

Response of Jujube Fruits to Exogenous Oxalic Acid Treatment Based on Proteomic Analysis

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In this study, we found that oxalic acid (OA) at the concentration of 5 mM could delay jujube fruit senescence by reducing ethylene production, repressing fruit reddening and reducing alcohol content, which consequently increased fruit resistance against blue mold caused by *Penicillium expansum*. In order to gain a further understanding of the mechanism by which OA delays senescence and increases disease resistance of jujube fruit, we used a proteomics approach to compare soluble proteome of jujube fruits treated with water or 5 mM OA for 10 min. A total of 25 differentially expressed proteins were identified by using electrospray ionization quadrupole time-of-flight tandem mass spectrometry (ESI-Q-TOF-MS/MS). Among these proteins, alcohol dehydrogenase 1, which plays a direct role in ethanol metabolism, was repressed, and the abundances of three photosynthesis-related proteins was enhanced in jujube fruit after OA treatment. The protein identified as a cystathionine β -synthase domain-containing protein, which can regulate ethylene precursors, was also induced by OA treatment. The activity of 1-aminocyclopropane-1-carboxylic acid synthase was significantly suppressed in OA-treated jujube fruit. In addition, three proteins related to the defense/stress response were up-regulated by OA, and contributed to the establishment of systemic resistance induced by OA in jujube fruits. These results indicated that OA treatment might affect ethanol and ethylene metabolism, resulting in delaying senescence, and increase resistance of jujube fruits against fungal pathogens.

Keywords: Defense response • Ethylene release • Jujube fruits • Oxalic acid • Senescence.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ADH, alcohol dehydrogenase; CBB, Coomassie Brilliant Blue; CBS, cystathionine β -synthase; DTT, dithiothreitol; ESI-Q-TOF-MS/MS, electrospray ionization quadrupole time-of-flight tandem mass spectrometry; FID, flame ionization detector; IEF, isoelectric focusing; OA, oxalic acid; PDA, potato dextrose agar; PR proteins, pathogenesis-related proteins; ROS, reactive oxygen species; RuBisCO, ribulose biphosphate carboxylase/oxygenase; SAM, S-adenosyl-methionine; SOD, superoxide dismutase; TFA, trifluoroacetic acid.

Introduction

Oxalic acid (OA), as an organic acid, is distributed widely in plants, fungi and animals, and plays different roles in different living organisms (Shimada et al. 1997). In several phytopathogenic fungi, OA is considered a pathogenicity factor. Marciano et al. (1983) reported the significance of OA in the virulence of two *Sclerotinia sclerotiorum* isolates from sunflower. Godoy et al. (1990) demonstrated the role of OA in the pathogenicity of *S. sclerotiorum* on *Phaseolus vulgaris*. In order to test the hypothesis that oxalate induces foliar wilting during fungal infection by manipulating guard cells, Guimarães and Stotz (2004) investigated the mechanism by which OA affects host cells and tissues, and found that guard cells treated with OA accumulated potassium and broke down starch, both of which are known to contribute to stomatal opening. Livingstone et al. (2005) recovered transgenic peanut plants expressing an OA-degrading oxalate oxidase and evaluated them for increased resistance to *Sclerotinia minor*. Their study demonstrated the importance of OA

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in infection of peanuts by *S. minor*, and suggested that through increasing oxalate oxidase expression, transformed peanuts could enhance resistance to injury and fungal infection. In addition, Kim et al. (2008) found that OA could induce the programmed cell death response in plant tissue not by reducing the pH value but by inducing increased reactive oxygen species (ROS) levels in the plant.

Meanwhile, some evidence showed that OA has a potential effect on inducing resistance against pathogen and antioxidant systems. Kayashima and Katayama (2002) considered that OA was available as a natural antioxidant and played an important role in the natural and artificial preservation of oxidized materials. OA has also been shown to be an anti-browning agent for harvested vegetables (Castaner et al. 1997) and banana slices (Yoruk et al. 2002). Zhang et al. (1998) observed that OA treatment induced the activity of chitinase in cucumber leaves. In our previous studies, we reported that exogenous OA treatment could serve as an anti-browning agent for litchi fruit to prevent the pericarp from browning (Zheng and Tian 2006). Treatment with OA at 5 mM slowed the respiration of peach fruit, increased the activities of antioxidant enzymes, maintained membrane integrity and delayed the fruit ripening process (Zheng et al. 2007a). We also proved that mango fruit dipped in 5 mM OA solution for 10 min showed lower ethylene production and a lower incidence of decay as compared with control fruit (Zheng et al. 2007b). However, little is known about the biochemical or molecular mechanisms involved in this response.

In recent years, proteomics has been proven to be a powerful method to explore the protein changes of tomato fruit (Rocco et al. 2006, Faurobert et al. 2007) and grape berry (Giribaldi et al. 2007) during development and ripening. On the other hand, there is still a lack of information about harvested fruit senescence using proteomic analysis. Jujube has been traditionally considered to be functional fruit with a very high marketing value in China, because of its nutrition value, special components and delicious taste. However, jujube fruit is highly perishable, extremely susceptible to decay and easily loses its commercial value after harvest (Lin et al. 2004). In our previous studies, we found that OA could effectively delay peach and mango fruit ripening during storage (Zheng et al. 2007a, Zheng et al. 2007b). In order to provide further new evidence for the function of OA in regulating fruit ripening, in this study we investigated the physiological response of jujube fruit to OA treatment and employed a proteomics approach to study the mechanism involved.

Results

OA inhibited jujube fruit reddening

The peel color of jujube fruits represents one of the symbols of fruit senescence. In general, the color of the pericarp

changes from green at harvest to red at the end of storage. OA-treated fruit reddened slowly and showed a lower reddening index than control fruit during storage (Fig. 1A, C). Furthermore, OA treatment could significantly delay the decrease in chlorophyll content of peel, although the chlorophyll content of peel gradually decreased during the whole storage period (Fig. 1B).

OA induced the resistance of jujube fruit

OA treatment at a concentration of 5 mM could inhibit blue mold rot caused by *Penicillium expansum* in jujube fruit, resulting in significantly lower disease incidence in OA-treated fruit and smaller lesion diameter compared with control fruit (Fig. 2). This indicated that OA treatment enhanced the resistance of jujube fruits to *P. expansum*.

Change of OA levels in fruits

The OA level in control fruit showed a slight increase during storage periods at 20°C, but exogenous OA treatment could significantly increase endogenous OA content in jujube fruit. The OA level reached the highest level 1 d after treatment and was about 2-fold higher than that in control fruit (Fig. 3A). Although the OA concentration in treated fruit declined in the following storage period, it was still significantly higher compared with control fruit (Fig. 3A).

OA decreased ethanol content and the activity of ADH in jujube fruit

Ethanol content gradually increased with prolonged storage time in both OA-treated and control fruits. The ethanol content of OA-treated fruit showed an obviously lower level than that in control fruit during the whole storage period (Fig. 3B).

As shown in Fig. 3C, alcohol dehydrogenase (ADH) activity was first found to increase, and then to decrease subsequently both in water- and OA-treated jujube fruit during storage at 20°C. The level of ADH activity in OA-treated fruit was greatly repressed after 5 mM OA treatment.

Effect of OA on ethylene release, CO₂ production, ACC synthase and ACC oxidase

OA treatment significantly inhibited ethylene release and delayed the ethylene release peak (Fig. 4A). The ethylene production in OA-treated fruit was reduced about 3-fold compared with control fruit. Similarly, fruit respiration was obviously limited after OA treatment. CO₂ production in OA-treated fruit was shown to be lower than in the control fruit at 1 d after OA treatment (Fig. 4B). OA treatment significantly limited 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity, particularly at 2 d after OA treatment (Fig. 4C), but ACC oxidase activity in OA-treated fruit

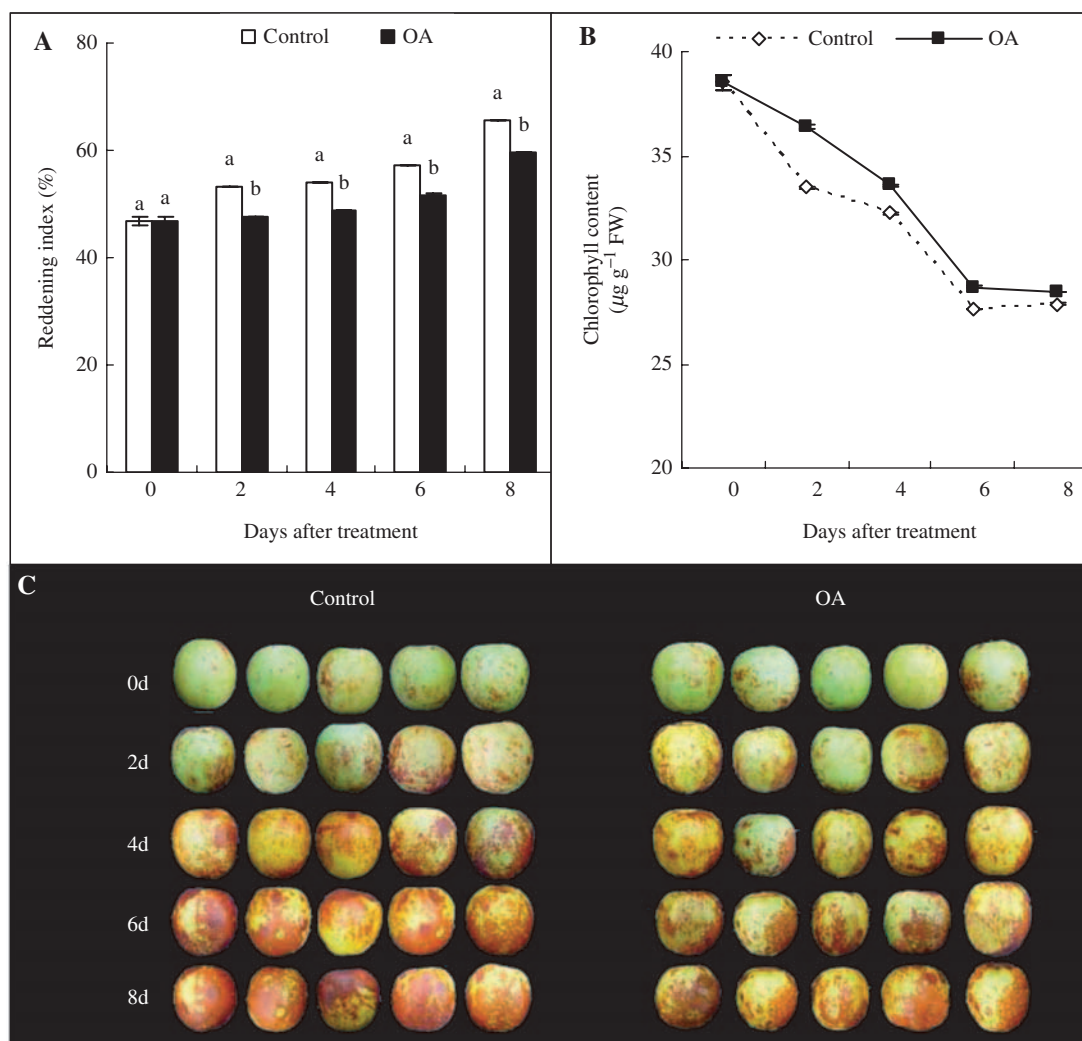


Fig. 1 Effect of OA on the peel color of jujube fruits turning to red during storage at 20°C. (A) Reddening index of jujube fruits after water and OA treatment during storage at 20°C. (B) Chlorophyll content in jujube fruits after treatment with water or 5 mM OA during storage at 20°C. (C) Changes in peel color of jujube fruits after water and 5 mM OA treatment during storage at 20°C. Control and OA represent fruits treated with water and OA, respectively. Data are the means \pm SE of three replications. Bars represent the standard deviation of the treatment means of pooled data.

was shown to be higher at 2 and 4 d after OA treatment as compared with the control (Fig. 4D).

Differently expressed proteins in response to OA

To study the effects of OA treatment on protein expression, we used two-dimensional electrophoresis to display and compare the proteins in OA-treated and untreated fruit. Analysis of the 2D gels revealed >600 protein spots after ignoring very faint spots and spots with undefined shapes and areas (Fig. 5). To analyze the proteins induced by OA, the changes in spot intensity between water- and OA-treated samples were quantified by ImageMaster 2D Elite software. Changes in the abundance of protein spots were scored only when they were reproducibly observed in three

independent replications. A total of 30 proteins whose expression was induced or repressed after OA treatment were detected in this study. Protein identification was an analytical challenge because of the lack of genome sequence information for jujube. Tandem mass spectrometry (MS/MS), which has been reported to be the most successful technique to identify proteins correctly from organisms where the whole DNA sequence is unknown (Joubert *et al.* 2001), was used to identify these proteins. MS/MS spectra were submitted for database searching in the Mascot search engine, and 25 proteins which scored greater than the threshold were identified with Mowse (Table 1).

Of these 25 identified proteins, 14 were found to be up-regulated with OA treatment, while 11 were down-regulated

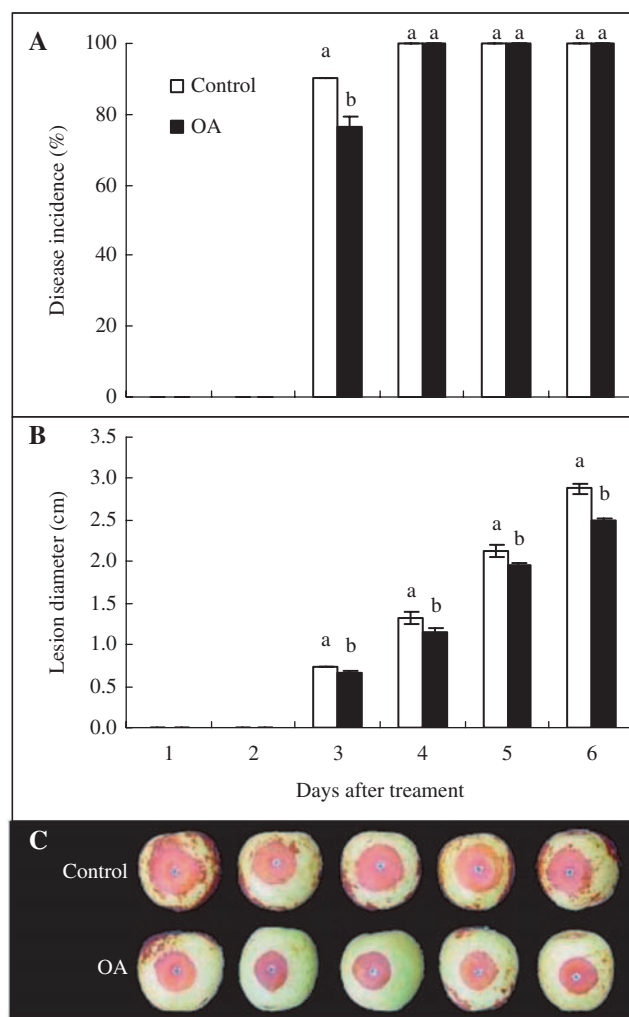


Fig. 2 Effects of OA on disease development in jujube fruits. (A) Disease incidence of blue mold in jujube fruits after water and OA treatment. (B) Lesion diameter of blue mold in jujube fruits after treatment with water or OA solution. (C) Symptom of blue mold in jujube fruits after water and 5 mM OA treatment for 10 min, respectively. Data are the means \pm SE of three replications. Bars represent the standard deviation of the treatment means of pooled data.

in comparison with the control. The identified proteins were classified into several functional categories according to Bevan et al. (1988) and Schiltz et al. (2004), including metabolism and energy, protein synthesis, modification, degradation, defense and stress, transcription, transporters, cell structure and others (Fig. 6). Of the differentially expressed proteins, eight were related to metabolism and energy, including adenine phosphoribosyltransferase (J-6), ribulose biphosphate carboxylase/oxygenase (RuBisCO) activase (J-7), RuBisCO large subunit-binding protein subunit β (J-8), formate dehydrogenase (J-9), cystathionine β -synthase (CBS) domain-containing protein (J-13), the dihydrolipoamide acetyltransferase (E2) subunit of the pyruvate dehydrogenase

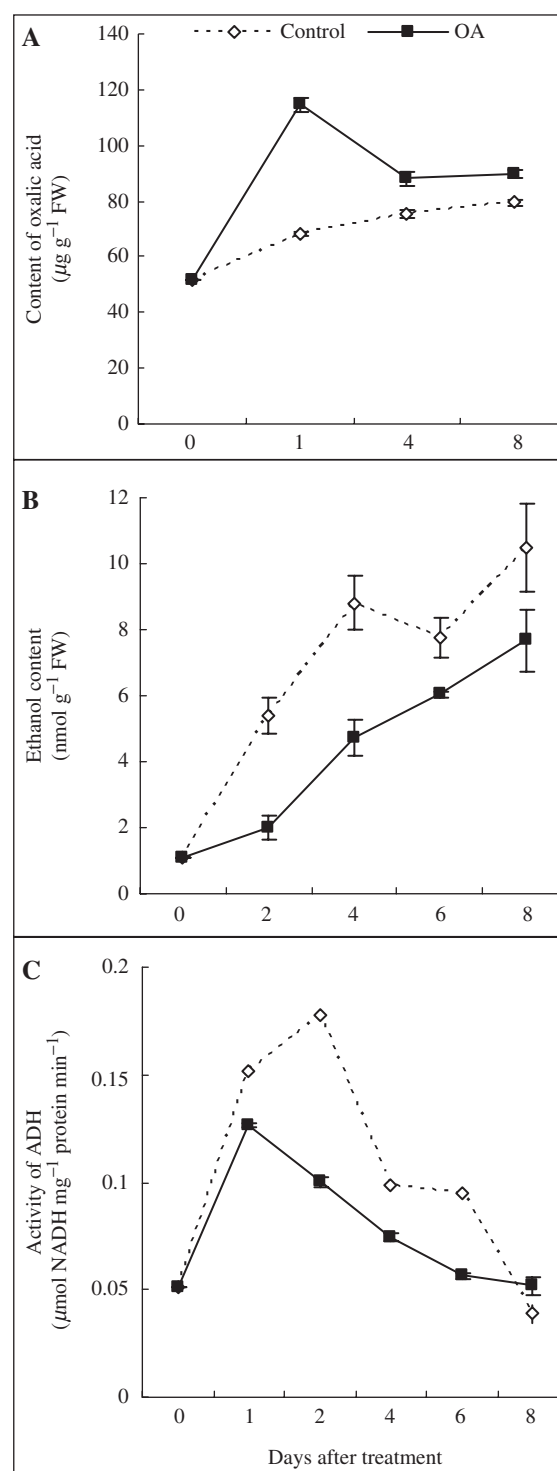


Fig. 3 Changes in OA content, ethanol content and activity of ADH of jujube fruits after treatment with water or 5 mM OA during storage at 20°C. Data are the means \pm SE of three replications. Bars represent the standard deviation of the treatment means of pooled data.

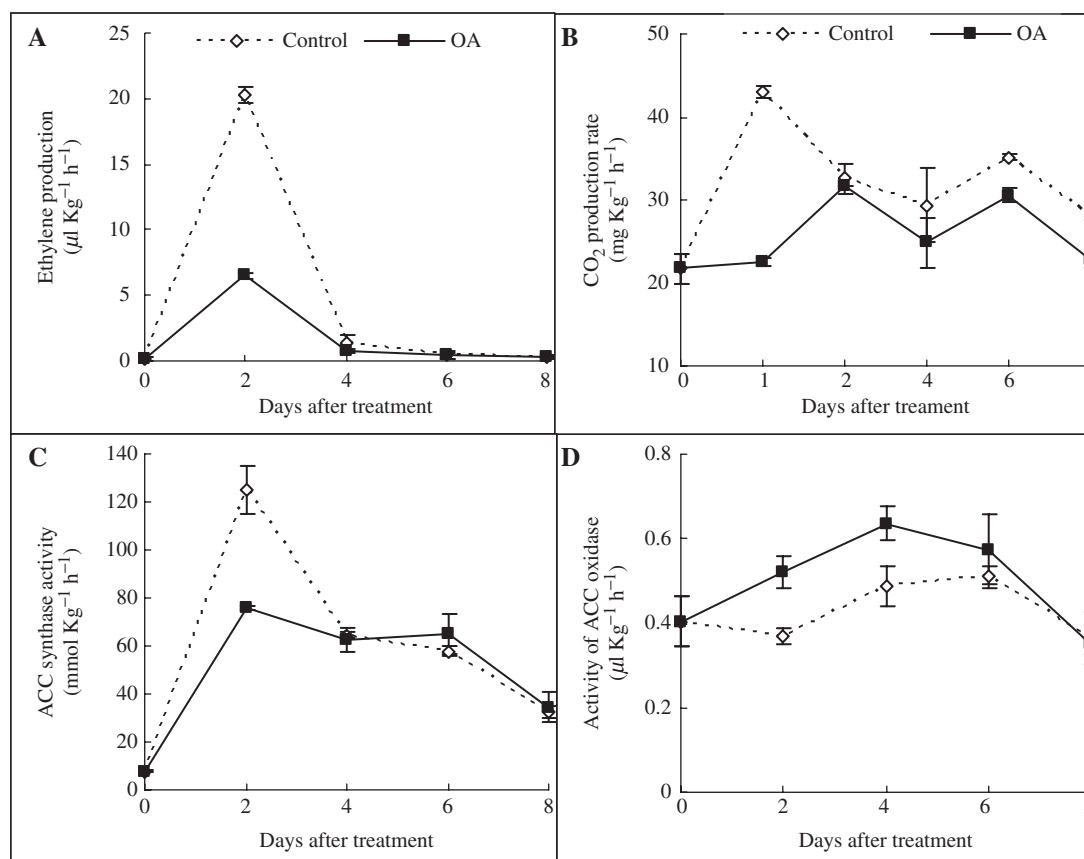


Fig. 4 Effect of OA on ethylene production, CO₂ production rate, and activities of ACC synthase and ACC oxidase in jujube fruits during storage at 20°C. Data are the means \pm SE of three replications. Bars represent the standard deviation of the treatment means of pooled data.

complex (J-14), PSII oxygen-evolving complex protein 2 (J-15) and ADH1 (J-20). Most notably, we found that the expression levels of four proteins, i.e. RuBisCO activase, RuBisCO large subunit-binding protein subunit β , PSII oxygen-evolving complex protein 2 and CBS domain-containing protein, were apparently up-regulated, but the abundance of ADH1 was down-regulated when the fruits were treated by OA. Of the remaining proteins, three were involved in defense and stress, including major allergen (J-1), Cu/Zn superoxide dismutase (Cu/Zn-SOD; J-2) and heat shock protein 70 (HSP70; J-28). It was notable that the abundance of defense and stress proteins was obviously increased after OA treatment. In order to determine the details of protein spots in Fig. 5, we magnified the areas of protein spots of interest in Fig. 7A, in which the marked numbers of the protein spots are the same as those in Fig. 5. Fig. 7B shows the abundance of the proteins which are involved in metabolism and energy, and defense and stress responses in a time-dependent manner and indicates that these proteins were also differentially expressed when the storage time was prolonged.

In addition, we identified proteins involved in protein synthesis, modification, degradation (J-4, 12, 16, 17, 18 and 29),

transcription (J-5, 10 and 25), transport (J-23 and 24) and cell structure (Fig. 5 and Table 1).

Discussion

Jujube fruit is one of climacteric fruits and senesces rapidly at room temperature after harvest. In general, pericarp reddening and flesh fermentation softening are two major parameters of jujube fruit senescence. During fruit ripening, oxalate also plays important roles such as in the oxalate-soluble pectin which is related to firmness of tomato (Guillon et al. 2008) and banana (Happi Emaga et al. 2008). Results from preliminary experiment indicated that OA solutions at 2, 5 and 10 mM could affect ethylene release and fruit respiration, and inhibit disease development, but OA at 5 mM showed the best effect (data not shown). We found that OA treatment at 5 mM could obviously inhibit pericarp reddening and effectively delay chlorophyll degradation in jujube fruit (Fig. 1). Fruit treated with 5 mM OA showed an obvious decrease in ethylene release peak (Fig. 4A) and delay in fruit respiration (Fig. 4B) as compared with control fruit. We previously found that OA treatment could lower respiration of peach fruit (Zheng et al. 2007a) and reduce ethylene

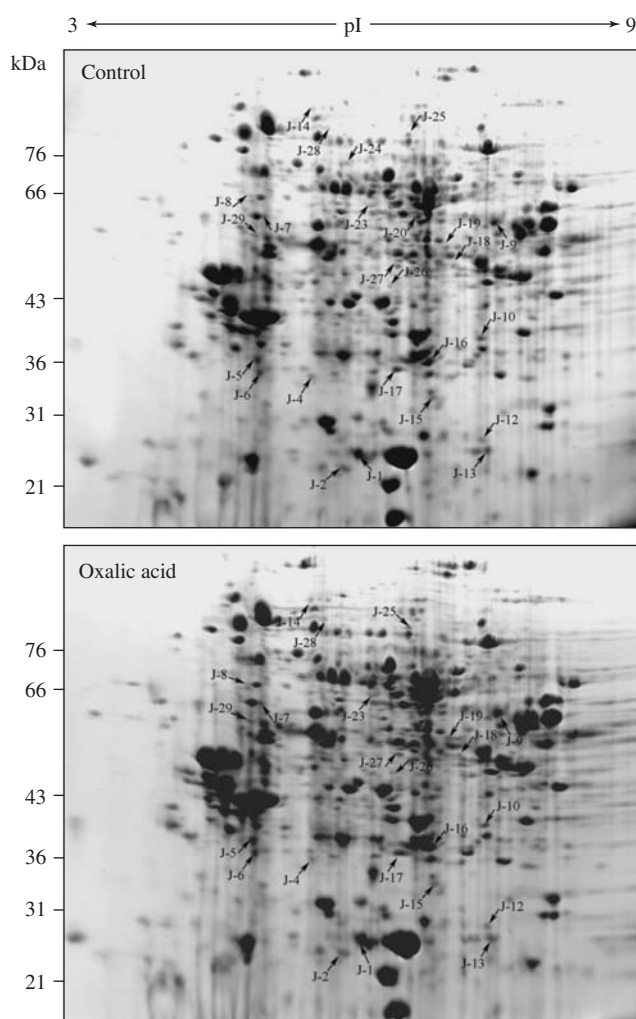


Fig. 5 Comparison of proteome patterns of jujube fruits in response to OA. Protein extraction and 2D PAGE protocol were described in Materials and Methods. The numbers indicate differentially expressed proteins and correspond to the numbers in Table 1. Arrows indicate proteins that were differentially expressed after treatment with 5 mM OA for 10 min.

production in mango fruit (Zheng et al. 2007b). The mechanism by which OA affects fruit senescence is related to the inhibition of ethylene release and fruit respiration. The fact that the activity of ACC synthase in OA-treated fruit was lower than that in control fruit (Fig. 2C) indicates that biosynthesis of ethylene can be inhibited by exogenous OA treatment. It is well known that ACC synthase, as a key regulatory enzyme of ethylene biosynthesis, catalyzes the production of the ethylene precursor ACC from S-adenosyl-methionine (SAM) (Yang and Hoffmann 1984). According to the results from electrospray ionization-MS/MS (ESI-MS/MS) analysis (Table 1), we found that a CBS domain-containing protein (J-13) was up-regulated by OA treatment (Fig. 7). CBS domains are generally found in cytosolic or membrane proteins performing different functions, including metabolic

enzymes, kinases and channels (Ignoul and Eggermont 2005). CBS has been considered to perform a crucial step in the biosynthetic pathway of cysteine by providing a regulatory control point for SAM (Finkelstein 1998), an important precursor of ethylene production (Wang et al. 2002), as it catalyzes the condensation of L-serine and L-homocysteine to form L,L-cystathionine, which is subsequently cleaved to cysteine (Miles and Kraus 2004). He et al. (2006) investigated the synergistic effect on the production of SAM in *Pichia pastoris* by knocking-in of SAM synthase and knocking-out of CBS, and proved that disruption of CBS resulted in a robust increase of SAM production in *P. pastoris*.

Application of OA can significantly delay the infection process of *P. expansum* in jujube fruit (Fig. 2), which is consistent with a previous report that OA treatment induced resistance of mango fruit against fungal pathogens (Zheng et al. 2007b). On the basis of proteome analysis, three defense-related proteins, i.e. a major allergen (J-1), Cu/Zn-SOD (J-2) and HSP70 (J-28), were identified and up-regulated by OA treatment (Fig. 7B). Some reports indicated that the major allergen (J-1) belongs to the ubiquitous group of pathogenesis-related proteins (PR-10) in plants (Hoffmann-Sommergruber 2000; Marković-Housley et al. 2003). Cu/Zn-SOD (J-2) is a major cellular isoform of SODs, and catalyzes the dismutation of superoxide ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) and dioxygen (O_2) (Fridovich 1995). SODs prevent damage to cellular membranes caused by ROS, acting as a primary defense during oxidative stresses to which organisms are exposed (Natvig et al. 1996). Due to their superoxide-detoxifying capacities, SODs have a hallmark of plant defense responses to pathogens (Lanfranco et al. 2005). Meanwhile, HSP70 is one of the major classes of chaperone molecules and plays many roles in eukaryotic cells (Bukau and Horwich 1998). In addition to its part in responses to abiotic stress, HSP70 also functions in response to pathogen attack, is an essential component of the hypersensitive response defense mechanism and plays an important role in the plant defense signal upstream transduction pathway (Kanzaki et al. 2003). Our experimental results proved that OA stimulated the expression of the three defense-related proteins (J-1, J-2 and J-28) (Fig. 7B), resulting in an increase in fruit resistant against fungal pathogens (Fig. 2). In a recent study, we also reported that six antioxidants and three PR proteins induced by *Pichia membranaefaciens* and salicylic acid were involved in the defense response of peach fruit against fungal pathogens (Chan et al. 2007). However, the pH value of OA solution can affect OA toxicity to fungal pathogens. In general, the pH value of 1–100 mM OA solutions is about 1–3, which can significantly inhibit the growth of *Colletotrichum gloeosporioides* on potato dextrose agar (PDA), but mycelial growth of *P. expansum* on PDA was not affected when the pH value of 5 and 10 mM solutions was altered to pH 6 with 1 N NaOH (data not showed).

Table 1 Identification of differentially expressed proteins from jujube fruits using MS/MS analysis

Spot ^a	Homologous protein	Organism ^b	Score ^c	SC ^d	Mr	pI	Accession no. ^f
Metabolism and energy							
↑J-6	Adenine phosphoribosyltransferase	<i>Brassica napus</i>	186	3	20%	21.79	gi 32492063
↑J-7	Ribulose biphosphate carboxylase/oxygenase activase, chloroplast precursor	<i>Malus × omestica</i>	58	5	3%	48.05	gi 3914605
↑J-8	RuBisCO large subunit-binding protein subunit beta, chloroplast precursor	<i>Brassica napus</i>	189	4	10%	62.43	gi 134104
↑J-9	Formate dehydrogenase, mitochondrial precursor	<i>Hordeum vulgare</i>	62	2	2%	41.52	gi 21263612
↑J-13	CBS domain-containing protein	<i>Solenostemon scutellarioides</i>	223	10	13%	22.23	gi 118162023
↑J-14	Dihydrolipoamide acetyltransferase (E2) subunit of PDC	<i>Arabidopsis thaliana</i>	80	3	3%	65.9	gi 559395
↑J-15	PSII oxygen-evolving complex protein 2	<i>Arabidopsis thaliana</i>	157	6	92%	1.434	gi 1076373
↓J-20	Alcohol dehydrogenase 1	<i>Paeonia officinalis</i>	174	9	21%	22.72	gi 13958651
Protein synthesis, modification, degradation							
↑J-4	Proteasome subunit beta type 3	<i>Oryza sativa</i>	150	6	11%	22.77	gi 49388033
↓J-12	Ubiquitin conjugating enzyme/ubiquitin-like activating enzyme	<i>Arabidopsis thaliana</i>	71	3	10%	16.52	gi 18409633
↓J-16	Methionine sulfoxide reductase	<i>Fragaria × ananassa</i>	92	3	10%	21.5	gi 11342533
↑J-17	GroES-like protein	<i>Arachis hypogaea</i>	92	2	5%	21.21	gi 194466087
↓J-18	Eukaryotic translation initiation factor 2 beta subunit-like	<i>Solanum tuberosum</i>	79	3	9%	29.83	gi 82621136
↓J-29	Peptidase aspartic, catalytic; UBA-like	<i>Medicago truncatula</i>	207	8	16%	44.99	gi 92874926
Defense and stress response							
↑J-1	Major allergen	<i>Malus × domestica</i>	88	2	7%	17.55	gi 886683
↑J-2	Cu/Zn superoxide dismutase	<i>Triticum aestivum</i>	52	2	6%	20.31	gi 1568639
↑J-28	Heat shock protein 70	<i>Cucumis sativus</i>	133	11	9%	70.78	gi 6911553
Transcription							
↑J-5	Ribonucleoprotein	<i>Pisum sativum</i>	51	3	3%	32.13	gi 2330647
↓J-25	Aspartyl-tRNA synthetase, class IIb	<i>Medicago truncatula</i>	187	19	8%	60.52	gi 92897799
↓J-10	GTP-binding nuclear protein Ran-A1	<i>Nicotiana tabacum</i>	271	22	26%	25	gi 1172835
Transporters							
↑J-23	ATP synthase, B subunit	<i>Trevoa trinervis</i>	1058	57	53%	53.39	gi 7578493
↓J-24	ATP binding/protein binding	<i>Arabidopsis thaliana</i>	128	8	5%	59.35	gi 15221770
Cell structure							
↓J-19	Type IIIa membrane protein cp-wap13	<i>Vigna unguiculata</i>	108	7	8%	39.4	gi 2218152
Others							
↓J-26	Unknown protein	<i>Zea mays</i>	95	4	7%	42.67	gi 194702788
↓J-27	Putative aldo/keto reductase 1	<i>Salvia miltiorrhiza</i>	127	5	6%	40.05	gi 151301846

^aThe spot number as given in Fig. 5.^bThe species of the matched protein.^cThe score obtained from Mascot for each match.^dThe number of matched peptides.^eAmino acid sequence coverage for the identified proteins.^fAccession number from the NCBI database of matched protein.

↑represents proteins up-regulated in response to OA; ↓represents proteins down-regulated in response to OA.

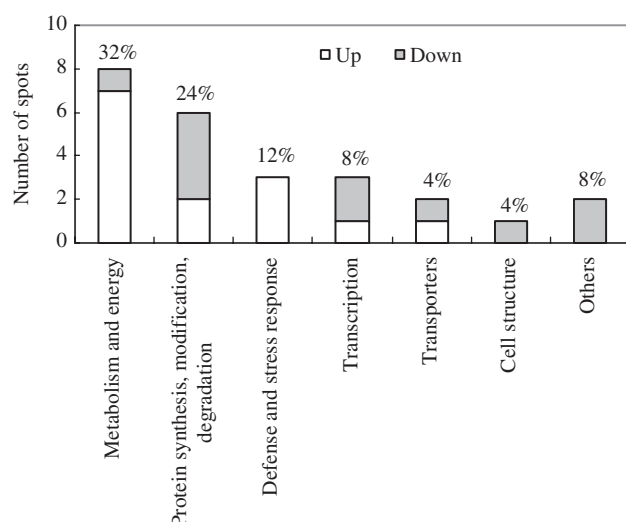


Fig. 6 Functional classification of identified protein spots of jujube fruits. Proteins were categorized into seven classes according to Bevan et al. (1998) and Schiltz et al. (2004). The distribution of proteins up- or down-regulated after OA treatment is given for each category. White, proteins whose abundance was up-regulated after OA treatment; gray, proteins whose abundance was down-regulated after OA treatment.

Fermentation softening occurs due to the overaccumulation of ethanol in jujube fruit and induces bad flavors and color, seriously destroy the qualities of fruits. Treatment with OA can reduce the ethanol content of jujube fruit (Fig. 3B). According to the results from proteome analysis, we found that the expression of ADH1 (J-20), a main enzyme responsible for catalyzing the reduction of acetaldehyde to ethanol during the fermentation of sugars (glycolysis) (Du Preez et al. 2007), was repressed by OA treatment (Fig. 7B). Exogenous OA application decreased ADH activity (Fig. 3C) in jujube fruit during storage periods at 20°C. In the ripening fruit, the ADH plays an important role in the development of several flavour and aroma compounds (Speirs et al. 2002). In some plants, ADH accumulation is involved in the response to a variety of stresses, such as cold, dehydration and hypoxia (Dolferus et al. 1994, Dolferus et al. 1997, Imahori et al. 2001, Kato-Noguchi and Yasuda 2007). During ethanolic fermentation, ADH is one of only two enzymes (pyruvate decarboxylase and ADH) which are required to produce ethanol from pyruvate (Tadege et al. 1999). In Arabidopsis, Peng et al. (2001) reported that ethylene is necessary, but not solely responsible, for the induction of ADH during hypoxia. Our results indicate that OA may affect ethanol metabolism via repression of the expression of ADH.

OA treatment enhanced the abundances of three chloroplastic proteins, i.e. RuBisCO activase (J-7), RuBisCO large subunit-binding protein subunit β (J-8) and PSII oxygen-evolving complex protein 2 (J-15) (Fig. 7B). In general, photosynthesis-related proteins are actively degraded and

are hardly synthesized again during leaf senescence (Lim et al. 2003, Yoshida 2003). Chlorophyll degradation also dramatically increases during tissue senescence (Thomas and Howarth 2000, Ren et al. 2007), which corresponds to the loss of green color in senescent leaves and ripening fruits (Pružinská et al. 2005). In this study, we found that the expression levels of J-7 and J-8 declined during storage, but OA treatment alleviated the tendency to decline (Fig. 7B), which suggests that exogenous OA treatment can inhibit chloroplast degradation and pericarp reddening of jujube fruit.

Taken together, the novel findings of this study were that OA treatment (i) repressed ethylene production and increased the expression of a CBS domain-containing protein; (ii) induced the expression of defense response proteins; and (iii) repressed the expression of ADH1 and the activity of ADH, resulting in lower ethanol content in fruit. These results provide new evidence for the function of OA in mediating fruit senescence and inducing fruit resistance against fungal pathogens.

Materials and Methods

Fruit and pathogen

Jujube (*Ziziphus jujuba* Mill. cv Dongzao) fruit were harvested when they developed for 110 d after full blooming from an orchard in Beijing, PR China, and were immediately transported to our laboratory at the Institute of Botany, Chinese Academy of Sciences. The fruits were sorted based on size and the absence of physical damage or infections. The selected fruits were surface-disinfested with 2% (v/v) sodium hypochlorite for 2 min, washed with sterile water, and air-dried prior to use.

Penicillium expansum Link was initially isolated from the naturally infected jujube fruits, and cultured routinely on PDA plates for 14 d at 25°C. Spores were obtained by flooding the surface of the culture with sterile distilled water containing 0.05% (v/v) Tween-80 and gently scrubbing with a sterile spatula. The spore suspension was filtered through four layers of sterile cheesecloth to remove any hyphal fragments. The concentration of the suspension was adjusted to 1×10^5 spores ml^{-1} with the aid of a hemocytometer.

Treatment with OA

Jujube fruits were immersed in 5 mM OA solution or in distilled water as control for 10 min, then dried in air at 20°C for 2 h and divided into two groups. For the first group, OA-treated fruits were wounded (4 mm deep, 3 mm wide) at the equator of the fruit using a sterile nail after 24 h at 20°C. Aliquots of 10 μl of a suspension of *P. expansum* at 1×10^5 spores ml^{-1} were inoculated into each wound site. Fruits were put into 200 mm \times 130 mm \times 50 mm plastic boxes with plastic film to maintain a high relative humidity (95%), and stored

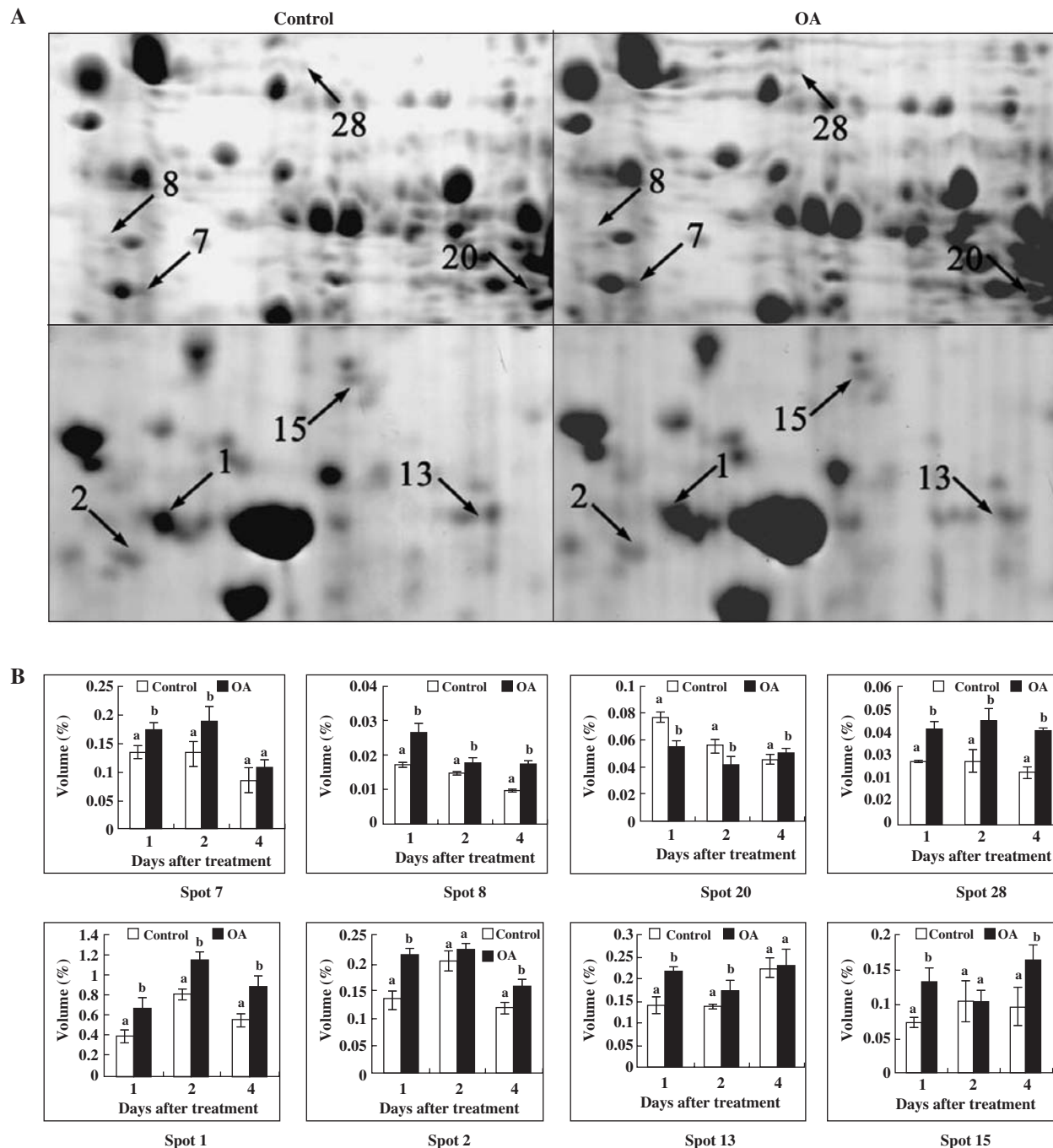


Fig. 7 (A) Enlargement of the regions containing the changed proteins which were involved in metabolism and energy, and defense and stress response on the gel shown in Fig. 5. (B) Time-dependent accumulation of the proteins mentioned above in jujube fruits for 1, 2 and 4 d after 5 mM OA treatment. Protein extraction and the 2D PAGE protocol were described in Materials and Methods. The number of each protein spot corresponds to its listing in Table 1. The spot volume was normalized as a percentage of the total volume of all spots on the corresponding gel. The relative abundance ratio in jujube fruits treated with OA was divided by that in fruits treated with water, from three different gels and independent extractions. The protein spots with significant changes ($P < 0.05$) were considered to be different. Data are the means \pm SE of three biological replications. Bars represent the standard errors of the mean.

at 20°C. Disease incidence and lesion diameter were measured daily after treatment according to the following formulas:

$$\text{Disease incidence (\%)} = \frac{\text{Number of decayed jujube fruit}}{\text{Total number of treated fruit}} \times 100$$

$$\text{Lesion diameter (cm)} = \frac{\sum \text{Lesion diameter of decayed jujube fruit}}{\text{Total number of treated fruit}}$$

For the second group, fruits were put directly into plastic boxes as described above without pathogen inoculation. At various time intervals after treatments, samples from fruit flesh were obtained for the following assays. Three replications for each treatment were performed, and each replicate contained 15 fruits. The entire experiment was repeated twice.

Assessment of reddening index

The reddening index of the fruits was assessed according to the reddening area on the fruit surface with the following scales: 0 = no red; 1 = less than 1/3 red; 2 = 1/3–2/3 red; 3 = more than 2/3 red; 4 = fully red. The reddening index was calculated using the following formula: $[(1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4) \times 100 / (4 \times N)]$, where N = total number of fruits measured and N₁, N₂, N₃ and N₄ were the numbers of fruits showing the different scales of reddening.

Analysis of chlorophyll content

Peel (3 g; approximately 1 mm thick) was cut from the equatorial surface of each jujube fruit and rapidly transferred to a tube with 25 ml of 80% acetone. The acetone extracts were read at 645 and 663 nm for determination of Chl *a* and *b* on a UV-160 spectrophotometer (UV-160A, Shimadzu Scientific Instruments, Kyoto, Japan) by the method of Arnon (1949). Three replicates with 15 fruits each were performed with different storage times.

OA concentration measurement

The OA was extracted from control and treated fruits according to the method of Prusky et al. (2004). OA concentrations of the extracts were measured using a gas chromatograph (Agilent 6890N, Vernon Hills, IL, USA) equipped with a DB-5 fused capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Agilent) connected to a flame ionization detector (FID). The injector temperature was 280°C and the column temperature was held at 80°C for 1 min, increased by 20°C min⁻¹ to 200°C and held for 1 min, and by 30°C min⁻¹ to a final temperature of 280°C for 5 min. Experiments were conducted three times.

Determination of ethylene biosynthesis and CO₂ production

Ethylene release from fruits was measured by enclosing samples in an airtight container for 2 h at 20°C, withdrawing

a 1 ml air sample, and injecting it into a gas chromatograph (SQ-206, Beifenruili, Beijing, PR China) equipped with an FID and an activated alumina column. The experimental conditions were: 80°C oven temperature, 150°C injector temperature, 380°C detector temperature. The rate of CO₂ production was determined according to the method of Zhu et al. (2006). ACC synthase was assayed using 3.5 g of fruit flesh from 10 fruits as described by Fan et al. (1998). For the measurement of ACC oxidase activity, flesh fruit slices of 1 mm thickness (2 g) were floated on 4 ml of 4 mM ACC, 50 mM MES, pH 6.5, in a 10 ml vial with gentle rotation for 2 h at 20°C according to the method described by Nadeau et al. (1993). These vials were sealed with a rubber stopper and incubated for 1 h, and then the ethylene formed was determined as described above. The activity was expressed as ethylene produced per kg FW per h. Three replications for each treatment were performed.

Analysis of ethanol content

Contents of ethanol were determined by gas chromatography (Agilent 6890N) according to the method described by Tian et al. (2004). A fruit sample of about 15 g was put in an Omni-mixer (Omni International Inc., Waterbury, CT, USA) and an equal amount of 20% trichloroacetic acid was added. The mixture was homogenized for 2 min in an ice water bath. A 3 g sample of the mixture was sealed in a 10 ml vial, and then incubated in a thermostated bath at 40°C for 60 min. A 1 ml sample was removed by syringe and injected into the gas chromatograph (Agilent 6890N) equipped with an FID and a wax™10 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; SUPELCO, Bellefonte, PA, USA). The injector temperature was 200°C and the column temperature of 37°C was held for 1 min, increased by 1°C min⁻¹ to 70°C and held for 1 min, and increased by 80°C min⁻¹ to a final temperature of 200°C for 5 min.

Assay of ADH enzyme activity

The ADH activity was measured according to the protocols of Kumutha et al. (2008) with some modifications. A 5 g aliquot of fruit tissue from 10 fruit was pulverized with liquid nitrogen, then homogenized with 5 ml of extraction buffer [100 mM Tris–HCl, pH 8.0 and 15 mM dithiothreitol (DTT)]. The extract was centrifuged at 12,000×g for 15 min at 4°C. The enzyme reaction mixture contained 1.375 ml of Tris–HCl (100 mM pH 8.0), 15 μl of 0.5 mM NAD⁺ and 100 μl of extract. The enzyme reaction was initiated by addition of 10 μl of 95% ethanol, and the A₃₄₀ was measured every 30 s for 5 min. The protein concentration of the extract was determined as described by the method of Bradford (1976). Activity was expressed as μmol NADH formed per min per mg of protein.

Protein extraction from fruits

Total protein extracts were prepared from jujube fruits according to the method of Saravanan and Rose (2004) with some modifications. All procedures described below were carried out at 4°C. Briefly, 8 g of flesh from 10 fruits was ground in liquid nitrogen using a mortar and pestle, homogenized in 8 ml of an extraction buffer containing 20 mM Tris-HCl (pH 7.5), 250 mM sucrose, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT and 1% Triton X-100 on ice, and subsequently extracted with an equal volume of Tris-HCl pH 7.8 buffered phenol for 30 min. After centrifugation at 10,000×g for 40 min, the phenol phase was re-extracted three times with extraction buffer as above. Proteins were precipitated from the final phenol phase with 5 vols. of ice-cold saturated ammonium acetate in methanol overnight at -20°C. The proteins were collected by centrifugation (10,000×g, 30 min) and washed twice with cold saturated ammonium acetate in methanol and twice with acetone. The precipitate was air-dried and solubilized in thiourea/urea lysis buffer containing 2 M thiourea, 7 M urea, 4% (w/v) CHAPS, 1% (w/v) DTT and a 2% (v/v) mixture of carrier ampholytes of pH 5–8 and pH 3.5–10 in a ratio of 1:1. Protein was kept at -80°C until use.

Two-dimensional gel electrophoresis analysis

Two-dimensional gel electrophoresis was carried out according to the method of O'Farrell (1975) with minor modifications. The isoelectric focusing (IEF) gel solution contained 10% NP-40, 30% (w/v) acrylamide, 9.5 M urea, 10% ammonium persulfate and an equal mixture of 2% carrier ampholytes (pH 3.5–10 and 5–8). The gels were polymerized in glass tubes (Daiichi Pure Chemicals, Tokyo, Japan) to obtain gels of 13 cm length and 3 mm diameter. Approximately 600 µg of proteins determined by the method of Bradford (1976) were applied to the gel. IEF was performed at 200 V for 30 min, 400 V for 14 h and then 800 V for 2 h. After being run in the first dimension, the IEF gels were transferred to equilibration buffer containing 10% (v/v) glycerol, 2.5% (w/v) SDS, 125 mM Tris-HCl (pH 6.8) and 5% (v/v) β-mercaptoethanol, and incubated for 15 min at room temperature twice. The equilibrated strip was placed onto the stacking gel and sealed with 0.5% agarose prepared in equilibration buffer. SDS-PAGE in the second dimension was performed with 15% separation gels and 5% stacking gels at a constant current of 30 mA. After electrophoresis, the 2D gels were stained with Coomassie Brilliant Blue (CBB) R-250 solution containing 50% (v/v) methanol, 15% (v/v) acetic acid and 0.1% (w/v) CBB R-250.

Gel images were acquired using a flatbed scanner (Amersham Biosciences, Uppsala, Sweden) with 300 dpi resolution and saved in TIF format. Image analysis was performed by Image Master 2D Elite software (Amersham Biosciences,

Uppsala, Sweden). For each treatment analyzed, at least triplicate gels were prepared from three different protein extractions. Protein spots were selected for quantitative analysis if they were consistently visible in three replicates. To correct experimental variations in 2D gels, the spot volumes were normalized as a percentage of the total volume in all of the spots in the corresponding gel. The relative change between control and 5 mM OA-treated samples was subjected to statistical analysis with SPSS (SPSS Inc., Chicago, IL, USA), and only spots that changed significantly in averaged normalized spot volume were excised for protein identification.

In-gel digestion

In-gel digestion was performed as described by Chan et al. (2007). Protein spots were excised from the gels and cut into about 1 mm² pieces, then destained with a 1:1 (v/v) solution of methanol and 50 mM NH₄HCO₃, changing the solutions every 1 h until the blue color of CBB was removed. Proteins were reduced with 10 mM DTT and 10 mM EDTA in 100 mM NH₄HCO₃ for 1 h at 60 °C. After drying in a vacuum centrifuge for 30 min, the gels were incubated with 40 mM iodoacetamide in 100 mM NH₄HCO₃ for 30 min at room temperature in the dark, then washed several times with water and completely dried in a vacuum centrifuge. Gel pieces were rehydrated with a digestion buffer of 5 ng µl⁻¹ trypsin (sequencing grade, modified; Promega, Madison, WI, USA) in 100 mM NH₄HCO₃ and digested for 14–16 h at 37°C. After digestion, the gel slices were washed three times with 0.1% trifluoroacetic acid (TFA) in 50% (v/v) acetonitrile to extract the peptides. The collected solutions were concentrated to 10 µl, and then desalted with ZipTipC18 (Millipore, Bedford, MA, USA). Peptides were eluted from the column in 2 µl of 0.1% TFA in 50% acetonitrile.

Protein spot identification by ESI-Q-TOF-MS/MS

ESI-MS/MS analysis of the digests was performed using an ESI quadrupole time-of-flight hybrid mass spectrometer (ESI Q-TOF; micro, Micromass, Altrincham, UK) with a z-spray source (Nouwens et al. 2002). Before loading the digested peptides, the instrument was externally calibrated with the fragmentation spectrum of the doubly charged 1,571.68 Da (785.84 *m/z*) ion of fibrinopeptide B. The peptides were introduced by nanoelectrospray needle (gold-coated borosilicate glass capillaries, Micromass). The applied spray voltage was 800 V, with a sample cone working on 30 V. The collision energy was varied from 14 to 40 V dependent on the mass and charge state of the peptides. MS/MS data were processed using the MaxEnt3 (Micromass), and the data searched in NCBI nr protein databases (version October 10, 2008; 7,109,111 sequences; 2,453,259,876 residues) with the Mascot MS/MS Ions Search program on the Matrix Science public web site. A peptide tolerance of ±2.0 Da for the

precursor ions and a MS/MS tolerance of ± 0.8 Da for the fragment ions was set. Peptide charges of +2 and +3 monoisotopic mass were chosen, and the instrument type was set to ESI-Q-TOF. Viridiplantae was chosen for the taxonomic category, trypsin was specified as the proteolytic enzyme, and one missed cleavage site was allowed for each search. Carbamidomethylation of cysteine (+57.02 Da) and oxidation of methionine (+16 Da) were designated as variable modifications for searching.

Statistical analysis

All data were analyzed as a one-variable general linear model procedure (analysis of variance) by SPSS. Mean separations were performed with the least significant difference test, and differences at $P < 0.05$ were considered to be significant. Results presented were pooled across repeated experiments.

Supplementary data

Supplementary data are available at PCP online.

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