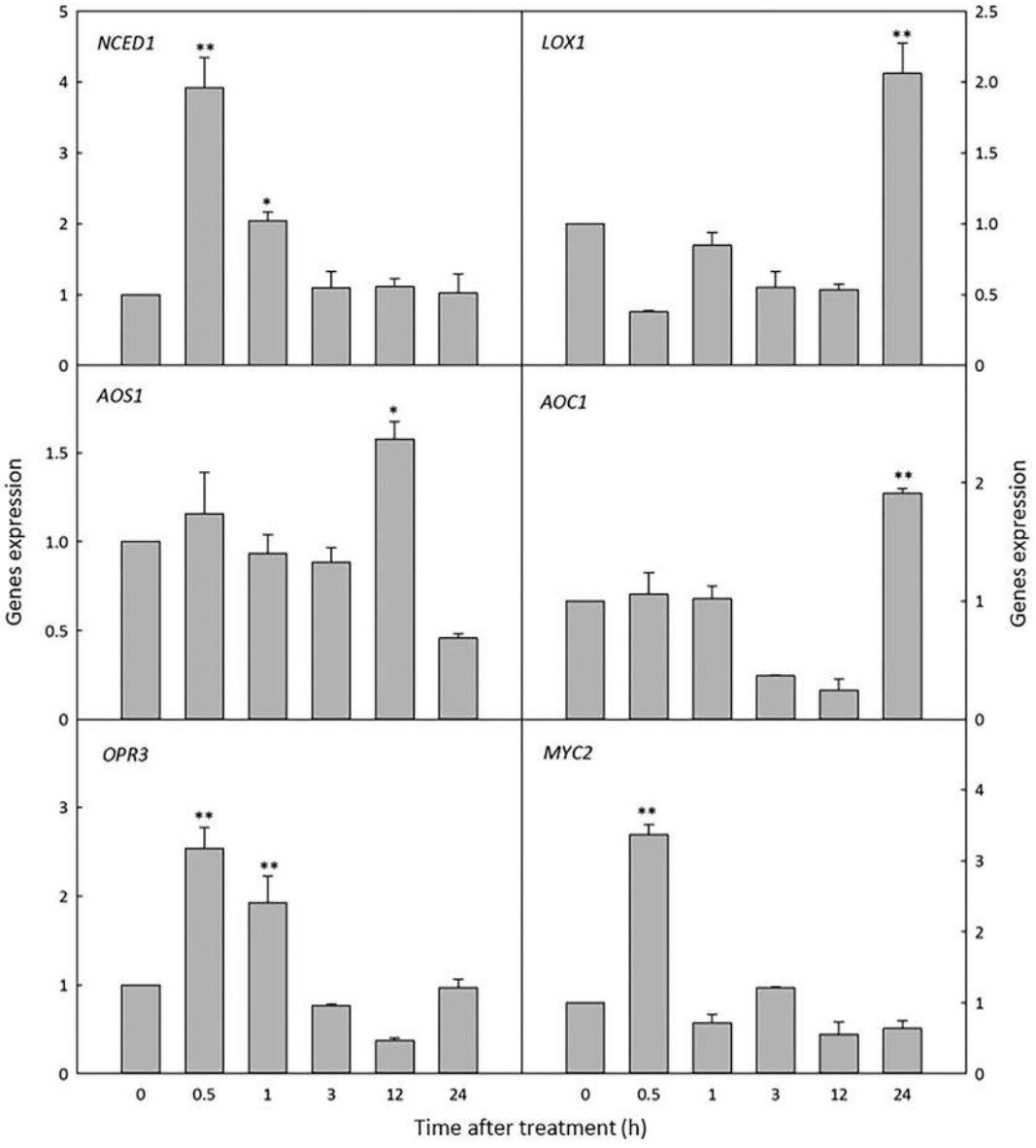

Fig. 4 – Time courses of expression of genes encoding enzymes involved in the biosynthesis pathways of ABA and JA in wheat leaves under drought priming. *NCED1*, 9-*cis*-epoxycarotenoid dioxygenase; *LOX1*, lipoxygenase; *AOS1*, allene oxide synthase; *AOC1*, allene oxide cyclase; *OPR3*, 12-oxo-phytodienoic acid reductase; *MYC2*, myelocytomatosis proteins. Three biological replicates were measured. *, $P < 0.05$; **, $P < 0.01$.

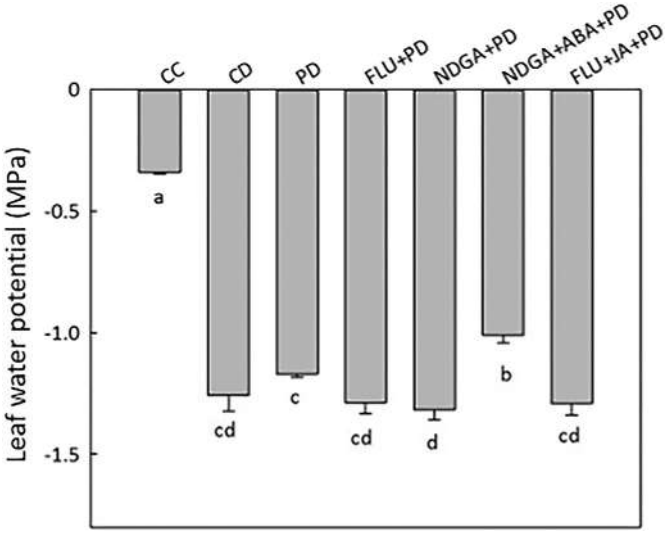

Fig. 5 – Leaf relative water potential in wheat under drought stress. FLU means spraying with the ABA biosynthesis inhibitor fluridone; NDGA means spraying with the JA biosynthesis inhibitor nordihydroguaiaretic acid. CC, no drought priming + no drought stress; CD, no drought priming + drought stress; PD, drought priming + drought stress. FLU + PD, spraying FLU before drought priming + drought stress; NDGA+PD, spray NDGA before drought priming + drought stress; NDGA + ABA + PD, spraying NDGA and ABA before drought priming + drought stress; FLU + JA + PD, spraying FLU and JA before drought priming + drought stress. Three biological replicates were measured. Different letters indicate statistical significance at $P < 0.05$ within treatments.

FLU + PD, and NDGA + ABA + PD showed higher Φ_{PSII} than NDGA + PD. NDGA + ABA + PD showed higher Φ_{PSII} than CD, and no significant difference compared with PD. F_v/F_m showed a tendency similar to that of Φ_{PSII} (Fig. 6).

REC and MDA contents were increased by drought stress, PD showed lower than CD. REC showed no differences among FLU + PD, NDGA + PD and CD, and was higher than in the PD treatment. No significant differences between NDGA + PD, FLU + PD, compared with PD were found. FLU + JA + PD showed no differences compared with FLU + PD, and NDGA + ABA + PD showed lower REC values compared to NDGA + PD. The contents of MDA were not significantly different between FLU + JA + PD and FLU + PD, NDGA + ABA + PD and NDGA + PD, respectively.

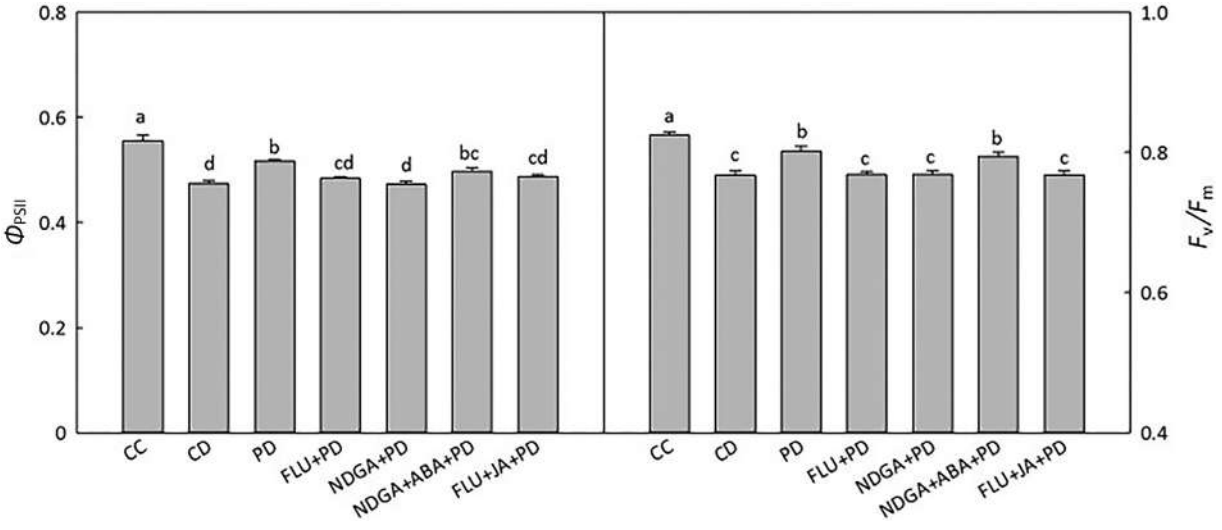

Fig. 6 – Chlorophyll fluorescence parameters in wheat under drought stress. Φ_{PSII}, actual PSII photochemical efficiency; F_v/F_m, maximum efficiency of PSII photochemistry. FLU means spraying with the ABA biosynthesis inhibitor fluridone; NDGA means spraying with the JA biosynthesis inhibitor nordihydroguaiaretic acid. CC, no drought priming + no drought stress; CD, no drought priming + drought stress; PD, drought priming + drought stress. FLU + PD, spraying FLU before drought priming + drought stress; NDGA+PD, spray NDGA before drought priming + drought stress; FLU + JA + PD, spraying FLU and JA before drought priming + drought stress; NDGA + ABA + PD, spraying NDGA and ABA before drought priming + drought stress. Three biological replicates were measured. Different letters indicate statistical significance at $P < 0.05$ within treatments.

Table 2 – Effects of drought priming on the antioxidant enzyme activities of wheat leaves under drought stress.

Treatment	REC (%)	MDA (mmol g^{-1} FW)	H_2O_2 (mmol g^{-1} FW)	SOD (U min^{-1} mg^{-1} protein)	CAT (U min^{-1} mg^{-1} protein)	APX (U min^{-1} mg^{-1} protein)	GR (U min^{-1} mg^{-1} protein)
CC	21.2 ± 5.9 c	2.4 ± 0.1 c	0.78 ± 0.06 b	21.2 ± 1.8 e	5.2 ± 0.1 c	5.0 ± 0.2 c	1.7 ± 0.04 d
CD	36.0 ± 3.5 a	3.6 ± 0.2 a	1.1 ± 0.02 a	25.5 ± 0.4 c	6.2 ± 0.1 ab	6.2 ± 0.2 ab	1.9 ± 0.02 c
PD	25.5 ± 2.2 c	2.8 ± 0.2 b	1.0 ± 0.01 a	35.3 ± 0.2 a	6.7 ± 0.1 a	7.1 ± 0.7 a	2.2 ± 0.02 a
FLU + PD	35.7 ± 0.5 a	3.4 ± 0.2 ab	1.1 ± 0.02 a	23.8 ± 2.0 cde	5.9 ± 0.4 ab	5.9 ± 0.2 bc	1.8 ± 0.11 cd
NDGA + PD	36.8 ± 0.8 a	3.0 ± 0.1 bc	1.1 ± 0.01 a	21.9 ± 0.9 de	5.7 ± 0.4 b	5.5 ± 0.1 bc	1.7 ± 0.02 d
FLU + JA + PD	33.7 ± 2.0 ab	3.1 ± 0.4 abc	1.1 ± 0.01 a	24.5 ± 0.1 cd	6.0 ± 0.4 b	5.4 ± 0.2 bc	2.0 ± 0.1 bc
NDGA + ABA + PD	27.5 ± 0.2 bc	2.8 ± 0.9 bc	1.0 ± 0.01 a	29.0 ± 0.3 b	6.7 ± 0.2 b	6.2 ± 0.2 ab	2.2 ± 0.02 ab

FLU means spraying with the ABA biosynthesis inhibitor fluridone, NDGA means spraying with the JA biosynthesis inhibitor nordihydroguaiaretic acid. CC, no drought priming + no drought stress; CD, no drought priming + drought stress; PD, drought priming + drought stress. FLU + PD, spraying FLU before drought priming + drought stress; NDGA+PD, spray NDGA before drought priming + drought stress; FLU + JA + PD, spraying FLU and JA before drought priming + drought stress; NDGA + ABA + PD, spraying NDGA and ABA before drought priming + drought stress. Three biological replicates were measured. REC, relative electrical conductivity; MDA, malondialdehyde; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; GR, glutathione reductase. Different letters indicate statistical significance at $P < 0.05$ within treatments.

Drought stress increased the content of H_2O_2, and there were no significant differences among the treatments with drought stress. H_2O_2 content was increased by drought priming, while FLU and NDGA treatments reduced H_2O_2 content (Fig. S3). The activities of SOD, CAT, APX and GR all increased by drought stress. The activities of SOD and GR in PD showed higher than in CD. The activities of SOD and GR showed no differences among FLU + PD, NDGA + PD and CD, but were lower than PD. FLU + JA + PD showed no differences compared with FLU + PD, and NDGA + ABA + PD were higher than NDGA + PD. The activities of CAT and APX showed no significant differences between PD and CD treatments. The activity of CAT in FLU + PD showed no significant differences compared with PD, while NDGA + PD showed lower CAT activity than PD. No significant differences between FLU + JA + PD and FLU + PD, NDGA + ABA + PD and NDGA + PD in CAT and APX activities were observed (Table 2).

Application of FLU and NDGA resulted in no significant differences in the contents of ABA and JA under control conditions. Under drought priming, the FLU and NDGA treatments significantly decreased endogenous ABA content, which was much lower with NDGA than with FLU. The endogenous JA content decreased only in NDGA, with no significant differences in FLU, compared with H_2O under

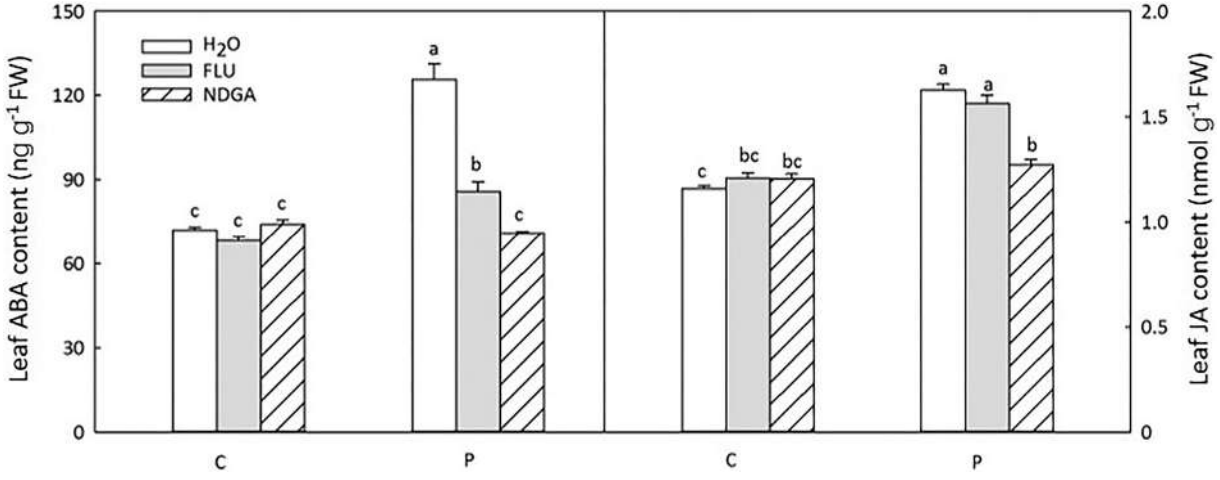

Fig. 7 – Contents of ABA and JA in wheat leaves under drought priming. C, no drought priming; P, drought priming. H_2O means no chemical spray; FLU means spraying with the ABA inhibitor fluridone; NDGA means spraying with the JA biosynthesis inhibitor nordihydroguaiaretic acid. Three biological replicates were measured. Different letters indicate statistical significance at $P < 0.05$ within treatments.

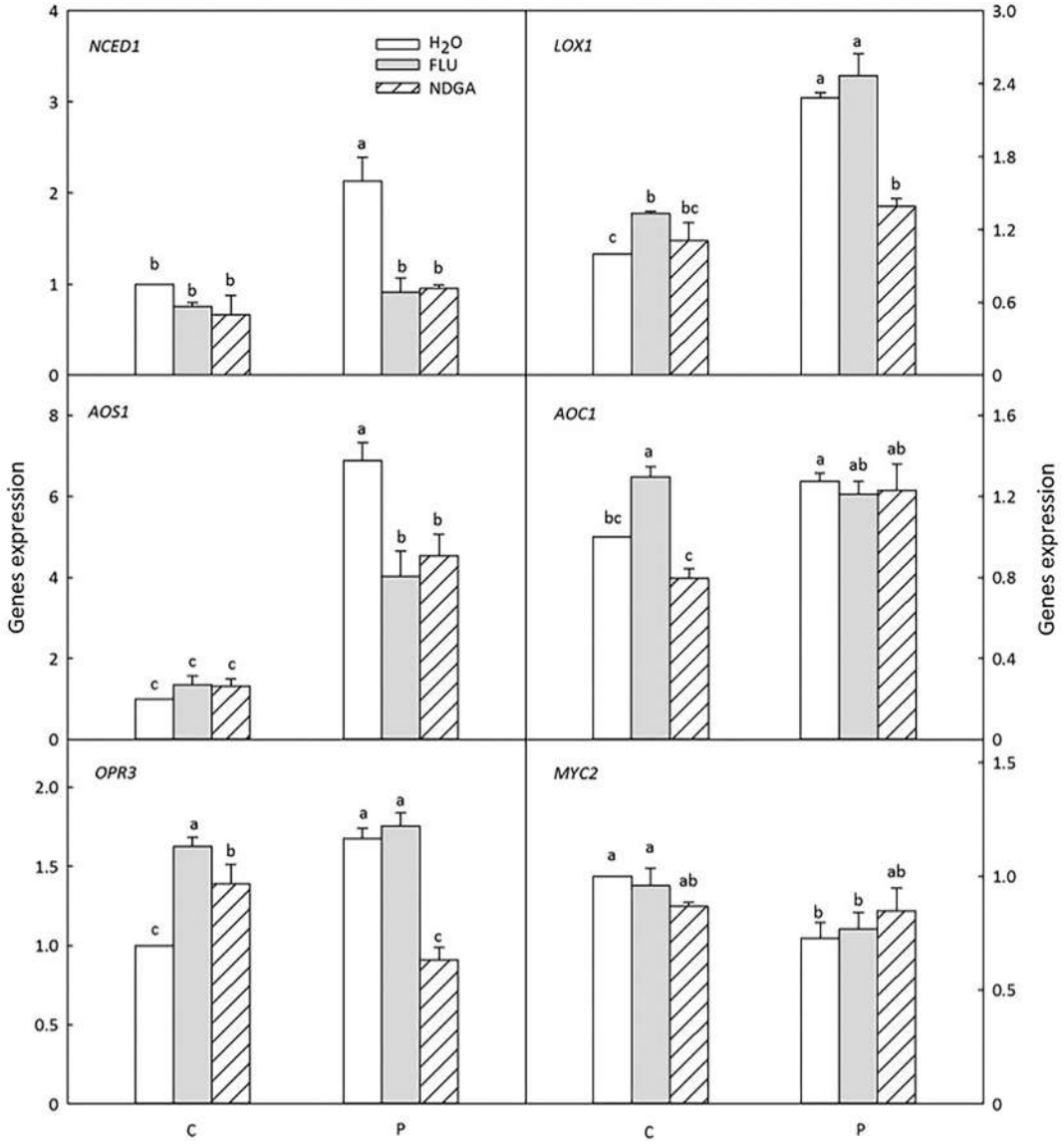

Fig. 8 – Expression of genes encoding enzymes involved in the biosynthesis pathways of ABA and JA in wheat leaves under drought priming. C, no drought priming; P, drought priming. *NCED*, 9-*cis*-epoxycarotenoid dioxygenase; *LOX*, lipoxygenase; *AOS*, allene oxide synthase; *AOC*, allene oxide cyclase; *OPR*, 12-oxo-phytodienoic acid reductase; *MYC*, myelocytomatosis proteins. H_2O means no chemical spray, FLU means spraying with the ABA inhibitor fluridone, NDGA means spraying with the JA biosynthesis inhibitor nordihydroguaiaretic acid. Three biological replicates were measured. Different letters indicate statistical significance at $P < 0.05$ within treatments.

drought priming (Fig. 7). The expression of *NCED1* gene showed no significant difference under control conditions, and was suppressed by FLU and NDGA application under drought priming, with no significant difference between FLU and NDGA treatments. The expression of *LOX1* was up-regulated in FLU under control, but down-regulated in NDGA compared with H_2O under drought priming. No differences between FLU and H_2O under drought priming were found. The expression of *AOS1* showed no significant differences under control, and was up-regulated by drought priming, with no significant differences between FLU and NDGA. The expression of *AOC1* showed higher in FLU under control, and showed no significant difference in FLU and NDGA compared with H_2O under drought priming. The expression of *OPR3* was induced by FLU and NDGA under non-stressed conditions, and induced by drought priming and down-regulated only in NDGA, compared with H_2O treatment. No significant differences among treatments in the expression of *MYC2* gene were found (Fig. 8).

4. Discussion

4.1. *Endogenous ABA and JA induced by drought priming contributed to increased tolerance to drought stress*

Drought priming induced plant tolerance to recurring drought stress, as evidenced by increased plant and root biomass

accumulation, and rates of photosynthesis, i.e. F_v/F_m in primed relative to non-primed plants (Fig. 1). Most DEGs were down-regulated in response to drought priming, relative to the control; for example, genes encoding proteins clustered as plant hormone signaling transduction involved in auxin signaling transduction (IAA and SAUR), the gibberellin-signaling pathway (phytochrome-interacting factor 4), ethylene-signaling pathway (EIN3 and ERF1/2), brassinosteroid signaling pathway (TCH4), salicylic acid signaling pathway (NPR1 and PR1).

Genes induced by drought priming contributed to increased tolerance to subsequent drought stress episodes. Among the up-regulated DEGs clustered in plant hormone signaling transduction were genes encoding proteins related to ABA biosynthesis and metabolism and JA biosynthesis as well as genes involved in ABA and JA signaling pathways. NCED is the key biosynthetic enzyme in the pathway of ABA biosynthesis [44] and overexpressed *NCED* correlated with increased ABA content [45]. ABA concentration is a balance between biosynthesis and catabolism [46]. In the present study, *NCED* was up-regulated, and down-regulated the expression of ABA metabolism *abscisic acid 8′-hydroxylase* in primed plants. Drought priming increased the endogenous contents of ABA and JA. The expression of ABA biosynthesis *NCED1* were significantly induced 0.5 h after drought priming, compared with control, whereas no significant differences after 3 h following drought priming occurred. The LOX6 is required for 12-OPDA biosynthesis, and the *lox6* mutant showed lower survival rates than wild type plants under drought stress [47]. In the present study, the expressions of *LOX*, *AOS* and *OPR*, which are involved in the JA biosynthetic pathway, were induced by drought priming. *LOX1*, *AOS1*, and *AOC1* were expressed more highly during 12 or 24 h, except that *OPR3* was expressed more highly after 0.5 h and 1 h by drought priming, compared with control (Fig. 4). The above results might suggest that the content of JA during early growth stages results from the release of JA from the bound state, but that during the later stages JA accumulation results from JA biosynthesis.

The ABA and JA signaling pathways are involved in priming-induced stress tolerance in *Arabidopsis* [48]. In our study, the expression of *SnRK2* which is involved in ABA signaling, was up-regulated, and the expression of *jasmonate ZIM-domain* (*JAZ*), a repressor of JA, was down-regulated, suggesting that both ABA and JA signaling pathways were up-regulated by drought priming. MYC2 transcription factors can activate the downstream JA target gene expressions [49] and also function in an ABA-dependent manner, as overexpression of AtMYC2 transcription factors improved drought tolerance in transgenic plants [50]. In our study, the expression of *MYC2* gene showed a similar pattern to that of *NCED* gene. *Calmodulin* gene and *disease resistance protein RPM1* gene, which are involved in the pathways of calcium signaling and

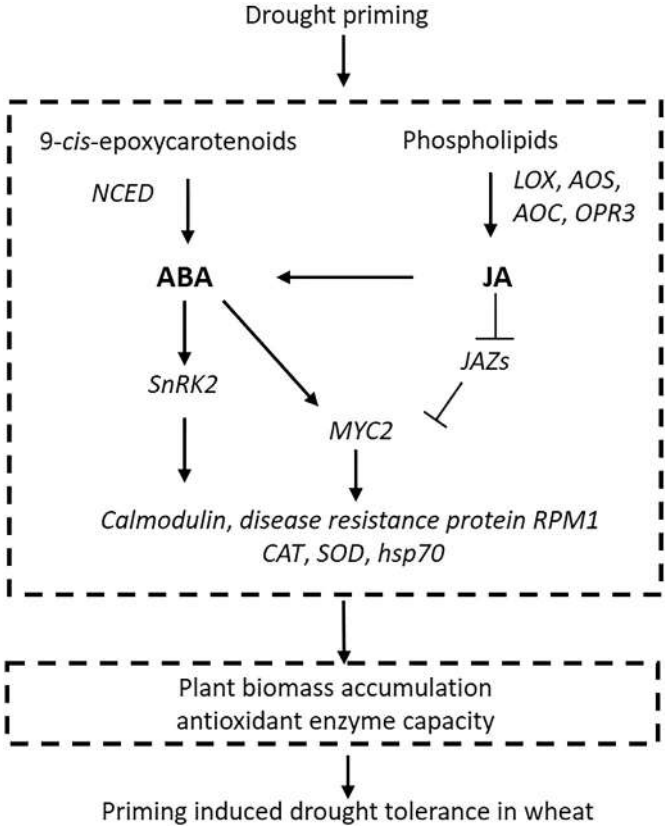

Fig. 9 – Proposed model of drought priming induced tolerance to drought stress in wheat. *NCED*, 9-*cis*-epoxycarotenoid dioxygenase; *LOX*, lipoxygenase; *AOS*, allene oxide synthase; *AOC*, allene oxide cyclase; *OPR*, 12-oxo-phytodienoic acid reductase; *MYC*, myelocytomatosis proteins; *SnRK2*, Snf1-related kinases; *JAZs*, jasmonate ZIM-domain; *SOD*, superoxide dismutase; *CAT*, catalase; *hsp 70*, heat shock 70 kDa.

stress tolerance, were more highly expressed in primed plants than in the control treatment.

Genes involved in stress response including *CAT*, *SOD*, and *heat shock 70 kDa*, which all are important for oxidative stress tolerance, were up-regulated by drought priming. Genes encoding sucrose synthase were up-regulated by drought priming, in agreement with the finding that the accumulation of sucrose and amino acids was significantly increased after priming. The accumulation of these compounds reached a peak after 48 h in the drought priming treatment and decreased after recovery (Fig. S3). Therefore, plants showed increases in both concentrations of ABA and JA and the abundance of genes encoding biosynthesis enzymes following drought priming in order to cope with recurring drought stress episodes.

4.2. ABA and JA were required for drought priming-induced drought tolerance

It is necessary to understand the hormonal crosstalk and physiological responses in drought priming and drought stress. ABA and JA have been reported to be involved in enhanced plant tolerance to drought stress [51,52], while the role of ABA and JA in priming induced drought tolerance are far from clear. Here, the inhibitors of ABA and JA were used to elucidate the roles of ABA and JA in priming-induced drought tolerance. F_v/F_m, Φ_{PSII}, and activities of SOD and GR were significantly higher in PD than in CD, while the leaf REC and MDA contents were lower in PD than in CD, with no significant differences among NDGA + PD, FLU + PD and CD treatments. The content of H_2O_2 was induced by drought priming as well as the content of ABA. The finding of no significant differences in the content of H_2O_2 among the drought stress treatment were found, suggesting that H_2O_2 could function as a stress signal involved in drought priming, while causing damage to cell membranes at higher concentrations under drought stress. This finding might be a result of increased activities of antioxidant enzymes, as indicated by less cell membrane damage in primed than in non-primed plants. These results suggest that induced resistance and tolerance to drought stress by priming requires JA and ABA.

JA regulates plant responses to drought by regulating ABA biosynthesis and accumulation via ABA-dependent signaling [52]. Application of JA increased foliar ABA concentrations [21] and JA deficiency diminished ABA accumulation [53]. In the present study, the NDGA + ABA + PD showed a tendency similar to that of PD, while FLU + JA + PD showed no significant difference from CD. These results suggest that blocking JA accumulation and applying ABA, but not vice versa, could restore the priming effects. Blocking ABA had no effect on endogenous JA contents, whereas blocking JA led to the reduction of endogenous ABA contents under priming. The blocking of ABA and JA suppressed the expression of *NCED1*, while the expression of *LOX1* and *OPR3* decreased only with the blocking of JA treatment under drought priming. These results suggest that both the ABA content and transcription of genes involved in ABA biosynthesis were reduced by blocking JA accumulation. Therefore, JA acts upstream of ABA in drought priming-induced stress tolerance.

5. Conclusions

ABA and JA were required, and JA acted upstream of ABA, in response to priming-induced drought tolerance in wheat. A proposed working model is shown in Fig. 9. Genes (*NCED*, *LOX*, *AOS*, *AOC*, *OPR3*) involved in biosynthesis of ABA and JA were induced by drought priming, and the contents of endogenous ABA and JA showed the same tendency. *SnRKs*, *JAZs*, and *MYC2*, which are involved in the ABA and JA signaling pathways, induced the downstream gene expression of *calmodulin*, *disease resistance protein RPM1*, *CAT*, *SOD*, *hsp70*, which contribute to drought priming-induced drought tolerance in wheat.

Declaration of competing interest

Authors declare that there are no conflicts of interest.

Acknowledgments

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Author contributions

Xiao Wang and Dong Jiang designed the experiments. Xiao Wang interpreted data and wrote the manuscript. Qing Li and Jinging Xie performed the experiments and analyzed data. Mei Huang, Jian Cai, Qin Zhou, Tingbo Dai, and Dong Jiang participated in the critical reading and discussion of the manuscript.

Appendix A. Supplementary data

Supplementary data for this article can be found online at https://doi.org/10.1016/j.cj.2020.06.002.

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