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Fatty acid metabolic flux and lipid peroxidation homeostasis maintain the biomembrane stability to improve citrus fruit storage performance

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ABSTRACT

Little is known about the variations of fresh fruit biomembrane and its physiological and biochemical characteristics during storage. A navel orange mutant 'Gannan No.1' (*Citrus sinensis* Osbeck) showed higher membrane stability and titratable acid while lower calyx senescence compared with wild-type 'Newhall'. The membrane damage was significantly reduced in 'Gannan No.1' under 10% polyethylene-glycol (41.16% *vs.* 8.77%) and 30% polyethylene-glycol (52.59% *vs.*16.11%) treatments on day 45 after harvest. Consistently, membrane electrolyte leakage and malondialdehyde were significantly decreased in 'Gannan No.1', and superoxide dismutase and glutathione reductase were activated. A metabolic analysis was performed to evaluate membrane fatty acid unsaturation and peroxidation. Linolenic acid and hexadecylenic acid contributed to the higher degree of unsaturated fatty acids in 'Gannan No.1'. Furthermore, 'Gannan No.1' accumulated stress-resistant metabolites such as proline, α-tocopherol and glutathione. Correlation analysis of membrane homeostasis indexes with quality parameters showed the importance of biomembrane stability in maintaining citrus fruit quality.

1. Introduction

Postharvest quality deterioration of fruit, mainly including physiological disorder and pathogenic diseases (Ding et al., 2015; Lin et al., 2016; Porat, Lichter, Terry, Harker, & Buzby, 2018; Zhang et al., 2018), causes serious loss of 5–50% in the total production (Porat et al., 2018). Biomembranes are essential constituents of plant cells, and play important roles in metabolic processes, nutritional maintenance and signal transductions (Li, Xu, Libeisson, & Philippar, 2016; Lin et al., 2018). To date, the enhancement of biomembrane stability is widely applied to help the maintenance of fruit quality for commercial purposes. However, there has been no systematic research on the rhythmic variations and biological basis of fruit biomembranes during storage.

Biomembranes are laterally heterogeneous, and their stability is reflected by biophysical characteristics such as membrane fluidity and integrity, potential and energy state (Kachroo & Kachroo, 2009; Lin et al., 2018; Nakamura, 2018). These properties of biomembranes are established under the dynamic equilibrium of metabolic flux control such as the fatty acid composition of membrane lipids and the incorporated polysaccharides, tocopherols and flavonoids in Arabidopsis (Ballweg & Ernst, 2017; Nakamura, 2018; Selvaraj, Krishnaswamy, Devashya, Sethuraman, & Krishnan, 2015). In plants, long chain fatty acids (LCFAs), which are synthesized from acetyl-CoA in plastids, are partially desaturated into unsaturated fatty acids (UFAs) with one to three double bonds, or are processed into very long chain fatty acids (VLCFAs) ranging from 20 to 34 carbon straight chains (Li et al., 2016; Libeisson et al., 2013). Saturated fatty acids especially VLCFAs and UFAs are major substrates for cuticle lipid and membrane lipid, respectively (Kachroo & Kachroo, 2009; Martin & Rose, 2014). Therefore, modifying the balance between UFAs and saturated fatty acids can mediate the phase transition and fluidity of membrane, and in turn tune fruit metabolite homeostasis (Ballweg & Ernst, 2017). Carbon flux is

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also the metabolic pools of fatty acid and lipid synthesis in Arabidopsis (Baud et al., 2010). Cytosolic citrate is cracked into acetyl-CoA, which is the precursor of fatty acid synthesis but is membrane-impermeable (Fatland, Nikolau, & Wurtele, 2005). Saccharides provide cytosolic substrates such as pyruvate for plastidial fatty acid and lipid synthesis (Baud et al., 2010), and are widely conjuncted to aglycones to form glycoconjugates such as flavonoids in plants (Yang, Liu, Yang, Gupta, & Jiang, 2018).

Besides membrane lipid synthesis, lipid peroxidation indexes such as malondialdehyde (MDA) can indicate the biomembrane stability of fruit. Under chilling and pathogen stresses, lipid peroxidation might be accelerated by reactive oxygen species (ROS) burst, resulting in serious damages to membrane lipids, proteins and DNA (Gill & Tuteja, 2010; Lin et al., 2016; Wang et al., 2018). To equilibrate oxidative bursts and scavengers, excessive ROS is scavenged by antioxidases such as superoxide dismutase, peroxidase and glutathione reductase (Karuppanapandian, Juncheol, Changsoo, Manoharan, & Wook, 2011). Moreover, lipophilic tocopherols and flavonoids, which are both derived from pyruvate in plants, play important roles in anti-lipid peroxidation and nonspecific interactions with phospholipids, which induce changes in membrane features (e.g., fluidity) (Foyer & Noctor, 2005; Gill & Tuteja, 2010; Wang et al., 2018). Similarly, ascorbic acid and glutathione are small-molecular weight antioxidants and alleviate free radical stress to maintain biomembrane stability (Foyer & Noctor, 2005; Karuppanapandian et al., 2011). However, few variations in fruit biomembranes have been reported, and the relation between metabolic homeostasis and membrane stability needs to be elaborated for improvement of fruit quality and stress tolerance.

Modern breeding objective of citrus is to develop highly nutritious and stress-resistant varieties. A naturally occurring mutant of 'Newhall' navel orange has been patented as new citrus variety ('Gannan No.1'). In this study, we performed comparative analyses of membrane property, physiological quality and metabolic homeostasis between 'Gannan No.1' and wild-type cultivar 'Newhall', and aimed to clarify the importance of biomembrane stability in maintaining the quality and improving the storage performance of citrus fruit.

2. Materials and methods

2.1. Materials

Blemish-free fruits of Newhall orange (*Citrus sinensis* Osbeck) ('Newhall') and its mutant ('Gannan No.1') were harvested at 210 days after anthesis (with a fruit peel color of orange) from the same orchard under normal cultural practices in Anyuan county, Jiangxi province, China. All the experimental fruits were subjected to commercial packaging and stored in a ventilated warehouse (temperature: 12–20 °C; relative humidity (RH): 85%–90%). Twenty fruits from each of 'Newhall' and 'Gannan No.1' fruits were selected for the measurement of decay incidence and calyx senescence during storage. Ten fruits from each of 'Newhall' and 'Gannan No.1' were used for the determination of water loss rate during storage. Three fruits from each of 'Newhall' and 'Gannan No.1' were selected for measurement of respiration rate during storage. All the above experiments were performed with three biological replicates. Juice sacs of ten fruits at mature stage as well as at 45, 135 and 180 days after harvest (DAH) were sampled and frozen in liquid nitrogen and stored at −80 °C for further analysis with three biological replicates. Ten 'Newhall' and 'Gannan No.1' fruits at 45 and 135 DAH were used to evaluate the sensory quality. Ten 'Newhall' and 'Gannan No.1' fruits at each stage were chosen for determination of the total soluble solid (TSS), titratable acid (TA), ascorbic acid, juice yield, as well as water content in the pericarp and juice sacs with three biological replicates.

2.2. Measurement of membrane stability, membrane potential and ATP level

Juice sacs from three fruits per cultivar (1.5 g) were collected, washed in sterile deionized water for 15 min, and then exposed to 15 mL 0% (control), 10% and 30% polyethylene glycol (PEG) 6000 for 16 h in the dark according to the method of Bajji, Kinet, and Lutts (2002). Electrolyte leakage was measured before (ECi) and after (ECf) 4 h of rehydration, and ultimately after autoclaving (ECt). The index of cell membrane injuries was calculated as (Rs − Rc)/(1 − Rc) * 100%, where Rs and Rc represent (ECf − ECi)/(ECt − ECi) for the control and PEG 6000-treated tissues, respectively (Flint, Boyce, & Beattie, 1967). During storage, the electrolyte leakage of fruit juice sacs was also measured based on the procedure described by Huang, Wang, Zhang, and Liu (2013).

Membrane potential of juice sacs was measured by the current clamp mode of patch clamp (Axoclamp 900A, MD (AXON), USA) according to the methods of Martinez-Cortina, Ullrich, and Sanz (1992). Flesh sample was immobilized in a dish and then immersed into 5 mL of 4 °C bath solution (5 mM $CaCl_2$, 0.5 mM KCl, 5 mM 2-(*N*-morpholino) ethanesulfonic acid), which was adjusted to pH 5.8 with 1 M Tris. The pipette was filled with 3 M KCl solution (v/v, 3:2) as an internal solution and pricked into the tissue using Micromanipulator MP-285. The value of potential was recorded by software Clampex 10.6 in the protocol of Gap-free, which was connected to Axon Digidata 1550B.

For ATP measurement, 100 mg of ground juice sacs was extracted using 0.6 mL of trichloroacetic acid solution (5% w/v) as described by Liu et al. (2007). After the centrifugation at 10,000 *g* for 10 min, 10 μL of supernatant was added to 490 μL of 25 mM tris-acetate buffer (pH 7.95), and then detected using the ENLITEN ATP assay kit (Promega; http://www.promega.com). Bioluminescence was quantified using an infinite F200/M200 microplate reader (TECAN, Mannedorf, Switzerland). A standard curve was established using the ATP standard solution for calculating the ATP levels in the samples according to the kit instruction.

2.3. Determination of lipid peroxide and MDA production, and enzyme activities

Lipid peroxide was extracted as described by Hara and Radin (1978). Half gram of thoroughly ground juice sacs was mixed with 7 mL of hexane/isopropanol (v/v, 3:2). The extract was shaken in the fume hood for 1 h and spun down at 10,000 rpm for 10 min. The supernatant was collected and the pellet was centrifuged with an additional 2 mL of hexane/isopropanol (v/v, 3:2). The supernatant was combined and transferred to evaporate by a speed vacuum concentrator. The dried extract was re-dissolved in 3.6 mL of 90% methanol. Lipid peroxide was measured using the PeroxiDetect kit (Sigma-Aldrich) according to the manufacturer's instructions.

The MDA was measured by a thiobarbituric acid-based colorimetric method according to the methods of Schmedes and Holmer (1989). To quantify the activities of superoxide dismutase, peroxidase, ascorbate peroxidase and glutathione reductase, 0.5 g of juice sacs was extracted with 4.5 mL of 100 mM phosphate-buffered saline at pH 7.4 with the addition of 0.5 g of polyvinylpolypyrrolidone overnight. All these parameters were measured using a kit (Nanjing Jiancheng Bioengineering Institute) following the manufacturer's instructions as described by Huang et al. (2013).

2.4. Extraction and analysis of total lipids, primary and second metabolites

Total lipids were extracted with 2.4 mL isopropanol containing 0.01% butylated hydroxytoluene and pentadecanoate (as an internal standard) from the frozen pulp (300 mg) as described by He et al. (2018). Free fatty acids were transesterified using 2 mL of 2.5% H_2SO_4 (v/v) in methanol at 80 °C for 1 h, followed by the addition of 1 mL

hexane and 3 mL 0.9% NaCl (w/v). The hexane-containing fatty acid methyl esters (FAME) were collected, concentrated with stream of N_2, re-dissolved with hexane and analyzed using gas chromatograph–mass spectrometry (GC–MS) with a Thermo Scientific TRACE TR-FAME GC column as described by Libeisson et al. (2013).

The primary metabolites were extracted with 2700 μL methanol and 300 μL ribitol in water (0.2 mg mL^{-1}, as an internal standard) from fruit pulp (300 mg) as described by He et al. (2018). The extracts (100 μL) were incubated in 20 mg mL^{-1} methoxyamine hydrochloride in pyridine (50 μL) for 30 min at 50 °C, followed by the addition of N,O-bis (trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (50 μL) for 40 min at 60 °C. Each sample was detected using GC–MS (Thermo Fisher, ISQII, USA) equipped with a flame ionization detection and a TR-5 MS capillary column (30 m × 25 μm i.d. × 0.1 μm, Agilent Technologies). The programs of oven and column temperature as well as the metabolite identification and annotation were as reported by He et al. (2018).

The secondary metabolites were extracted according to Ding et al. (2015). The supernatant of mixture solution was detected using quadrupole time-of-flight 6520 mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) coupled to 1200 series rapid resolution high-performance liquid chromatography system (HPLC-QTOF-MS). To perform qualitative metabolic analysis, the identification was carried out by comparison of the accurate mass-to-charge ratio, retention time and the fragmentation patterns of standards, or similar fragmentation patterns with mass spectral data from literature or databases when no standards were available for the metabolites as shown in Table S1.

2.5. Fruit storage performance evaluation

Decay incidence and calyx senescence of fruits were visually evaluated. Sixty healthy fruits respectively from the 'Newhall' and 'Gannan No.1' were randomly selected and divided into three groups, respectively. Decay incidence was calculated according to the method of He et al. (2018). Calyx senescence rate was calculated according to the method reported by Carvalho, Salvador, Navarro, Monterde, and Martínezjávega (2008).

The water loss rate was measured according to He et al. (2018). Water contents in the pericarp and juice sacs were measured according to the method described by Ma et al. (2014). Pericarp disks from the equatorial plane of fruits were collected using a 1.2 cm diameter cork borer and juice sacs were sampled from six fruits, and dried by a Heto FD3 freeze-dryer (Heto-Holten, A/S, Allerød, Denmark) until constant weight. The difference between fresh and dry weight was calculated to assess the total water content. Three biological replicates were performed.

To measure the respiration rate, three fruits per genotype were sealed in 2.6-L glass jars and held at 25 °C for 3 h. Headspace gas samples (1 mL) were withdrawn with a syringe. The carbon dioxide level of samples was measured using a gas chromatograph according to the method of Lurie and Pesis (1992).

2.6. Fruit quality determination and evaluation

TSS and TA of 'Newhall' and 'Gannan No.1' fruits were determined as described by Ma et al. (2014). The content of α-tocopherol was measured using a plant vitamin E enzyme-linked immunosorbent assay kit [Rapidbio (RB)] according to the manufacturer's protocol as described by Zhu et al. (2015). Ascorbic acid content was measured according to the method of Ponting (1943). Fruit juice of 5 mL was 10-fold diluted with 1% (v/v) oxalic acid. Then, diluent of 2 mL was titrated with 3.73 mM 2, 6-dichlorophenolindophenol (DCPIP) until a pink color remained for 10 s. Based on the constructed standard curve, the volume of added DCPIP was used to calculate the ascorbic acid concentration.

The sensory quality of 'Newhall' and 'Gannan No.1' fruit was evaluated by 16 semi-trained judges (8 males and 8 females, aged 18–50) according to the method of Sdiri, Navarro, Monterde, Benabda, and Salvador (2012). First, each panelist assessed the peel glossiness of fruit by a centesimal grade with 0 being 'very low' and 100 being 'very high'. Second, the fruits were cut into four separated segments and placed into covered glass cups. Each panelist assessed the acidity, juiciness, flavor, mastication and sweetness of the fruit by a centesimal grade with 0 being 'very low' and 100 being 'very high'. The hedonic scale was rated using a 9-point scale, with 1 being extremely unpleasant, 5 being fair (commercially acceptable), and 9 being highly pleasant.

2.7. Statistical analysis

Principal component analysis (PCA) of metabolites was used to assess the metabolite variations in 'Newhall' and 'Gannan No.1' fruits using SIMCA-P 11.0 software (Umetrics, Umeå, Sweden). We performed the hierarchical clustering of metabolites, and analyzed the relationships between metabolites using a Pearson's correlation computed by R studio software. The statistical significance of the differences between 'Newhall' and 'Gannan No.1' fruit was determined by Duncan's test ($p < 0.05$) with the SAS software (SAS Institute, Cary, NC, USA). Mean values and standard error bars are provided.

3. Results

3.1. 'Gannan No.1' fruit showed the higher stability of biomembrane

The fruits of both 'Gannan No.1' and 'Newhall' were subjected to commercial packaging and then stored in a ventilated warehouse (temperature: 12–20 °C; RH: 85%–90%) (Fig. 1A). Biomembrane is a complex structure that participates in many important cellular functions of fruit. The membrane damage rate of 'Gannan No.1' after treatments with 10% and 30% PEG 6000 (8.77% and 16.11%) was lower than that of 'Newhall' (41.16% and 52.59%) at 45 DAH (Fig. 1B). Membrane stability of plants relies on membrane electrolyte leakage and potential, and ATP production in response to stresses. The electrolyte leakage of 'Gannan No.1' was significantly lower than that of 'Newhall' (Fig. 1C). The membrane potential of 'Gannan No.1' was also significantly lower than that of 'Newhall' at 45 DAH (−22.4 mV *vs.* −17.54 mV) and 135 DAH (−20.42 mV *vs.* −15.67 mV) (Fig. 1D). Consistently, the ATP level of 'Newhall' was lower than that of 'Gannan No.1' during storage (Fig. 1E). Furthermore, we observed lower levels of MDA and lipid peroxide in 'Gannan No.1' relative to those in 'Newhall'. From 0 DAH to 180 DAH, the MDA level of 'Gannan No.1' increased from 4.12 nmol g^{-1} to 10.34 nmol g^{-1}, while that of 'Newhall' increased from 7.95 nmol g^{-1} to 13.76 nmol g^{-1} (Fig. 1F). The lipid peroxide level of 'Gannan No.1' was lower than that of 'Newhall' at 0 DAH and 180 DAH (Fig. 1G). All these results of enhanced membrane physiological indexes and lower levels of peroxidate products indicated higher biomembrane stability in 'Gannan No.1'.

3.2. 'Gannan No.1' fruit had higher activities of antioxidant enzymes

Antioxidant enzymes can scavenge excessive ROS, possibly accelerating the peroxidation of membrane lipids and resulting in serious damages to biomembranes. To elucidate the mechanism underlying the higher membrane stability, we quantified the activities of antioxidases including superoxide dismutase, peroxidase, ascorbate peroxidase and glutathione reductase. From 0 DAH to 180 DAH, the relative superoxide dismutase activity of 'Newhall' decreased from 1.00 to 0.79, while that of 'Gannan No. 1' decreased from 1.07 to 0.82 (Fig. 2). The relative peroxidase activity of 'Gannan No.1' was significantly higher than that of 'Newhall' at both 0 DAH and 180 DAH (Fig. 2). Additionally, ascorbate peroxidase and glutathione reductase are involved in the ascorbic acid-glutathione antioxidant system. We found that from 0 DAH to

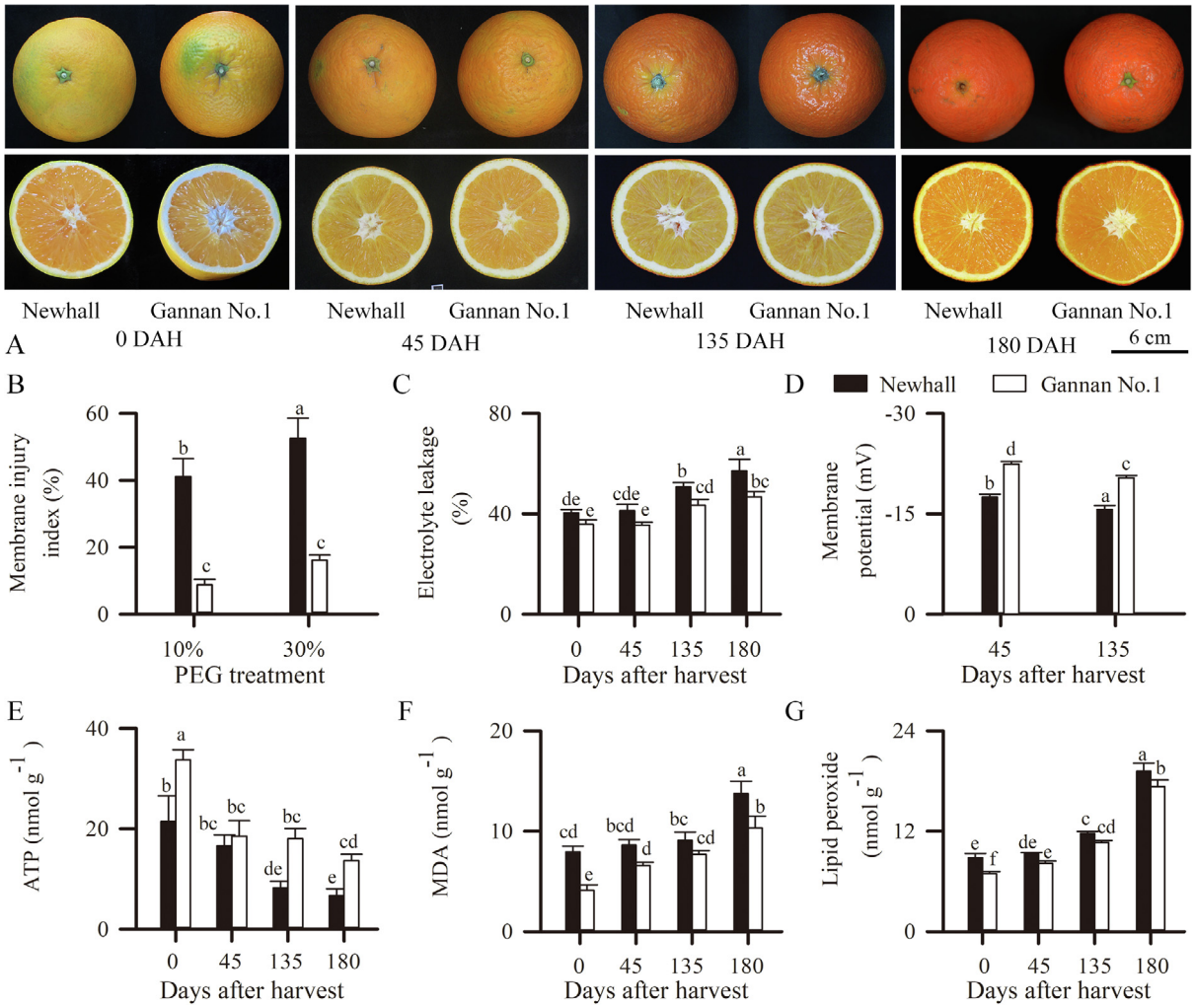

Fig. 1. Changes in phenotype and biomembrane stability of 'Newhall' and 'Gannan No.1' fruits during storage. (A) Fruit phenotype. (B) Membrane damage by PEG-treatment. (C) Membrane electrolyte leakage. (D) Membrane potential. (E) ATP level. (F) MDA level. (G) Lipid peroxide level. Values with different letters are significantly different according to *t*-test ($p < 0.05$). Data are shown as means ± SE (n = 3).

180 DAH, the relative ascorbate peroxidase activity of 'Newhall' increased from 1.00 to 2.26, and that of 'Gannan No.1' rose from 1.30 to 4.28 (Fig. 2). Furthermore, the relative glutathione reductase activity in 'Gannan No.1' was higher than that in 'Newhall' fruit, especially at 45 DAH and 180 DAH (Fig. 2). All the above results showed that the higher levels of superoxide dismutase, peroxidase, ascorbate peroxidase and glutathione reductase enzyme mitigated the membrane lipid peroxidation and further enhanced membrane stability in 'Gannan No.1'. In addition to antioxidase system, fruit metabolite homeostasis such as fatty acid unsaturation balance and stress-metabolite accumulation can also contribute to biomembrane stability.

3.3. 'Gannan No.1' fruit showed higher degrees of unsaturation of fatty acids

We measured the fatty acids of total lipids, including large amounts of straight chain fatty acids with chain lengths ranging from C14 to C24 and 0–3 double bonds in both 'Gannan No.1' and 'Newhall' fruit (Fig. 3A). In 'Gannan No.1', the contents of saturated fatty acids such as hexadecanoic acid and stearic acid were almost unchanged, but those of UFAs were elevated, especially hexadecenoic acid, vaccenic acid and linolenic acid (Fig. 3B). The relative content of hexadecenoic acid in 'Gannan No.1' was significantly higher than that in 'Newhall' during storage (Fig. 3B). The relative content of hexadienoic acid of 'Gannan No.1' was also higher than that of 'Newhall' at 135 DAH (1.11 *vs.* 2.05) (Fig. 3B). Similarly, from 0 DAH to 180 DAH, the relative content of linolenic acid dramatically increased from 1.21 to 2.34 in 'Gannan No.1', but only gradually increased from 1 to 1.81 in 'Newhall'. During storage, vaccenic acid and octadecadienoic acid showed similar decreasing trends but obvious quantitative differences in 'Gannan No.1' and 'Newhall'. In 'Gannan No.1', the contents of vaccenic acid and octadecadienoic acid were respectively 13.83% and 35.68% higher at 0 DAH, and 14.17% and 10.24% higher at 45 DAH compared with in 'Newhall' (Fig. 3B). Furthermore, the relative ratio of UFAs in 'Gannan No.1' was higher than that in 'Newhall' fruit, especially at 0 DAH (1.145 *vs.* 1.00) and 45 DAH (1.38 *vs.* 1.21) (Fig. 3C). We also calculated the index of unsaturated fatty acids (IUFA) of both 'Gannan No.1' and 'Newhall' fruit. The IUFA of 'Gannan No.1' was higher than that of 'Newhall' fruit at 0 DAH (1.10 *vs.*1.00), 45 DAH (1.28 *vs.* 1.13) and 180 DAH (1.13 *vs.* 1.01) (Fig. 3D). These results showed that the UFAs content and IUFA level in 'Gannan No.1' were higher than those in 'Newhall', and fluctuated less significantly during storage. Previous studies have indicated that stress-resistant metabolites play important roles in modulating membrane stability.

3.4. 'Gannan No.1' fruit accumulated stress-resistant metabolites

Anti-oxidative metabolites such as proline, flavonoids, tocopherol and glutathione were significantly accumulated in 'Gannan No.1'. The results from PCA and hierarchical cluster analysis of primary

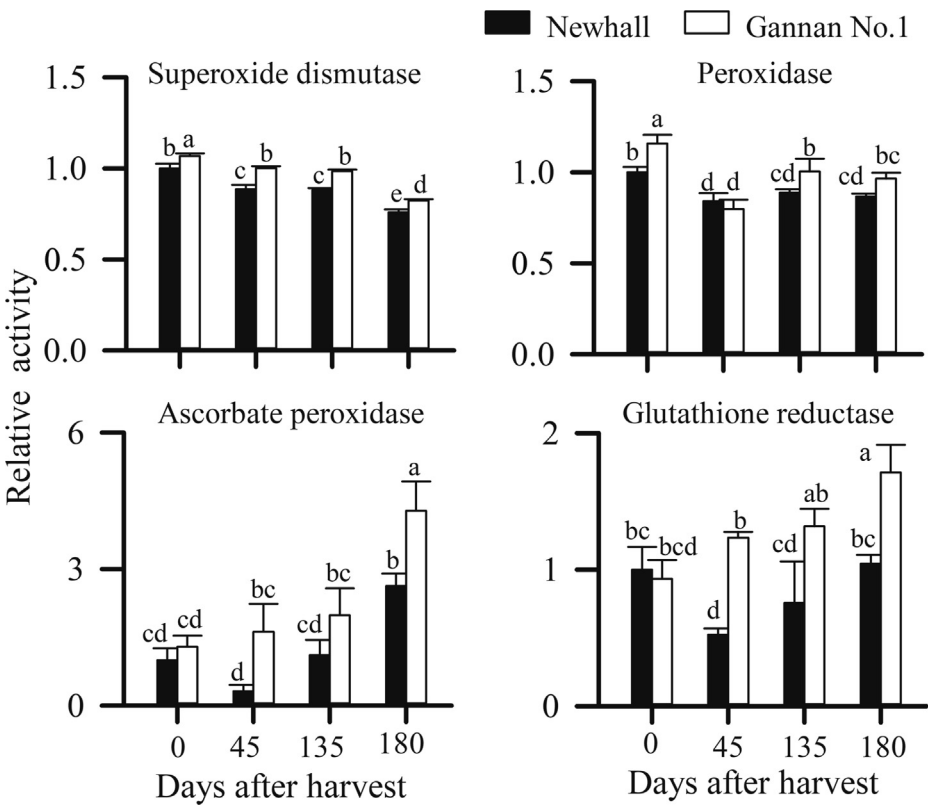

Fig. 2. Changes of anti-oxidase enzyme activities in 'Newhall' and 'Gannan No.1' fruits during storage. (A) Superoxide dismutase activity. (B) Peroxidase activity. (C) Ascorbate peroxidase activity. (D) Glutathione reductase activity. The relative activities in 'Newhall' and 'Gannan No.1' fruits at different stages were calculated by comparison with those of 'Newhall' at 0 DAH. Values with different letters are significantly different according to *t*-test ($p < 0.05$). Data are shown as means ± SE (n = 3).

metabolites such as amino acids and secondary metabolites mainly such as flavonoids showed major differences at the variety and storage stage levels (Figs. 4A, S1). We found that in 'Gannan No.1', the percentage of up-regulated metabolites increased to 41.54% (45 DAH) and then decreased to 17.45% (180 DAH), while the percentage of down-regulated metabolites only ranged from 3.17% (0 DAH) to 6.35% (180 DAH) (Fig. 4B). Amino acids are the precursors of compounds that are important for the defense system of plants. Although phenylalanine was lower in 'Gannan No.1' relative to in 'Newhall', there was a significantly higher accumulation of proline in 'Gannan No.1' (Fig. 4C; Table S2). The relative content of proline in 'Gannan No.1' decreased from 1.44 at 0 DAH to 1.28 at 180 DAH, while that of 'Newhall' decreased to 0.99 at 180 DAH. The contents of glutamic acid and isoleucine were also significantly higher in 'Gannan No.1' than in 'Newhall' at both 0 DAH and 135 DAH (Fig. 4C). We further found that the relative content of the flavonoid hesperetin-O-hexoside was significantly higher in 'Gannan No.1' than in 'Newhall' at 45 DAH (1.43 *vs.* 0.96), 135 DAH (1.26 *vs.* 0.94) and 180 DAH (1.07 *vs.* 1.51) (Fig. 4C). Similar significant accumulations of heptamethoxyflavone, luteolin-O-rutinoside and O-hexoside were observed in 'Gannan No.1' during storage (Fig. 4C). More importantly, the relative content of α-tocopherol in 'Gannan No.1' was significantly higher than that in 'Newhall' at both 45 DAH (0.97 *vs.* 0.77) and 180 DAH (0.78 *vs.* 0.63) (Fig. 4C). The ascorbic acid content of 'Gannan No.1' was slightly higher than that of 'Newhall' during storage (Fig. 4C). The relative glutathione content of 'Gannan No.1' was significantly higher than that in 'Newhall' at 45 DAH (0.84 *vs.* 0.74), 135 DAH (0.61 *vs.* 0.49) and 180 DAH (0.78 *vs.* 0.63) (Fig. 4C). Besides, saccharides and organic acids have important functions in acetyl-CoA pools for membrane FA synthesis and can also reflect fruit quality.

3.5. 'Gannan No.1' fruit exhibited better storage performance and inner quality

We further found that organic acids and saccharides were significantly higher in 'Gannan No.1' than in 'Newhall' (Fig. 5A; Table S2). Besides, the relative content of citric acid in 'Gannan No.1' was significantly higher than that in 'Newhall' (Fig. 5A), and similarly, 'Gannan No.1' had a significantly higher relative content of quininic acid than 'Newhall' at both 45 DAH (1.23 *vs.* 0.97) and 180 DAH (1.99 *vs.* 0.88) (Fig. 5A). In addition, the relative glucose content in 'Gannan No.1' continuously decreased from 0 DAH (1.19) to 180 DAH (0.98), which was higher than that in 'Newhall' at 180 DAH (0.81). The relative content of fructose in 'Gannan No.1' gradually decreased from 1.15 (0 DAH) to 0.92 (180 DAH), but in 'Newhall', it decreased from 1 (0 DAH) to 0.75 (180 DAH) (Fig. 5A). Similar results were obtained for galactose, total inositol and starch (Fig. 5A). Consistently, TA content in 'Gannan No.1' was significantly higher than that in 'Newhall' during storage (Figs. 5B; S2). Sensory analysis also confirmed the higher degree of tartness of 'Gannan No.1' fruit (Fig. S3). TSS content in 'Gannan No.1' was significantly higher than that in 'Newhall' at 135 DAH and 180 DAH (Fig. 5B). The above results had been verified by repeated experiments (Fig. S2; Table S2).

Besides, the decay incidence and calyx freshness of fruit should be taken into consideration during storage. From 45 DAH to 180 DAH, the decay incidence of 'Newhall' fruit rapidly increased from 6.67% to 90%, and that of 'Gannan No.1' fruit increased from 0% to 76.67% (Fig. 5B). The calyx senescence rate of 'Newhall' was significantly higher than that of 'Gannan No.1' at 45 DAH and 135 DAH (Fig. 5B). Although 'Gannan No.1' had a higher respiration rate than 'Newhall' at 0 DAH, the respiration rate of 'Newhall' was significantly higher than that of 'Gannan No.1' at 180 DAH (Fig. 5B). Additionally, the juice yield, weight loss and water content of 'Gannan No.1' were not significantly different from those of 'Newhall' during storage (Figs. 5B; S3), indicating significantly lower decay incidence and stalk browning, and higher inner quality of 'Gannan No.1' fruit during storage.

3.6. Relationship of membrane stability with storage performance and quality

To reveal the effects of biomembrane stability on the storage

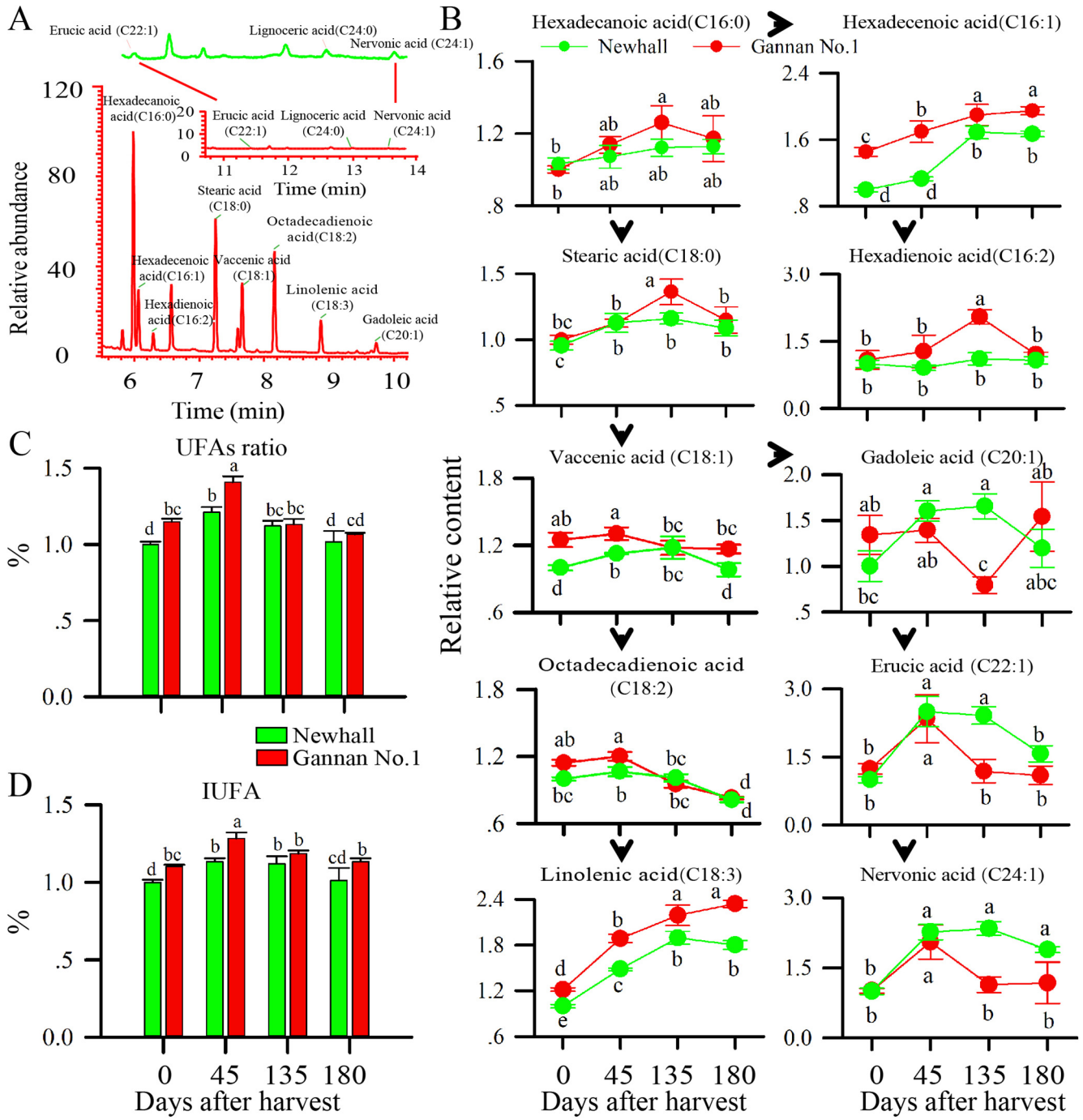

Fig. 3. Changes in the composition of fatty acids and indexes of unsaturated fatty acids in 'Newhall' and 'Gannan No.1' fruits. (A) Separation of the methyl ester derivatives of fatty acids. The numbers in brackets after fatty acids indicate the length of carbon chain. (B) Relative content of fatty acid components. The numbers in brackets after fatty acids indicate the length of carbon chain. (C) Ratio and (D) index of unsaturated fatty acids. The relative values of the metabolites in 'Newhall' and 'Gannan No.1' fruits at different stages were calculated by comparison with those in 'Newhall' at 0 DAH. Values with different letters are significantly different according to *t*-test ($p < 0.05$). Data are shown as means ± SE (n = 3).

performance and quality of fruit, Pearson's correlation analysis was performed between membrane parameters (degradation products such as MDA and lipid peroxide, energy status (ATP) and unsaturation indexes such as IUFA and UFAs ratio) and physiological indexes (decay incidence, calyx senescence, TSS and TA, and saccharides and citrate) (Tables 1; S3). MDA was positively correlated with lipid peroxide, but negatively correlated with ATP (Table 1). The correlation coefficients of MDA with decay incidence, calyx senescence, TA and TSS were 0.80 ($p < 0.05$), 0.85 ($p < 0.01$), −0.89 ($p < 0.01$), and −0.97 ($p < 0.01$), respectively (Table 1). Similarly, we observed significant negative correlations of MDA with citric acid, glucose, and fructose (Table 1). Similar results of correlation analysis were obtained for lipid peroxide (Tables 1; S3). We further observed significant positive correlations among α-tocopherol, ascorbic acid and glutathione. More importantly, α-tocopherol, ascorbic acid and glutathione showed significant negative correlations with MDA and lipid peroxide, but were positively correlated with ATP, indicating that they contribute to the anti-lipid peroxidation of biomembranes (Tables 1; S3). Subsequent correlation analysis indicated that IUFA contributes to the quality maintenance of fruit, as indicted by its positive correlations with

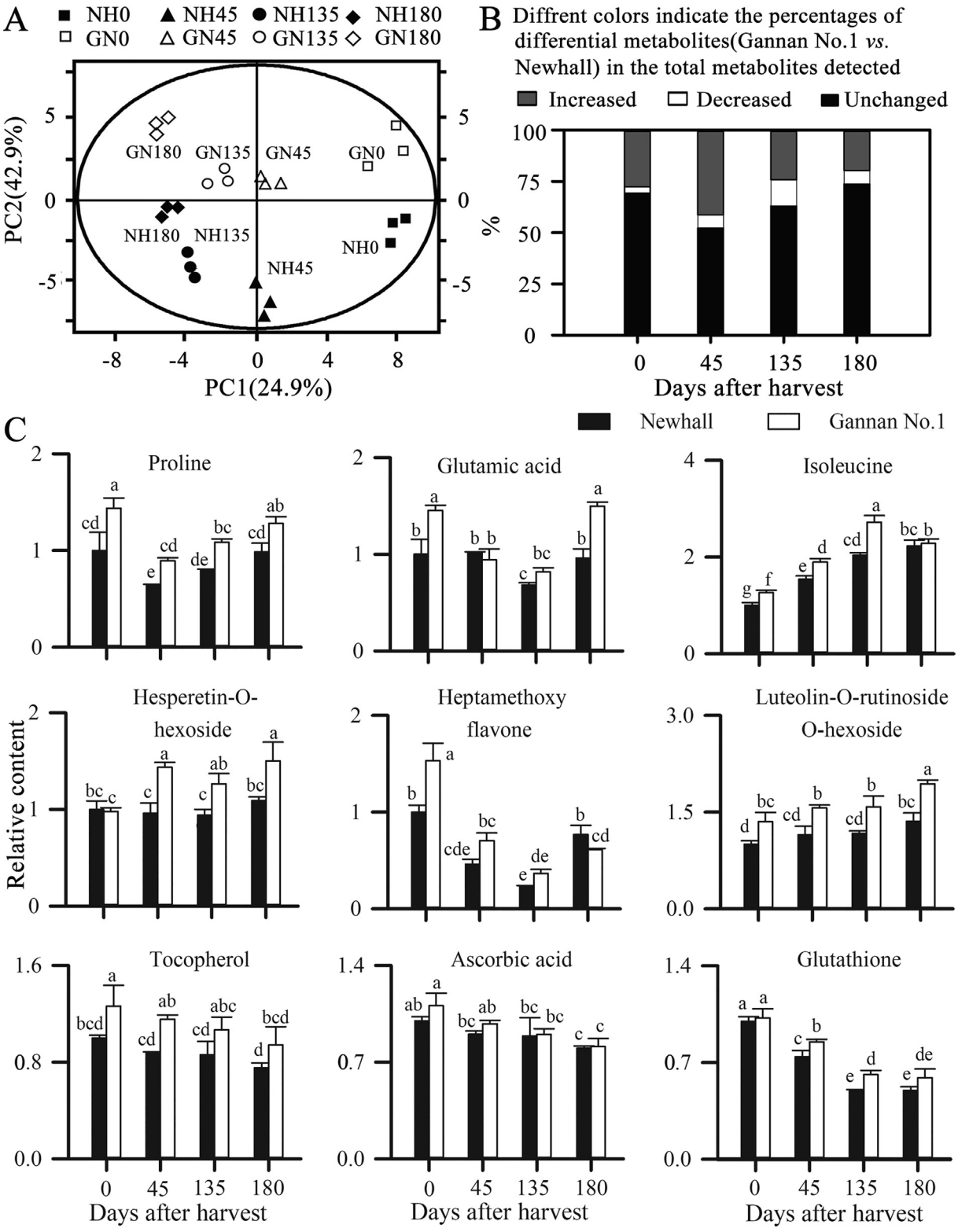

Fig. 4. Changes of resistance-related metabolites in 'Newhall' and 'Gannan No.1' fruits during postharvest storage. (A) Principal component analysis of metabolites: different varieties during postharvest storage are indicated by different kind of shapes: solid shape, Newhall (NH); air-cored shapes, Gannan No.1 (GN). The numbers before the shapes indicate the days after harvest. (B) Percentages of differential metabolites (Gannan No.1 *vs.* Newhall) in the total metabolites detected. (C) Contents of stress-related metabolites including proline, glutamic acid, isoleucine, heptamethoxyflavone, hesperetin-O-hexoside, luteolin-O-rutinoside, O-hexoside, α-tocopherol, ascorbic acid and glutathione. The relative metabolic contents of 'Newhall' and 'Gannan No.1' fruits at different stages were calculated by comparison with those of 'Newhall' at 0 DAH. Values with different letters are significantly different according to *t*-test ($p < 0.05$). Data are shown as means ± SE (n = 3).

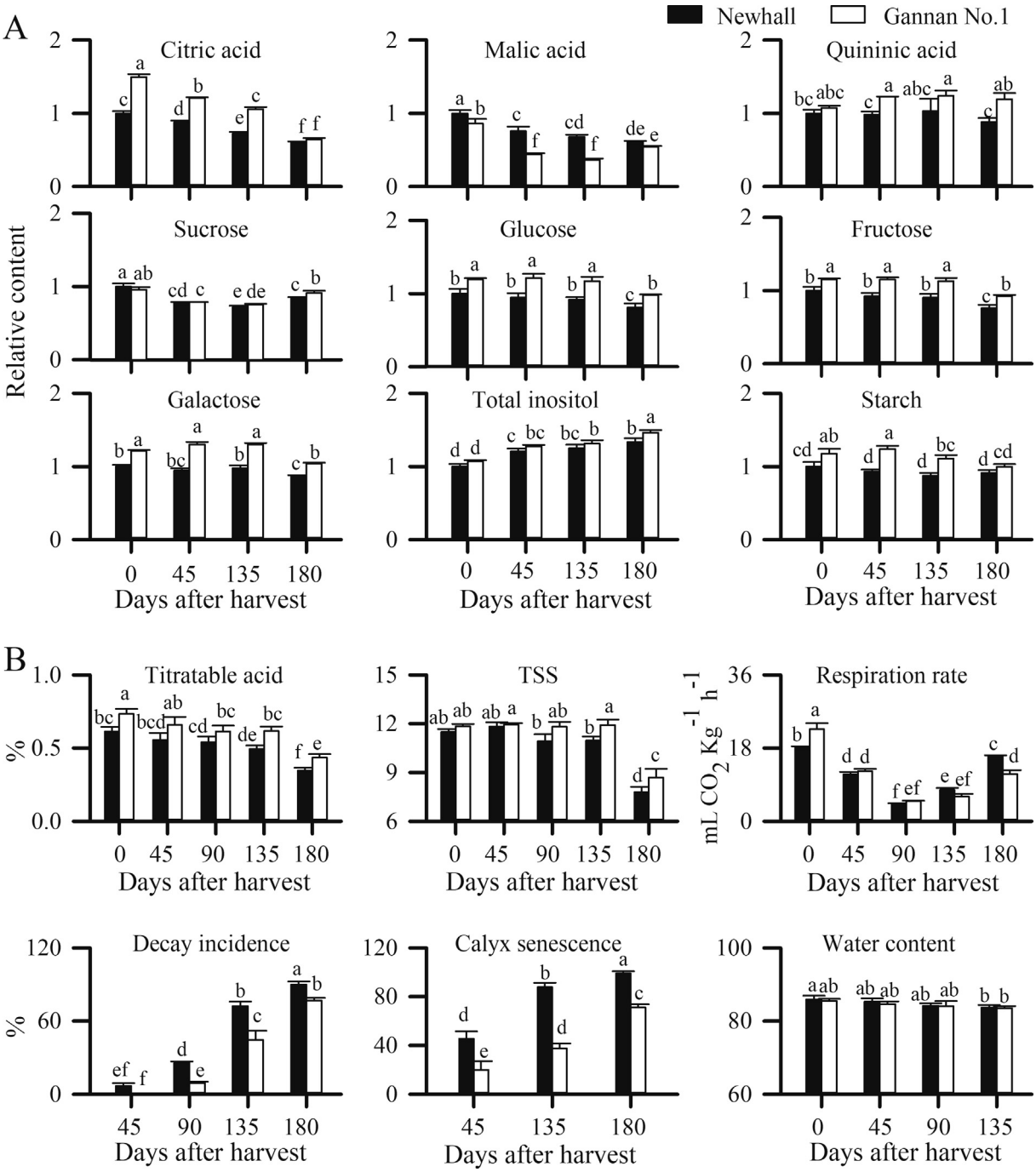

Fig. 5. Changes of fruit quality in 'Gannan No.1' and 'Newhall' during storage. (A) Organic acids and saccharides in 'Newhall' and 'Gannan No.1' fruits. The relative metabolic contents of 'Newhall' and 'Gannan No.1' fruits at different stages were calculated by comparison with those of 'Newhall' at 0 DAH. (B) Fruit performance and quality parameters of 'Gannan No.1' and 'Newhall' fruits. Different letters indicate significant differences ($p < 0.05$). Data are shown as means ± SE (n = 3).

soluble sugars (fructose and glucose) (Table 1). Similar results of correlation analysis were obtained for the ratio of UFAs (Tables 1; S3). All the results indicated that in 'Gannan No.1', the enhanced biomembrane stability by metabolic flux of fatty acids and anti-lipid peroxidation contribute to the maintenance of fruit postharvest quality.

4. Discussion

'Gannan No.1' fruit exhibits a high commercial value for its better performance and higher quality compared with 'Newhall' fruit during storage, as indicated by its lower decay incidence, calyx senescence and metabolite-consumption. Our results indicate that the lower membrane potential, membrane damage and lipid peroxidation enhance the biomembrane stability of 'Gannan No.1', which improves the stress tolerance of the fruit and thus contributes to better maintenance of fruit quality. The conventional packaging with physical barrier, which resembles the wax layer, protects citrus fruit from cross-infection of pathogens and non-stomatal water loss (D'Aquino, Piga, Agabbio, & Mccollum, 1998; Martin & Rose, 2014). Under the conventional packaging, 'Gannan No.1' fruit showed no significant differences from 'Newhall' fruit in weight loss, water content and juice yield. Consistently, the variations of surface membrane or seed lipids could increase the biomass efflux, central metabolisms such as tricarboxylic acid cycle and glycolysis, and synthesis of fatty acids and flavonoids in

Table 1
Pearson's correlation analysis between biomembrane stability and quality parameters of fruit.

	MDA	Lipid peroxide	ATP	IUFA	Decay incidence	Calyx senescence	TSS	TA	Citric acid	Fructose	Glucose	α-tocopherol	Ascorbic acid	Glutathione
MDA	1.00													
Lipid peroxide	0.91[a]	1.00												
ATP	−0.88[a]	−0.74[b]	1.00											
IUFA	−0.64	−0.59	0.35	1.00										
Decay incidence	0.80[b]	0.90[b]	−0.78[a]	−0.53	1.00									
Calyx senescence	0.85 [a]	0.84[a]	−0.90[a]	−0.40	0.92[a]	1.00								
TSS	−0.89[a]	−0.95[a]	0.65	0.60	−0.78[b]	−0.77[b]	1.00							
TA	−0.97[a]	−0.94[a]	0.88[a]	0.61	−0.87[a]	−0.87[a]	0.92[a]	1.00						
Citric acid	−0.93[a]	−0.84[a]	0.92[a]	0.56	−0.81[b]	−0.81[a]	0.78[b]	0.95[a]	1.00					
Fructose	−0.91[a]	−0.78[b]	0.78[b]	0.71[b]	−0.70[b]	−0.81[b]	0.84[a]	0.92[a]	0.89[a]	1.00				
Glucose	−0.86[a]	−0.70[b]	0.74[b]	0.75[b]	−0.64	−0.75[b]	0.76[b]	0.88[a]	0.87[a]	0.99[a]	1.00			
α-tocopherol	−0.90[a]	−0.71[b]	0.88[a]	0.61	−0.68	−0.83[a]	0.70	0.90[a]	0.94[a]	0.95[a]	0.95[a]	1.00		
Ascorbic acid	−0.90[a]	−0.93[a]	0.85[a]	0.54	−0.92[a]	−0.88[b]	0.80[b]	0.93[a]	0.92[a]	0.75[b]	0.70	0.80[b]	1.00	
Glutathione	−0.76[b]	−0.76[b]	0.89[a]	0.28	−0.91[a]	−0.87[a]	0.61	0.81[b]	0.81[b]	0.64	0.59	0.73[b]	0.89[a]	1.00

[a] $p < 0.01$.
[b] $p < 0.05$.

Arabidopsis, citrus and tomato (Kimbara et al., 2013; Lippold et al., 2009; Lonien & Schwender, 2009; Wang et al., 2016). These data suggest that 'Gannan No.1' has stable biomembranes to maintain fruit quality and adjust to the surrounding stresses.

4.1. Effect of fatty acid metabolic flux and lipid oxidation on the membrane stability of 'Gannan No.1'

Previous studies have reported the reversible transformations of UFAs and saturated fatty acids in membrane lipids, and the corresponding changes from a fluid (disordered) state to a non-fluid (ordered) state under surrounding stresses (Ballweg & Ernst, 2017; Lin et al., 2016). Here, we found higher UFAs content and IUFA level in 'Gannan No.1', indicating its higher biomembrane fluidity compared with that of 'Newhall'. Besides the physical-chemical adaptations of biomembranes, membrane lipid oxidation was caused by free radical attack, indicating the occurrence of a series of adverse reactions especially ROS-mediated membrane damages (Gill & Tuteja, 2010; Karuppanapandian et al., 2011; Lin et al., 2018). The slighter fluctuation of IUFA during storage indicated lower lipid oxidation in 'Gannan No.1', which is supported by the lower levels of lipid-oxidation products including MDA and lipid peroxide, and the higher energy status of 'Gannan No.1'. The activity of antioxidant enzymes is the biological basis of stress resistance and anti-lipid peroxidation of biomembranes (Foyer & Noctor, 2005; Karuppanapandian et al., 2011; Lin et al., 2016; Wang et al., 2018). Consistently, 'Gannan No.1' fruit exhibited higher activities of antioxidases such as superoxide dismutase, peroxidase, ascorbate peroxidase and glutathione reductase. Besides, flavonoids, especially heptamethoxyflavone and luteolin-O-rutinoside, were significantly accumulated in 'Gannan No.1' relative to 'Newhall'. Flavonoids can be incorporated into the membrane structure and mediate its fluidity, and also scavenge the excessive ROS to decrease lipid oxidation and maintain fruit storage performance (Selvaraj et al., 2015). The higher contents of lipophilic compounds of glycosylated flavonoids such as hesperetin-O-hexoside were consistent with the accumulation of saccharides in 'Gannan No.1' to strengthen the membrane structure. More importantly, the total content of α-tocopherol in 'Gannan No.1' was higher than that in 'Newhall'. Tocopherols have strong abilities to protect polyunsaturated fatty acids from oxidative damage, as they are localized within biomembranes and allow the donation of a hydrogen atom while maintaining a resonance-stabilized configuration (Sattler et al., 2006). We also observed higher contents of water-soluble antioxidant ascorbic acid and glutathione in 'Gannan No.1', which is consistent with the higher activities of glutathione reductase and ascorbate peroxidase in 'Gannan No.1'. Donation of a hydrogen atom generates a tocopherol radical which is recycled back to the original tocopherol by the interaction with ascorbic acid, and the dehydrogenated ascorbic acid is restored into the original state by glutathione (Sattler et al., 2006; Szarka, Tomasskovics, & Bánhegyi, 2012). The higher ascorbic acid-glutathione-tocopherol triad is involved in the anti-peroxidation of lipids in 'Gannan No.1'.

4.2. Effect of biomembrane stability on quality maintenance and stress response of 'Gannan No.1'

Biomembranes are the boundaries of the living protoplasm and the environment, and compose numerous organelles such as cytomembranes. With a higher biomembrane stability, 'Gannan No.1' exhibited better fruit quality and performance in storage, which is supported by the previously reported results of Lippold et al. (2009) and Kimbara et al. (2013). We observed negative correlations of lipid peroxidation indexes such as MDA with quality parameters such as TA and TSS. The enhanced stability of biomembranes can effectively control proton leakage and nutrient permeability, mediating the cellular metabolic homeostasis. Consistently, we observed significant accumulations of saccharides, citrate acid and amino acids such as proline in 'Gannan No.1'. Similarly, there were significant negative correlations between MDA and citric acid, fructose and glucose. Higher contents of saccharides and citrate acid can provide substrates for respiration and energy consumption, indicating a good taste characteristic of 'Gannan No.1' fruit. Besides, saccharides and citrate acid can also be an important acetyl-CoA pool for plastidial fatty acid synthesis and cytosolic fatty acid elongation, respectively (Baud et al., 2010). We speculated that the decrease in wax and fatty acid elongation of 'Gannan No.1' might lead to the accumulations of saccharides and citrate in a feedback way, and might also reduce the recruiting of oxaloacetic acid from cytosol to mitochondria, resulting in a decrease in malate. As the main organic acid of citrus fruit, citric acid is also involved in various environmental adaptations as a known metal chelator, and increases the tolerance to aluminum and cadmium (Kaur et al., 2017; Mahmud, Hasanuzzaman, Nahar, Bhuyan, & Fujita, 2018). More importantly, we found that the rates of stalk browning and decay were positively correlated with MDA but negatively correlated with IUFA. The higher unsaturation degree of lipids decreases the potential energy of phase transition to stabilize or weaken the membrane fluid, resulting in a rapid response to surrounding stress (Gao et al., 2017; Sgobba, Paradiso, Dipierro, De, & de Pinto, 2015). Besides, the stable permeability of biomembrane in 'Gannan No.1' was accompanied by increases in soluble metabolites such as citric acid, glycerol and proline to maintain its tolerance to osmotic stress. A higher osmotic homeostasis is considered to be important for the defense system in desiccation tolerance and fruit senescence (Dar, Naikoo, Rehman, Naushin, & Khan,

2016). All these results indicate that the biomembrane stability of 'Gannan No.1' is enhanced by the metabolic flux of fatty acids and antioxidant lipid homeostasis, which contributes to the improvement of fruit quality and stress tolerance.

5. Conclusions

Our data demonstrate that 'Gannan No.1' fruit is an environment-friendly, stress resistant and nutritious variety of citrus. The enhanced biomembrane stability of 'Gannan No.1' improves its stress tolerance and physiological quality, and extends the storage life of fruit. Therefore, exploring the influence of biomembranes on fruit post-harvest quality is critical to environment and food safety in citrus industry, and can facilitate a better understanding of the biological basis of biomembranes.

Declaration of interests

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.04.009.

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