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Role of Brassinosteroids, Ethylene, Abscisic Acid, and Indole-3-Acetic Acid in Mango Fruit Ripening

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Abstract Rapid ripening of mango fruit limits its distribution to distant markets. To better understand and perhaps manipulate this process, we investigated the role of plant hormones in modulating climacteric ripening of 'Kensington Pride' mango fruits. Changes in endogenous levels of brassinosteroids (BRs), abscisic acid (ABA), indole-3-acetic acid (IAA), and ethylene and the respiration rate, pulp firmness, and skin color were determined at 2-day intervals during an 8-day ripening period at ambient temperature (21 ± 1°C). We also investigated the effects of exogenously applied epibrassinolide (Epi-BL), (+)-*cis*, *trans*-abscisic acid (ABA), and an inhibitor of ABA biosynthesis, nordihydroguaiaretic acid (NDGA), on fruit-ripening parameters such as respiration, ethylene production, fruit softening, and color. Climacteric ethylene production and the respiration peak occurred on the fourth day of ripening. Castasterone and brassinolide were present in only trace amounts in fruit pulp throughout the ripening period. However, the exogenous application of Epi-BL (45 and 60 ng g^{-1} FW) advanced the onset of the climacteric peaks of ethylene production and respiration rate by 2 and 1 day, respectively, and accelerated fruit color development and softening during the fruit-ripening period. The endogenous level of ABA rose during the climacteric rise stage on the second day of ripening and peaked on the fourth day of ripening. Exogenous ABA promoted fruit color development and softening during ripening compared with the control and the trend was reversed in NDGA-treated fruit. The endogenous IAA level in the fruit pulp was higher during the preclimacteric minimum stage and declined during the climacteric and postclimacteric stages. We speculate that higher levels of endogenous IAA in fruit pulp during the preclimacteric stage and the accumulation of ABA prior to the climacteric stage might switch on ethylene production that triggers fruit ripening. Whilst exogenous Epi-BL promoted fruit ripening, endogenous measurements suggest that changes in BRs levels are unlikely to modulate mango fruit ripening.

Keywords *Mangifera indica* L. · Brassinosteroids · Ethylene · Abscisic acid · Auxin · Respiration · Fruit ripening · Climacteric

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Introduction

Mango (*Mangifera indica* L.) is a climacteric fruit, in which ripening involves a preclimacteric minimum, climacteric rise, climacteric peak, and postclimacteric phases (Watada and others 1984). The ripening process of mango fruit involves numerous biochemical changes, including increased respiration, ethylene production, fruit softening, chlorophyll degradation, carotenoid synthesis, and several other metabolic activities leading to changes in carbohydrates, organic acids, lipids, phenolics, and volatile compounds (Gomez-Lim 1993; Singh and Singh 2011). Ethylene plays a pivotal role in the regulation of climacteric fruit ripening (Seymour and others 1993) and the role

of ethylene in controlling mango fruit ripening has been well established (Mann 1985; Cua and Lizada 1990; Singh and Janes 2001; Lalel and others 2003; Nair and Singh 2003). Under ambient temperature, 'Kensington Pride' mangoes harvested at the mature green stage exhibit a climacteric ethylene peak during day 3 of ripening, with a concomitant increase in respiratory production on the same day (Lalel and others 2003). Exogenous application of ethephon increased ethylene production and respiration rate in 'Kensington Pride' mangoes at ambient temperature (21 ± 1°C), whilst the application of inhibitors of ethylene biosynthesis [for example, aminoethoxyvinylglycine (AVG)] and action [for example, 1-methylcyclopropene (1-MCP)] suppressed these processes (Lalel and others 2003). Nguyen and others (2002) also claimed that 'Kensington Pride' mangoes took fewer days to reach the soft (eating) stage when treated with exogenous ethylene, although they retained the green skin color.

Abscisic acid (ABA) also plays an important role in fruit ripening (Vendrell and Palomer 1997). Increased levels of ABA during ripening have been reported in climacteric fruit such as tomato (Ruan and others 2005), peach (Wu and others 2003), and plum (Kitamura and others 1983), although there are no reports on changes in endogenous ABA levels during mango fruit ripening. Exogenous application of ABA enhances fruit ripening in several cultivars of mango such as 'Alphonso', 'Langra', and 'Zihua' (Palejwala and others 1988; Parikh and others 1990; Zhou and others 1996), although the exogenous application of ABA and an inhibitor of its biosynthesis, nordihydroguaiaretic acid (NDGA), in modulating fruit ripening of 'Kensington Pride' mango fruit has not yet been investigated. Exogenous application of ABA and/or NDGA has been reported to regulate the endogenous concentrations of ABA and consequently influenced fruit ripening in various climacteric fruits such as banana (Lohani and others 2004), kiwifruit (Chen and others 2005), and tomato (Zhu and others 2003; Zhang and others 2009).

In climacteric fruit, the level of auxin has been claimed to influence ethylene production through the induction of the activity of 1-aminocyclopropane-1-carboxylic acid synthase (ACS) during fruit ripening (Vendrell and Palomer 1997). A burst of climacteric ethylene resulted in the rapid degradation of endogenous IAA in 'Yulu' peach on the third day of ripening (Wu and others 2003). On the other hand, endogenous levels of IAA increased during the fruit-beaker and ripe-fruit stages in the *rin* (ripening inhibitor) tomato mutant (Hong and Lee 1993). However, it is not known how endogenous IAA levels change during the ripening of mango fruit.

Brassinosteroids (BRs) are a group of steroidal plant hormones implicated in numerous plant growth and development processes, including cell elongation, cell division, vascular differentiation, reproductive development, as well as pathogen and abiotic tolerance (Clouse 2002). Recent evidence suggests that BRs are also involved in the ripening of grapes, a nonclimacteric fruit (Symons and others 2006). In the climacteric tomato fruit, the exogenous application of BRs has been reported to promote ripening of tomato pericarp discs through increased ethylene production (Vardhini and Rao 2002). Later, Montoya and others (2005) reported higher concentrations of endogenous BRs in tomato fruit during the early stages of development. The intriguing questions are therefore whether the levels of endogenous BRs change during fruit ripening in mango and whether BRs play a key role in climacteric fruit ripening.

We examined whether the endogenous levels of BRs, ethylene, ABA, and IAA changed during the ripening process of 'Kensington Pride' mango fruit, and whether their exogenous application influenced the rate of ripening to further understand the role that plant growth regulators play in regulating fruit ripening.

Materials and Methods

In experiment I, changes in the endogenous levels of abscisic acid (ABA), indole-3-acetic acid (IAA), ethylene, and brassinosteroids (BRs) (castasterone and brassinolide) were measured along with fruit firmness and skin color development during ripening in 'Kensington Pride' mango fruit. In experiments II and III, the effects of exogenous epibrassinolide (Epi-BL) as well as ABA and nordihydroguaiaretic acid (NDGA) on fruit-ripening parameters such as respiration rate, ethylene production, fruit softening, and skin color development were examined in 'Kensington Pride' mango fruit during ripening at ambient temperature.

Fruit

Hard mature mango fruit (*Mangifera indica* L. cv. 'Kensington Pride'), characterized by green skin and light cream pulp color, uniform size, and free from visual blemishes and diseases, were used in all three experiments. In experiments I and II, fruit were obtained from a commercial orchard at Gingin (longitude 115°55′E and latitude 31°21′S), Western Australia (WA) on the 19 and 31 March 2008, respectively. In experiment III, hard mature green fruit was obtained from a commercial orchard located at Dongara, WA (longitude 114.93°55′E and latitude 29.26°15′S) on the 18 February 2009. In experiment I, the mature fruit were firm (92.92 ± 3.71 N) and had a respiration rate of 2.36 ± 0.01 mmol CO_2 kg^{-1} h^{-1}. In experiment II, the fruit were also firm (102.83 ± 18.48 N) and had a respiration rate of

1.64 ± 0.04 mmol CO_2 kg^{-1} h^{-1}. In experiment III, the fruit were firm (75.6 ± 6.71 N) and had a respiration rate 2.68 ± 0.40 mmol CO_2 kg^{-1} h^{-1}. All the fruit were desapped (following harvest, sap from fruit was allowed to exude from the end of the stalk by physical inversion of the fruit on desapping trays to avoid sap burn injury over the skin), fungicide-treated (Sportak 0.55 ml L^{-1} with Prochloraz as an active ingredient), air-dried, packed in soft-board trays, and transported to Perth, WA, by a refrigerated truck (13°C).

Experiment I: Changes in Endogenous Levels of BRs, ABA, IAA, and Ethylene During Mango Fruit Ripening

The mango fruit were kept in soft-board trays and allowed to ripen at ambient temperature (21 ± 1°C) and relative humidity (RH) of 57.2 ± 11.1%. Pulp samples were collected and pooled from the inner and outer mesocarp at 2-day intervals during the 8-day ripening period. Pulp tissue was immersed in liquid nitrogen and stored at −80°C for determination of endogenous levels of BRs, ABA, and IAA. Ethylene production, respiration rate, fruit softness, and skin color development were determined every 2 days during fruit ripening. The experiment used a completely randomized design, including ripening period as a factor. Ten fruit were used for each of three replicates.

Experiment II: Effects of Exogenous Application of Epi-BL on Mango Fruit Ripening

Aqueous solution containing different doses (15, 30, 45, and 60 ng g^{-1} FW) of epibrassinolide [Epi-BL; (22R, 23R)-2α, 3α, 22, 23-tetrahydroxy-7-oxa-B-homo-5α-ergostan-6-one] was applied onto the skin of the whole, hard, mature mango fruit. The Epi-BL was purchased from Sigma-Aldrich Pty. Ltd., Castle Hill, Australia. Untreated fruit served as a control. Following treatment, the fruit were allowed to ripen at ambient temperature (21 ± 1°C) and RH of 54.1 ± 9.1%. Ethylene production, respiration rate, fruit softness, and skin color development were recorded daily during the ripening period. The time of onset of the climacteric peak and the rate of ethylene production and respiration rate at the climacteric peak were recorded. The experiment used a completely randomized design, including three replicates of ten fruit each.

Experiment III: Effects of Exogenous Application of ABA and NDGA on Modulating Mango Fruit Ripening

Hard mature green fruit were dipped for 5 min in an aqueous solution containing different concentrations (0.05, 0.1, and 0.2 mM) of (+)-*cis, trans*-abscisic acid (ABA) (Syntake Chemical, Shanghai, China) or (0.05, 0.01 and 0.02 mM) nordihydroguaiaretic acid (NDGA) (purchased from Sigma-Aldrich Pty. Ltd., Castle Hill, Australia). 'Tween 20' (0.05%) was present as a surfactant. NDGA is an inhibitor of ABA biosynthesis (Zhang and others 2009). Following treatments, the fruit were allowed to dry and ripen at ambient temperature (21 ± 1°C) and RH of 55.2 ± 11.1% in soft-board trays until reaching the soft (eating) stage (subjective firmness rating number 4), as previously described by Dang and others (2008). Control fruit were dipped in water containing 'Tween 20' (0.05%). Respiration rate, fruit softening, and skin color development were determined daily during the ripening period. The experiment used a completely randomized design, including three replicates of ten fruit.

Extraction, Purification, and Quantification of BRs, ABA, and IAA

Extraction of BRs, ABA, and IAA

Mango fruit pulp (200 g) was immersed and homogenized in 500 ml of cold methanol (−20°C) and distilled water (80:20 v/v) containing butylated hydroxytoluene (100 mg L^{-1}) to prevent oxidation of the hormones (Ross and others 1987). The extraction of plant hormones occurred in a 4°C refrigerator for 12 h. Extracts were then filtered using Whatman No. 1 filter paper and stored at −20°C.

Brassinosteroids (BRs) Purification and Quantification

An aliquot of each extract equivalent to 50 g fresh weight (FW) of pulp was taken and 50 ng of deuterated ($^2H_6$) brassinolide and castasterone (provided by Dr. Suguru Takatsuto and Prof. Takao Yokota from Japan) were added to each sample as internal standards. Sample purification and quantification of BRs using gas chromatography–mass spectrometry with selected ion monitoring (GC–MS–SIM) were performed as described by Symons and Reid (2003) and with the modification that these samples were not subjected to a C18-Sep Pak purification step.

Purification and Quantification of IAA and ABA

An aliquot of each extract equivalent to 1 g FW was taken and 50 ng of $^{13}C_6$ IAA (Cambridge Isotope Laboratories, Andover, MA, USA) and 40 ng of $^2H_4$ ABA (National Research Council of Canada, Saskatoon, Canada) were added to each sample. Samples were reduced in volume to less than 1 ml under vacuum at 35°C and taken up in 3 × 3-ml washes of 0.4% (v/v) acetic acid in distilled water (dH_2O). These washes were then passed through a

C18-Sep Pak cartridge, preconditioned with 10 ml of methanol, and followed by 10 ml of 0.4% (v/v) acetic acid in dH_2O. Hormones were then eluted with 15 ml of 70% (v/v) methanol in 0.4% (v/v) acetic acid. The elution was reduced to dryness under vacuum at 35°C, taken up in 400 µl of methanol, methylated with 1,500 µl of 0.2 M (trimethylsilyl) diazomethane (in diethyl ether), and then dried under a stream of nitrogen. As a final purification step, the sample was dissolved in 1 ml of dH_2O, then partitioned against 1 × 800 µl, followed by 2 × 400 µl of diethyl ether. The hormone-containing ether fraction was then divided in two. One half of the sample was reduced to dryness under nitrogen and then dissolved in 20 µl of chloroform for ABA methyl ester analysis using a triple quadrupole gas chromatograph–mass spectrometer (GC–MS–MS, 7000A, Agilent Technologies Inc., Santa Clara, CA, USA). The second half of the sample was reduced to dryness under nitrogen and the IAA-methyl ester was silylated twice, first in 10 µl pyridine and 40 µl *N*,*O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA, Sigma-Aldrich) at 80°C for 20 min, then dried under nitrogen, and again with 40 µl BSTFA at 80°C for 20 min. These samples were then reduced to dryness under nitrogen before being dissolved in 20 µl of chloroform for GC–MS–MS analysis. GC–MS–MS analysis was performed with a Varian 8400 autosampler and a Varian 3800 GC coupled to a Varian 1200 triple-quadrupole MS. The analyses of IAA and ABA samples were carried out using the methods described by Jager and others (2008) and were calculated as described by Ross and others (1995). Positive identification of IAA and ABA from mango pulp tissues was achieved by full-scan mass spectrometry.

Determination of Ethylene Production and Respiration Rate

The rates of ethylene production and respiration in mango fruit during ripening were determined according to Zaharah and Singh (2011a). The fruit was sealed in a 1,000-ml air-tight jar, fitted with a rubber septum, for 1 h at 20 ± 1°C. A headspace gas sample (1 ml) was then injected into a gas chromatograph to estimate the rate of ethylene production. The concentration of ethylene produced by fruit was quantified using a gas chromatograph (6890 N Network GC System; Agilent Technologies) fitted with a 2-m-long stainless-steel column filled with 80/100 mesh size Porapaq-Q (3.18-mm internal diameter; Supelco, Bellefonte, PA, USA) and a flame ionization detector (FID). Ethylene in the gas was identified by comparing its retention time and co-chromatography with authentic standards (0.9 ± 0.1 µl L^{-1} of ethylene in nitrogen) certified as β-standard and obtained from BOC Gases, Australia Ltd., Perth, Australia.

The rate of respiration was determined from the headspace gas (1 ml) using an infrared gas analyzer [Servomex Gas Analyser, Series 1450 Food Package Analyser; Servomex (UK) Ltd., Crowborough, UK]. The respiration rate of each sample was calculated based on the peak area of a 1-ml CO_2 standard (8.52 ± 0.17 µl L^{-1} of CO_2 in nitrogen; BOC Gases). The rates of ethylene production and respiration were expressed in nmol C_2H_4 kg^{-1} h^{-1} and in mmol CO_2 kg^{-1} h^{-1}, respectively. These estimations were repeated twice.

Fruit Firmness

Fruit (pulp) firmness was measured every 2 days during mango fruit ripening in experiment I, according to Zaharah and Singh (2011a), using a texture analyzer (TA Plus; AMETEK Lloyd Instruments Ltd., Fareham, UK). The machine was equipped with a horizontal square-base table (15 cm × 15 cm) linked to a personal computer via Nexygen™ software (ver. 4.6; AMETEK Lloyd Instruments Ltd., Fareham, UK). A Magness-Taylor probe (11 mm), with a 500-N load cell on, at a cross speed of 2 mm s^{-1}, a trigger force of 0.5 N, and compression of 25%, was used to puncture the pulp for all determinations. The pulp samples of outer mesocarp from the middle of the fully ripe fruit were collected and cut into cubes (5 cm × 2 cm × 2 cm) with a sharp knife. The pulp sample was placed on the top of the base table and the gap size between the pulp sample and probe was at least 2 mm. The pulp firmness was expressed in newtons (N). The rate of pulp firmness loss was calculated as percent firmness loss per 2-day interval during the ripening period.

Fruit Softness

Fruit softness of a sample from the middle of each fruit was recorded daily in experiments II and III using a rating scale of 1–5 (1 = hard, 2 = sprung, 3 = slightly soft, 4 = eating soft, and 5 = over soft) as described earlier by Zaharah and Singh (2011b).

Visual Skin Color

The visual fruit skin color was recorded every 2 days in the experiment I and daily in experiments II and III during the ripening period by following a rating of 1–5 according to the percentage of greenish and yellowish color: (1) 100% green, (2) 1–25% yellow, (3) 26–50% yellow, (4) 51–75% yellow, and (5) 76–100% yellow as described by Zaharah and Singh (2011a).

Statistical Analysis

The experimental data were subjected to one- or two-way analysis of variance (ANOVA) using SAS release 9.1 (SAS Institute Inc., Cary, NC, USA). Fisher's least significant differences (LSD) were calculated following a significant ($P \leq 0.05$) *F*-test. All the assumptions of ANOVA were checked to ensure validity of statistical analysis. Regression analysis was performed using the same program to determine the relationship between the endogenous level of ABA and IAA, and between ethylene production or respiration rate and the endogenous level of ABA and IAA and respiration, and between the endogenous level of ABA or IAA and fruit softness and skin color.

Results

Changes in Endogenous BRs, Ethylene, ABA, and IAA Levels During Ripening

Castasterone and brassinolide were detected in fruit pulp only in trace amounts during mango fruit ripening, with no clear changes in their accumulation throughout the ripening period (Table 1). However, the castasterone level may be slightly higher on day 8 (< 0.13 ng g^{-1} FW) and brassinolide was found (at trace levels) only after 6–8 days of ripening at ambient temperature (Table 1).

As expected, a typical climacteric ethylene production peak (8.24 nmol C_2H_4 kg^{-1} h^{-1}) was noticed on day 4 of the ripening period (Fig. 1a). Ethylene production was substantially lower (in a range of 0.72–1.30 nmol C_2H_4 kg^{-1} h^{-1}) during the preclimacteric and postclimacteric stages (Fig. 1a).

The endogenous level of ABA in the pulp tissue significantly ($P \leq 0.001$) increased (186.90%) during the climacteric rise stage (2 days of ripening) and peaked at the climacteric stage (277.26% increase on fourth day of ripening) compared with the preclimacteric minimum on 0 days of ripening (Fig. 1b). The endogenous ABA level in the pulp tissue significantly ($P \leq 0.001$) declined (20.23–37.05%) during the postclimacteric period (after 6 and 8 days of ripening), when compared with the endogenous level of ABA on day 4 of ripening.

The endogenous level of IAA in the pulp tissues was highest (3.30 ng g^{-1} FW, $P \leq 0.001$) at the preclimacteric minimum on day 0 of the ripening period and declined substantially during the climacteric and postclimacteric ripening periods. The endogenous level of IAA in the pulp

Table 1 Changes in the endogenous level of BRs (castasterone and brassinolide) in the fruit pulp of 'Kensington Pride' mangoes during ripening

Ripening period (days)	Brassinosteroid levels (ng g^{-1} FW)	
	Castasterone	Brassinolide
0	Trace <0.019	ND
2	ND	ND
4	Trace <0.016	ND
6	Trace <0.009	Possible trace
8	Trace <0.13	Possible trace

ND not detected

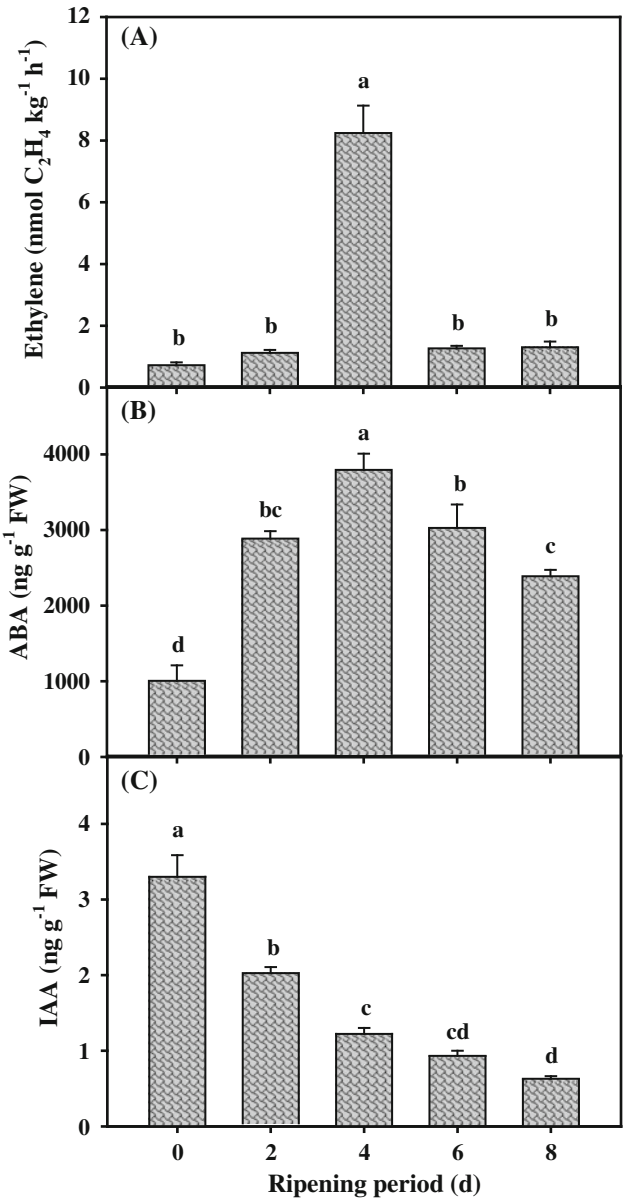

Fig. 1 Changes in the endogenous level of ethylene (**a**), ABA (**b**), and IAA (**c**) in the pulp during fruit ripening at ambient temperature. Vertical bars represent standard error of the mean (SEM). The same letters are not significantly different ($P > 0.001$). LSD ($P \leq 0.001$) for ethylene = 0.94, ABA = 631.84, and IAA = 0.45; $n = 3$

tissue was 80.91% lower after 8 days of ripening compared with its concentrations in the pulp tissue during the preclimacteric minimum stage (day 0) (Fig. 1c).

Respiration Rate, Fruit Firmness, and Skin Color During Fruit Ripening

The climacteric respiration peak (3.74 mmol CO_2 kg^{-1} h^{-1}) was noticed on the fourth day of ripening and was significantly ($P \leq 0.05$) higher (58.47%) compared with the rate at the preclimacteric minimum stage on day 0 of ripening (Fig. 2a). The rate of respiration declined substantially during the post-climacteric period (29.68 and 33.96% on days 6 and 8 of ripening, respectively) compared with its production during the climacteric respiration peak.

As expected, fruit firmness was highest (92.92 N) at the preclimacteric minimum stage on day 0 (Fig. 2b). The percentage reduction in pulp firmness was greater (61.72 and 60.11%) at the climacteric peak stage on days 4 and 6 of ripening, respectively, than at the climacteric rise stage on the second day of ripening (36.28%) and postclimacteric stage on the eighth day of ripening (31.64%).

The skin color was green at the preclimacteric minimum stage and at the climacteric rise stage (days 0 and 2, respectively, Fig. 2c). There was a dramatic increase (130.69%) in skin color development at the climacteric peak stage on day 4 of ripening compared with the color development at the climacteric minimum stage. The skin continued to yellow during the postclimacteric stage (after 6 and 8 days of ripening).

Effects of Exogenous Application of Epi-BL on the Onset and Climacteric Peak of Ethylene and Respiration

As expected, mango fruit exhibited climacteric ethylene and respiration peaks during the ripening period. The exogenous application of Epi-BL treatments (45 and 60 ng g^{-1} FW) significantly advanced the onset of the climacteric peak of ethylene production and respiration rate by 2 and 1 day(s), respectively (Table 2). Both of these treatments also had a higher climacteric ethylene production peak (4.81 and 5.74 nmol C_2H_4 kg^{-1} h^{-1}) and respiration rate (4.87 and 5.06 mmol CO_2 kg^{-1} h^{-1}) compared with the control (Table 2).

Effect of Exogenous Application of Epi-BL on Fruit Softening During Ripening

Exogenous applications of Epi-BL promoted fruit softening, particularly between days 3 and 7 of the ripening period compared with the control (Fig. 3a). Fruit softness

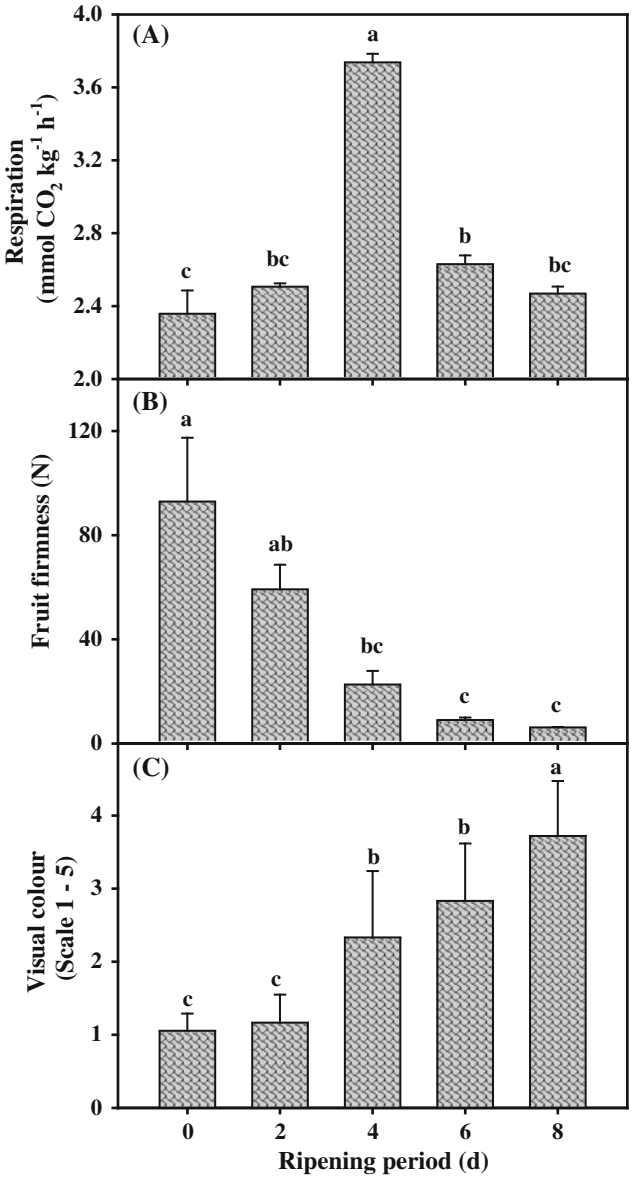

Fig. 2 Changes in the respiration rate (**a**), fruit firmness (**b**), and skin color (**c**) during fruit ripening at ambient temperature. Vertical bars represent standard error of the mean (SEM). Bars with the same letters are not significantly different ($P > 0.05$). LSD ($P \leq 0.01$) for respiration rate = 0.16, fruit firmness = 35.75, and skin color = 0.44. Respiration rate, $n = 3$; fruit firmness and skin color changes, $n = 15$

at the fully ripe stage did not significantly differ between Epi-BL-treated fruit and the control (Fig. 3a). Averaged over the ripening period, the mean of fruit softness was significantly ($P \leq 0.05$) higher (13.15, 15.96, 14.55, and 23.47%) after treatment with 15, 30, 45, and 60 ng g^{-1} FW of Epi-BL, respectively, compared with the control.

All Epi-BL treatments significantly ($P \leq 0.05$) improved skin color development between 2 and 7 days of ripening, irrespective of the concentration applied (Fig. 3b). Averaged over the ripening period, the mean of skin color development

Table 2 Changes in climacteric ethylene production (nmol C_2H_4 kg^{-1} h^{-1}) and respiration rate (mmol CO_2 kg^{-1} h^{-1}) during fruit ripening influenced by exogenous Epi-BL

Treatments (ng g^{-1} FW)	Ethylene climacteric		Respiration climacteric	
	Onset (days)	Peak rate	Onset (days)	Peak rate
Control	5.00a	1.58c	5.00a	3.55d
Epi-BL—15	5.00a	1.92c	4.00b	4.21c
Epi-BL—30	4.00b	3.06b	4.00b	4.45bc
Epi-BL—45	3.00c	4.81a	4.00b	4.87ab
Epi-BL—60	3.00c	5.74a	4.00b	5.06a
LSD ($P \leq 0.05$)	0.47***	1.05***	0.47**	0.48***

Means followed by the same letter within a column are not significantly different at $P > 0.05$; $n = 3$

Epi-BL = epibrassinolide

** and *** significant at $P \leq 0.01$ and 0.001, respectively

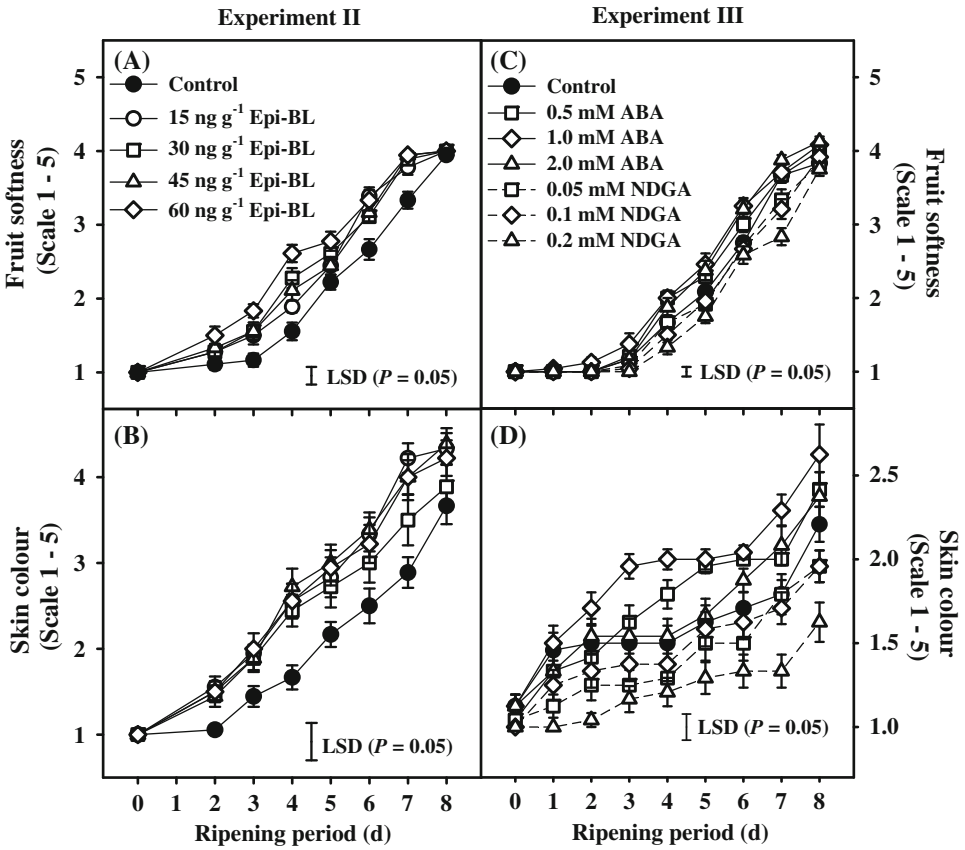

Fig. 3 Changes in fruit softening and skin color development by exogenous Epi-BL (**a**, **b**) and ABA and the ABA synthesis inhibitor NDGA (**c**, **d**) during ripening (RP) at ambient temperature. Vertical bars represent standard error of the mean (SEM) and are invisible when the values are smaller than the symbol; $n = 15$. LSDs for $P \leq 0.05$ are shown for fruit softness (**a**, **c**) and skin color (**b**, **d**)

on the fruit was significantly ($P \leq 0.05$) higher (29.76%) compared with the control.

Effect of Exogenous Application of ABA and NDGA on Fruit Softening and Skin Color Development During Ripening

As expected, in ABA-treated fruit, irrespective of the concentration applied, fruit softening was slightly promoted, particularly from day 3 to day 7 of the ripening period, compared with the control and all treatments of NDGA, an ABA biosynthesis inhibitor (Fig. 3c). The trends were the reverse in NDGA-treated mango fruit. Averaged over the ripening period, the mean of fruit softening was significantly ($P \leq 0.05$) higher (5.86, 10.36, and 9.46%) after 0.5, 1.0, and 2.0 mM of ABA treatment, respectively, compared with the control. Furthermore, averaged over the ripening period, mean fruit softening was significantly ($P \leq 0.05$) lower (7.66%) in 0.2 mM NDGA-treated fruit compared with the control.

Fruit treated with 1.0 mM ABA also showed more pronounced skin yellowing after 3–7 days of ripening compared with the control (Fig. 3d). The application of 0.2 mM NDGA inhibited skin color development from day 1 to day 8 of ripening compared with the control (Fig. 3d). Averaged over the ripening period, the mean of skin color development was significantly ($P \leq 0.05$) higher (8.33 and 20.83%) in 0.5 and 1.0 mM ABA-treated fruit, respectively, when compared with the control. The trend of fruit color development in the 0.05, 0.1, and 0.2 mM NDGA-treated fruit was the reverse.

Discussion

This study is the first time that endogenous levels of a wide range of plant hormones (BRs, IAA, ABA, and ethylene) have been investigated simultaneously during mango fruit ripening. Of particular interest is the finding that endogenous levels of the BRs castasterone and brassinolide were low and did not show pronounced changes during fruit ripening in 'Kensington Pride' mango (Table 1). This contrasts with the 13-fold increase in the level of castasterone that coincided with the onset of ripening in 'Cabernet Sauvignon' grape (Symons and others 2006). Grapes also contained much higher castasterone levels (>20 ng g^{-1} FW or >5 ng $berry^{-1}$) than those found in mango (Table 1), along with a consistent increase in the expression of the genes *VvDWF1* and *VvBRI1* that are involved in the biosynthesis of BRs (Symons and others 2006). This suggests that changes in the levels of endogenous BRs may not play a role in the ripening of climacteric fruits like mango, unlike their positive role in the nonclimacteric fruit, grape.

Although the changes in the levels of BRs were small in mango fruit, the exogenous application of Epi-BL (45 and 60 ng g^{-1} FW) did significantly ($P \leq 0.05$) advance the onset of the climacteric peak of ethylene production and respiration rate by 2 days and 1 day, respectively (Table 2). Both these treatments also increased the climacteric ethylene peak (4.81 and 5.74 nmol C_2H_4 kg^{-1} h^{-1}) and respiration rate (4.87 and 5.06 mmol CO_2 kg^{-1} h^{-1}) compared with the other Epi-BL treatments and the control (Table 2). Furthermore, all Epi-BL treatments significantly ($P \leq 0.05$) improved mango skin color development between the 2nd day and 7th day of fruit ripening (Fig. 3b). However, these results do not necessarily support a direct role for BRs in mango ripening because it has been suggested that applied BRs may stimulate ethylene biosynthesis (Schlagnhaufer and others 1984). Brassinosteroids (24-homobrassinolide and 28-epibrassinolide) have also been reported to stimulate ethylene production in pericarp discs of tomato fruit (Vardhini and Rao 2002). The response of plant tissues to an exogenous plant hormone can vary greatly depending on the type and age of the tissue. It may also be argued that the variation among experiments in ethylene production may be ascribed to the fruit lots obtained from different geographical locations and different harvest dates.

It is well established that ethylene plays a pivotal role in regulating the ripening of climacteric fruits, including mango (Brecht and Yahia 2009; Singh and Singh 2011). Consistent with previous findings, our results show a climacteric ethylene peak associated with the climacteric respiration peak on the fourth day of ripening (Figs. 1a, 2a), which we suggest triggers ripening of mango fruit. This increased ethylene production in 'Kensington Pride' mango fruit probably occurs due to increases in the 1-aminocyclopropane-1-carboxylic acid (ACC) content as well as the activities of ACS and ACC oxidase during the ripening period (Nair and others 2004), because ethylene production and respiration are also increased in ethephon-treated and reduced in 1-MCP treated fruit (Lalel and others 2003).

Climacteric ethylene production and the peak in respiration rate observed in mango fruit may be triggered by the higher concentration of ABA detected during the climacteric rise phase (day 2, Fig. 1b). Abscisic acid is known to promote fruit ripening in climacteric fruits (Vendrell and Palomer 1997). In the current study, the endogenous level of ABA rose on day 2 and peaked on day 4 of the ripening period, before declining in the postclimacteric stage (days 6 and 8, Fig. 1b). The increase in ABA levels preceded major changes in ethylene levels and there was a significant ($P \leq 0.01$) positive exponential relationship ($R^2 = 0.68$) between the endogenous level of ABA and ethylene production during the ripening period. High endogenous ABA levels have been previously reported to increase ethylene production during ripening of other climacteric fruits such as tomato (Ruan and others 2005) and peach (Wu and others 2003), as well as increase the climacteric respiration rate in plum (Kitamura and others 1983) and pear (Kochankov and others 1975). Exogenous application of NDGA has been shown to inhibit biosynthesis of ABA and decrease ethylene production and consequently retard the fruit ripening process in 'Alisa Craig' tomato (Zhang and others 2009).

Although part of the effect of ABA on mango ripening may be mediated by its proposed effect on ethylene production, a direct role cannot be discounted. For instance, exogenous applications of ABA promoted fruit softening from day 3 of ripening compared with the control and the trend was reversed in NDGA-treated fruit (Fig. 3c), prior to the climacteric stage in control fruit. Ethylene has been reported to be involved in increasing the activities of cell wall-modifying enzymes such as polygalacturonase (PG) and pectin esterase activity (Brecht and Yahia 2009; Singh

and Singh 2011). However, ABA may also directly affect the activity of fruit-softening enzymes; Zhou and others (1996) report that exogenous application of 5 mg L^{-1} ABA increased PG activity and consequently promoted fruit softening in 'Zihua' mango. Likewise, ABA has also been reported to induce the maturation of 'Nam Dokmai' and 'Nang Klangwan' mangoes (Kondo and others 2004). In the current study, there was a significant ($P \leq 0.05$) negative exponential relationship ($R^2 = 0.60$) between the endogenous level of ABA in pulp tissues and pulp firmness during the ripening period.

The exogenous application of ABA promoted coloration of the skin, and the application of its inhibitor, NDGA, retarded color development compared with the control (Fig. 3d). This change in the skin color of mangoes may be attributed to the ABA-induced accumulation of carotenoids as reported previously in mango cvs. 'Alphonso', 'Langra', and 'Zihua' (Palejwala and others 1988; Zhou and others 1996). In addition to the results obtained in mango, the exogenous application of ABA has also been reported to promote skin color during fruit development and ripening in cherry (Kondo and Gemma 1993), persimmon (Nakano and others 1997), and grape (Koyama and others 2010). However, it is important to note that in many of these studies (including the current investigation) it is difficult to distinguish between responses that are a direct effect of an exogenous (or applied) hormone and those responses that occur indirectly, and may be mediated by a secondary hormone signal(s).

The endogenous IAA level in the pulp was significantly ($P \leq 0.05$) higher during the initial ripening stage (day 0) and then declined substantially during the ripening period until the fully ripe stage (Fig. 1c). A similar reduction in the endogenous level of IAA during the ripening period has also been reported in other climacteric fruits such as tomato (Sheng and others 2000) and kiwifruit (Chen and others 1999). It may be argued that higher levels of endogenous IAA in fruit pulp during the preclimacteric stage and the accumulation of ABA prior to the climacteric stage might initiate ethylene production and consequently trigger fruit ripening. There was a significant ($P \leq 0.001$) positive exponential relationship ($R^2 = 0.76$) between the endogenous level of IAA in the pulp and pulp firmness during ripening, and a significant ($P \leq 0.001$) negative exponential relationship ($R^2 = 0.92$) between the endogenous level of IAA and skin color development. These results suggest that changes in IAA levels coupled with accumulation of ABA trigger ethylene production and consequently control these aspects of mango ripening. However, previous reports suggest that preharvest application of α-naphthalene acetic acid (NAA) alone or in combination with gibberellic acid (GA_3) promotes skin color development in 'Keshar' and 'Arumanis' mango during ripening (Wavhal and Athale 1988; Notodimedjo 2000). This result raises questions about the importance of the timing of changes in auxin levels in mango fruit ripening and warrants further investigation.

In conclusion, the highest levels of endogenous IAA in the fruit pulp were at the preclimacteric minimum stage (day 0), whereas ABA levels rose before the climacteric peak in ethylene levels and respiration rate. These changes are consistent with ABA promoting ethylene synthesis, leading to climacteric ethylene production and fruit ripening. Exogenous application of Epi-BL or ABA promoted fruit ripening and NDGA, an inhibitor of ABA biosynthesis, retarded ripening as measured by fruit softening and color development. In both cases we speculate that these responses may be, at least in part, mediated by Epi-BL- and ABA-induced increases in ethylene production. In contrast to the control of nonclimacteric ripening in grape, BRs levels remain very low and changed little during mango ripening. We suggest that endogenous BRs may not play a significant role in the climacteric ripening of mango fruit.

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