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ORIGINAL ARTICLES

Comparative Analysis of Endogenous Hormones in Leaves and Roots of Two Contrasting *Malus* Species in Response to Hypoxia Stress

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Abstract Plant hormones play important roles in regulating developmental processes and signaling networks involved in plant responses to biotic and abiotic stresses. We comparatively studied the growth and endogenous hormonal levels in leaves and roots in two *Malus* species (*M. sieversii* and *M. hupehensis*) differing in hypoxia tolerance under normoxic and hypoxia stress. The results showed that hypoxia stress inhibited growth of seedlings of both *Malus* species, but with significant differences in intensity. Exposure to hypoxia altered the levels of endogenous hormones in leaves and roots in both *Malus* seedlings. Leaf and root abscisic acid (ABA) contents increased in response to hypoxia stress in both genotypes despite different extents. Compared with *M. hupehensis*, *M. sieversii* was more responsive to hypoxia stress, resulting in larger increases in leaf and root ABA contents. The changes in leaf and root ABA contents correlating with the different tolerance levels of the genotypes confirm the involvement of this hormone in plant responses to hypoxia stress. Gibberellins (GAs; $GA_1 + GA_4$) continuously increased in leaves and roots during the whole period of stress, whereas indole-3-acetic acid (IAA) showed a sharp increase at the early stage in both *Malus* seedlings. In addition, zeatin riboside (ZR), dihydrozeatin riboside (DHZR), and isopentenyl adenine (IPA) differed in their pattern of changes in both *Malus* seedlings under hypoxia stress. Based on variations in endogenous hormonal levels in both *Malus* species that differ in their ability to tolerate hypoxia, we conclude that not a single hormone but multiple hormones and their interplay are responsible for hypoxia tolerance.

Keywords Hypoxia stress · *M. sieversii* · *M. hupehensis* · Abscisic acid · Indole-3-acetic acid · Gibberellins

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Introduction

Higher plants are aerobic organisms requiring oxygen for growth and metabolism. However, they frequently experience a lower oxygen (hypoxia) environment mainly due to soil waterlogging, soil compaction, overirrigation, or poor drainage (Drew 1997; Garnczarska and Bednarski 2004; Branco-Price and others 2005; Christianson and others 2010). Hypoxia is considered one of the major environmental stresses limiting plant growth and yield worldwide, especially in higher rainfall regions. Hypoxia stress inhibits root and shoot growth by affecting many plant physiological processes, including energy metabolism (Rawyler and others 2002), reactive oxygen species metabolism (Garnczarska and Bednarski 2004; Bai and others 2010), and hormone modulation (Arbona and Gómez-Cadenas 2008).

Plant hormones play important roles in regulating developmental processes and signaling networks involved in plant responses to a wide range of biotic and abiotic stresses (Robert-Seilaniantz and others 2007). When plants are subject to abiotic stress, some plant endogenous hormones have been found to be the key elements involved in signal transduction and in the regulation of gene expression in response to stress (Weyers and Paterson 2001; Xiong and others 2002). The plant endogenous hormones include mainly ethylene, abscisic acid (ABA), indole-3-acetic acid

(IAA), gibberellins (GA), and cytokinins (CTKs). The most active CTKs are zeatin riboside (ZR), dihydrozeatin riboside (DHZR), and isopentenyl adenine (IPA) (Weyers and Paterson 2001; Wang and others 2006). It is generally believed that ethylene and ABA are the major plant hormones associated with the plant response to water stress. Ethylene is a major regulator of submergence responses in rice (*Oryza sativa*). This gaseous phytohormone rapidly accumulates in tissues of submerged plants due to physical entrapment and active biosynthesis during the stress, triggering a range of acclimation responses, including shoot elongation, adventitious root formation, and carbohydrate metabolism (Fukao and Bailey-Serres 2008). Under flooding, increases in ABA content have been reported in leaves of pea (Jackson and others 1988), *Arabidopsis* (Ellis and others 1999), and citrus (Arbona and Gómez-Cadenas 2008) and in roots of *Gerbera jamesonii* (Olivella and others 2000), papaya (Mahouachi and others 2007), and citrus (Arbona and Gómez-Cadenas 2008). In addition, the exogenous application of ABA increased tolerance to anoxia in *Arabidopsis* (Dat and others 2004).

Other hormones also play a role in plant response to abiotic tress. Arbona and Gómez-Cadenas (2008) reported that flooding increased IAA and jasmonic acid (JA) levels in roots and leaves of citrus. However, some authors found that flooding decreased IAA. They have attributed this to a reduction in IAA transport because of a deficit of oxygen (Reid and Wample 1985). JA and its methyl ester methyl jasmonate (MJ), collectively named jasmonates, are natural hormones that regulate plant growth and development (Sasaki-Sekimoto and others 2005). They are involved in the signal transduction pathway of plant responses to several environmental stress factors. MJ increased plant resistance to stress (Sasaki-Sekimoto and others 2005; Wasternack 2007). Liu and Huang (2005) reported that ZR and DHZR content in both roots and shoots decreased under high soil temperatures. They think that early malfunctioning of CTK metabolism may be a major factor leading to shoot growth inhibition and leaf senescence under high soil temperature conditions.

Many studies have documented that plant hormones are involved in the regulation of the responses of plants to abiotic stresses. Thus, an understanding of the interplay between hormonal changes and stress tolerance is crucial for identifying key hormones involved in stress defense and manipulating stress tolerance in plants. Our previous study showed that exogenous salicylic acid alleviated growth inhibition and oxidative stress induced by hypoxia stress and increased tolerance of *Malus robusta* Rehd to hypoxia (Bai and others 2009). Unfortunately, our knowledge of the mechanisms that confer plant hypoxia tolerance is still limited. This is because of the differences in species, genotypes, and developmental stages of plants and varied plant–environment interactions. In addition, little attention was paid to hypoxia tolerance upon hormonal modulation, which essentially determines plant growth and survival in the environment.

Apple (*Malus domestica*) is one of the most economically important fruits worldwide. However, apple trees often encounter root-zone hypoxia stress in production, most notably due to transient flooding, soil and water mismanagement, and the use of heavy machines. Hypoxia stress in the root zone is thought to be one of the important factors restricting the development of apples. In our previous study, a considerable difference in hypoxia tolerance was observed among 12 *Malus* species, with *M. hupehensis* being the most tolerant to hypoxia and *M. sieversii* the most sensitive (Bai and others 2008). However, little is known about whether plant hormones are involved in hypoxia tolerance. Therefore, the aims of this study were to investigate the hormonal changes in leaves and roots of two *Malus* species that differ in hypoxia tolerance under normoxic and hypoxic conditions, and to determine whether and how the hormonal changes were correlated with hypoxia tolerance.

Materials and Methods

Plant Material and Experimental Design

Seeds of *M. hupehensis* and *M. sieversii* were collected from Pingyi Shandong (35°07′N, 117°25′E) and Gongliu Xinjiang (42°07′N, 86°37′E), respectively, in the field in autumn 2008. These research areas differ in hydrological conditions and vegetation composition (Table 1). *M. hupehensis* is from the wet climate region Pingyi of Shandong Province in eastern China and grows mostly in the margins of rivers and creeks. In contrast, *M. sieversii* is from the semiarid climate region Gongliu of Xinjiang Province in northwestern China and is distributed in the northwest mountains. In our previous study, a significant difference in tolerance to hypoxia was observed among 12 *Malus* species, among which *M. hupehensis* was the most tolerant of hypoxia and *M. sieversii* was the most sensitive to hypoxia (Bai and others 2008, 2010).

The experiment was conducted at the Northwest A & F University, Yangling (34°20′N, 108°24′E), China. Seeds (*M. hupehensis* and *M. sieversii*) were sterilized with 0.2% (v/v) potassium permanganate for 5 min and then thoroughly rinsed with distilled water. They were stratified with sand under 4°C for 35–40 days and then planted in plastic pots (12 cm × 12 cm) filled with sand. The plastic pots were placed in a greenhouse under natural light and temperature conditions. At the two true-leaf stage, the seedlings were watered with half-strength Hoagland's

Table 1 Origin of two *Malus* species used in the experiment on response to hypoxia stress

Species	Abbreviation	Locality	Climate	Mean annual precipitation	Mean annual temperature	Soil type
M. hupehensis	MH	Pingyi, Shangdong (35°07′N, 117°25′E)	Humid	785 mm	13.2°C	Clay
M. sieversii	MS	Gongliu, Xinjiang (42°07′N, 86°37′E)	Semiarid	256 mm	7.4°C	Mountain brown soil

nutrient solution (Hoagland and Arnon 1950) every other day. The pH of the nutrient solution was adjusted to 6.5 ± 0.1 by using NaOH or H_3PO_4. Forty days later, seedlings of similar size (7–8 leaves, about 5 cm in height) were transferred into plastic tubs (52 cm × 37 cm × 15 cm) containing 20 l half-strength Hoagland's nutrient solution. The plastic tubs were wrapped with black plastic to prevent exposure of the root systems to light and placed in a growth chamber, where the temperature was controlled at 23–25°C in the daytime and 15–18°C at night. The light was provided by sodium lamps at a photon flux density of 160 μmol m^{-2} s^{-1} with a 14-h light period. The solution was continuously aerated with an air pump and dissolved oxygen (DO) concentrations were maintained at 8.0–8.5 mg l^{-1} by a DO controller (FC-680, Corporation of Super, Shanghai, China). Seedlings were cultivated for 10 days to allow plants to adapt to the growth chamber conditions.

After the 10-day preculture, the seedlings were randomly divided into two groups: (1) control (CK): the solution was maintained at a DO concentration of 8.0–8.5 mg l^{-1} and (2) hypoxia (HY): aeration with compressed air was substituted for by N_2 gas and DO was maintained at 1.5–2.0 mg l^{-1} by another DO controller. Each treatment contained four replicates with 30 plants in each replicate, resulting in a total of 120 plants per treatment. At days 0, 3, 6, 9, 12, and 15 of the treatment, roots and leaves were collected, immediately frozen in liquid nitrogen, and stored at −70°C until use.

Growth Measurements

After 15 days of treatments, the lengths of shoots and roots were measured with a ruler and the number of leaves was manually counted for ten plants per treatment. Each plant was divided into leaves, shoots, and roots, and the tissues were oven-dried at 70°C for at least 72 h for dry weight.

Hormone Extraction and Purification

The extraction and purification of endogenous levels of ABA, IAA, GAs (GA_1 + GA_4), MJ, ZR, DHZR, and IPA were measured by an indirect ELISA technique that was a modification of that from He (1993) and Yang and others (2001). Briefly, the samples (0.5 g) were homogenized in liquid nitrogen and extracted in cold 80% (v/v) methanol containing 1 mm butylated hydroxytoluene as an antioxidant. The extract was incubated at 4°C for 4 h and centrifuged at 4000*g* for 15 min at the same temperature. The supernatant was passed through Chromosep C18 columns (C18 Sep-Pak Cartridge, Waters Corp., Millford, MA), prewashed with 10 ml 100% (w/v) and 5 ml 80% (v/v) methanol, respectively. The hormone fractions from the columns were eluted with 10 ml 100% (v/v) methanol and 10 ml ether. The resulting elution was dried under N_2 and dissolved in 2 ml phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20 and 0.1% (w/v) gelatin (pH 7.5) for ELISA analysis.

Quantification of Hormones

The mouse monoclonal antigens and antibodies against IAA, ABA, GAs (GA_1 + GA_4), MJ, ZR, DHZR, and IPA–IgG horseradish peroxidase used in ELISA were developed at the Phytohormones Research Institute, China Agricultural University (He 1993). ELISA was performed using a 96-well microtitration plate. Each well on the plate was coated with 100 μl of coating buffer (1.5 g l^{-1} Na_2CO_3, 2.93 g l^{-1} $NaHCO_3$, and 0.02 g l^{-1} NaN_3, pH 9.6) containing 0.25 μg ml^{-1} antigens against the hormones. The coated plates were incubated for 4 h at 37°C for GAs, ABA, ZR, DHZR, MJ, and IPA, and overnight at 4°C for IAA, and then kept at room temperature for 30–40 min. After washing four times with PBS + Tween 20 (0.1% [v/v]) buffer (pH 7.4), each well was filled with 50 μl of either extracts or GAs, ABA, ZR, DHZR, MJ, and IPA standards (0–2000 ng ml^{-1} dilution range), and 50 μl of 20 μg ml^{-1} antibodies against GAs, ABA, ZR, DHZR, MJ, and IPA, respectively. The plate was incubated for 3 h at 28°C for GAs, ABA, ZR, DHZR, MJ, and IPA, and overnight at 4°C for IAA, and then washed as described above. One hundred microliters of 1.25 μg ml^{-1} IgG–horseradish peroxidase substrate was added to each well and incubated for 1 h at 30°C. The plate was rinsed five times with the PBS + Tween 20 buffer, and 100 μl of color-appearing solution containing 1.5 mg ml^{-1} *o*-phenylenediamine and 0.008% (v/v) H_2O_2 was added to each well. The reaction progress was

stopped by adding 50 µl of 6 N H_2SO_4 per well when the 2000-ng ml^{-1} standard had a pale color and the 0-ng ml^{-1} standard had a deep color in the wells. Color development in each well was detected using an ELISA Reader (model EL310, Bio-TEK, Winooski, VT, USA) at optical density A_{490}. IAA, GAs, ABA, ZR, DHZR, MJ, and IPA contents were calculated following Weiler and others (1981) and Yang and others (2001).

Statistical Analysis

Analyses were carried out using SPSS v11 for Windows statistical software package (SPSS, Inc., Chicago, IL, USA). Results were represented as the mean ± standard error. Differences between treatments were separated by the least significant difference (LSD) test at the $P < 0.05$ probability level.

Results

Plant Growth

Root-zone hypoxia stress inhibited growth of seedlings of both *Malus* species studied, but with significant differences in intensity between genotypes (Table 2). The reduction in leaf number under hypoxia stress was more drastic in *M. sieversii* (21.0%) from the semiarid region than in *M. hupehensis* (8.1%) from the humid region. The decrease in plant height and root length was also greater in *M. sieversii* than in *M. hupehensis*. There were significant differences in leaf number, plant height, and root length between the controls and treated *M. sieversii*. However, there were no significant differences for these measurements in *M. hupehensis*. At the final harvest, the shoot and root dry masses were reduced by 24.9 and 42.4% in *M. sieversii* and by 7.3 and 15.9% in *M. hupehensis*, respectively, compared with those in the controls. In addition, there was a significant decline in the root/shoot ratio in both *Malus* genotypes under hypoxia stress compared to the control. However, *M. hupehensis* maintained a higher root/shoot ratio (0.28) and *M. sieversii* suffered a sharp decline in the root/shoot ratio (0.18). These differences in the growth characteristics may indicate different abilities of the two *Malus* species to adapt to hypoxia, which might be associated with their origins (Table 1).

ABA

Leaf ABA content increased in response to hypoxia stress in both genotypes but to different extents (Fig. 1a, b). Leaf ABA content in stressed *M. sieversii* remained significantly higher than in the controls from day 3 until the end of the experimental period. However, in *M. hupehensis*, significant increases were recorded only in the first 9 days of hypoxia stress, and there was no difference between the stressed *M. hupehensis* and the controls afterward.

Root ABA content (Fig. 2a, b) drastically and rapidly increased after hypoxia stress, peaked at 9 days, and then decreased later in both *Malus* genotypes. Continuous hypoxia stress, however, induced a significant decrease of this plant hormone in the stressed *M. sieversii* at the end of the experimental period, but it still remained significantly higher than the control. In *M. hupehensis*, no significant change in root ABA content was observed in response to hypoxia stress.

IAA

The immediate increase in leaf IAA content was observed in leaves of both *Malus* species in the first 3 days compared to their controls, and then decreased to a lower level than

Table 2 Leaf number (LN), plant height (PH), root length (RL), shoot dry weight (SDW), root dry weight (RDW), and root/shoot (R/S) ratio of two *Malus* species grown in normoxic and hypoxic nutrient solution for 15 days

	M. sieversii			*M. hupehensis*		
	CK	HY	HY/CK	CK	HY	HY/CK
LN	21.5 ± 2.92 a	17.0 ± 2.05 b	0.79	12.30 ± 1.34 a	11.30 ± 1.16 a	0.92
PH (cm)	18.4 ± 1.09 a	13.8 ± 0.75 b	0.74	16.15 ± 1.77 a	15.19 ± 2.53 a	0.94
RL (cm)	27.3 ± 4.81 a	17.6 ± 1.74 b	0.65	23.3 ± 3.29 a	20.7 ± 1.77 a	0.88
SDW (g $plant^{-1}$)	0.87 ± 0.08 a	0.65 ± 0.08 b	0.75	0.80 ± 0.18 a	0.74 ± 0.11 a	0.93
RDW (g $plant^{-1}$)	0.21 ± 0.05 a	0.12 ± 0.02 b	0.58	0.25 ± 0.23 a	0.21 ± 0.25 b	0.84
R/S	0.23 ± 0.03 a	0.18 ± 0.01 b	0.77	0.31 ± 0.04 a	0.28 ± 0.04 a	0.91

CK control, *HY* hypoxia

Data are mean ± standard error ($n = 10$). Different *letters* in the same row indicate a significant difference between the control and stressed plants at $P < 0.05$, LSD test

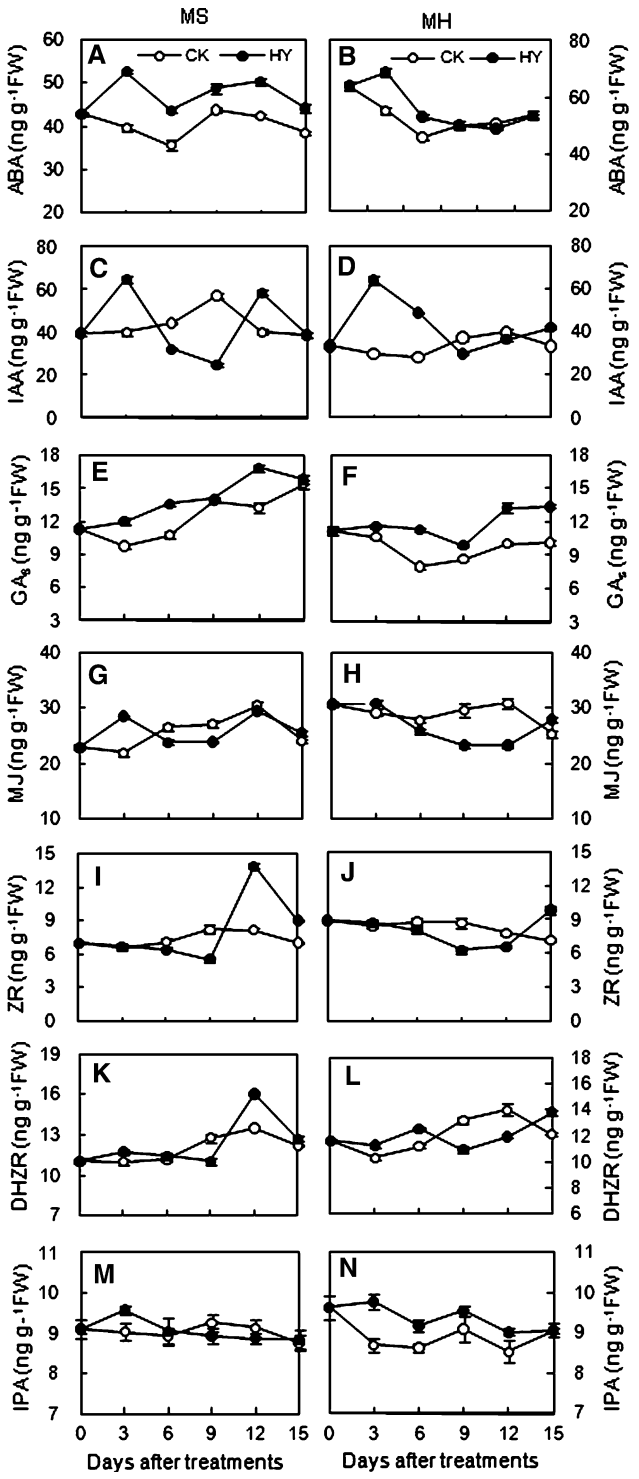

Fig. 1 Levels of endogenous plant hormones in leaves of two *Malus* species grown in normoxic and hypoxic nutrient solutions. From upper to lower: abscisic acid (ABA), indole-3-acetic acid (IAA), gibberellins (GAs), methyl jasmonate (MJ), zeatin riboside (ZR), dihydrozeatin riboside (DHZR), and isopentenyl adenine (IPA). Data are mean ± standard error of four independent determinations. MS *M. sieversii*, MH *M. hupehensis*, *CK* control, *HY* hypoxia

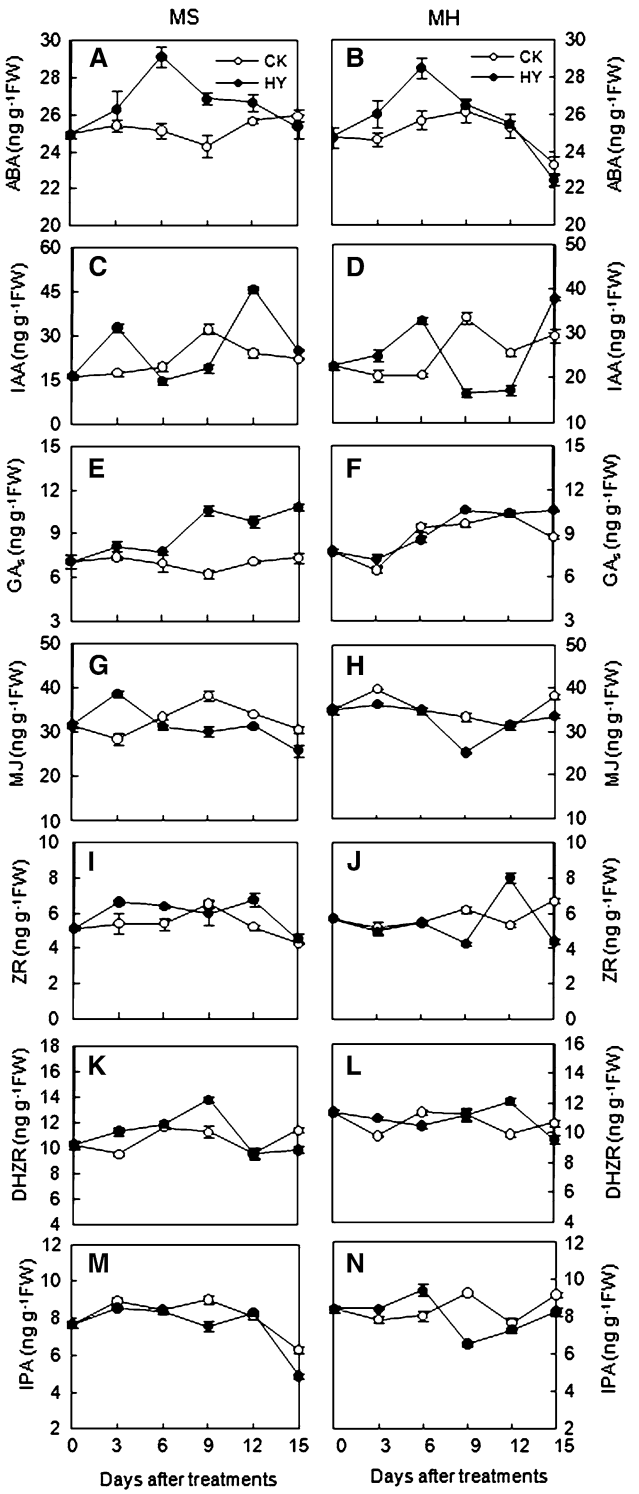

Fig. 2 Levels of endogenous plant hormones in roots of two *Malus* species grown in normoxic and hypoxic nutrient solution. From upper to lower: *ABA* abscisic acid, *IAA* indole-3-acetic acid, *GAs* gibberellins, *MJ* methyl jasmonate, *ZR* zeatin riboside, *DHZR* dihydrozeatin riboside, and *IPA* isopentenyl adenine. Data are mean ± standard error of four independent determinations. *MS M. sieversii*, *MH M. hupehensis*, *CK* control, *HY* hypoxia

that of the controls by day 9 (Fig. 1c, d). However, there were significant differences in IAA levels between *M. sieversii* and *M. hupehensi.* In *M. sieversii*, a biphasic scheme in leaf IAA accumulation was observed, with two transient accumulations that reached maximum levels at days 3 and 12. In *M. hupehensis*, no significant difference in leaf IAA content was observed in response to hypoxia stress after 9 days.

Root IAA content in the two species showed different changes in response to hypoxia stress. In *M. sieversii*, root IAA content showed similar patterns to leaf IAA under hypoxia stress, a biphasic scheme in root IAA accumulation was observed, with two transient accumulations that reached maximum levels at days 3 and 12 (Fig. 2c). In *M. hupehensis*, root IAA content increased in the first 6 days compared to the control, then decreased and reached a lower level than that of the control until 12 days. After this date, root IAA concentrations in stressed *M. hupehensis* increased to control levels (Fig. 2d).

GAs

Hypoxia stress significantly increased leaf GAs (GA_1 + GA_4) content (Fig. 1e, f) in both *M. sieversii* and *M. hupehensis*, but with differences in the patterns of accumulation. Increases in GAs content were observed in stressed *M. sieversii* and *M. hupehensis* at 12 and 6 days (26.9 and 32.3% increase, respectively), and levels in stressed plants remained very high until the end of the experimental period.

Root GAs content in the two species showed significantly different changes in response to hypoxia stress (Fig. 2e, f). Root GAs content increased in *M. sieversii* after the stress conditions, whereas GAs levels in *M. hupehensis* remained close to control levels most of the time or even decreased (in some cases reaching a 9.0% reduction). It was increased only at the end of the experimental period.

MJ

Hypoxia stress caused transient increases in leaf MJ concentrations in both genotypes, but with different patterns (Fig. 1g, h). Leaf MJ content in *M. sieversii* increased sharply, with a peak at 3 days, and then decreased and reached a lower level than that of the control during hypoxia treatment. Similarly, leaf MJ content in *M. hupehensis* decreased to lower levels than the controls. Root MJ content in *M. sieversii* showed similar patterns to leaves under hypoxia stress, whereas in *M. hupehensis* levels decreased below those of controls throughout the period of study (Fig. 2g, h).

ZR and DHZR

Leaf ZR and DHZR contents in *M. sieversii* showed similar patterns in response to hypoxia stress (Fig. 1i, k). No significant changes in leaf ZR and DHZR contents were observed in response to hypoxia stress in the first 6 days. Continuous hypoxia treatment, however, induced a significant decrease of the two plant hormones in *M. sieversii* from 6 to 9 days. After 9 days, there was a sharp increase in ZR and DHZR contents in *M. sieversii* to the end of the experimental period. Leaf ZR content decreased in *M. hupehensis*, reaching the minimum value after 9 days of hypoxia stress, and then gradually increased. DHZR content in *M. hupehensis* increased during the first 6 days of stress. After this date, DHZR content in leaves dropped below that of the controls (Fig. 1j, l).

Root ZR and DHZR contents increased in *M. sieversii*, with a peak at 12 and 9 days, respectively, and then decreased and reached a level similar to that of the controls (Fig. 2i, k). Root ZR levels in *M. hupehensis* showed no changes in the first 6 days, but the maximum levels were observed at 12 days, despite decreasing thereafter to levels below that of the controls (Fig. 2j). Root DHZR content in *M. hupehensis* remained close to control levels most of the time and increased only at the end of the experimental period (Fig. 2l).

IPA

There was a significant increase in leaf IPA content in *M. sieversii* at 3 days of hypoxia stress, but 6 days of hypoxia stress resulted in a decline, whereas leaf IPA content in *M. hupehensis* was higher than that in the controls throughout the period of study (Fig. 1m, n). Root IPA content in *M. sieversii* showed slight changes or a decrease (in some cases reaching a 16.1% reduction) after stress, whereas root IPA content in *M. hupehensis* increased to reach levels 1.2-fold higher than the controls 6 days after hypoxia stress, decreasing thereafter to lower levels than that of the controls (Fig. 2m, n).

Discussion

Molecular di-oxygen (O_2) is an absolute requirement for efficient production of ATP through oxidative phosphorylation in aerobic organisms (Bailey-Serres and Chang 2005). Oxygen deprivation in the root zone reduces mitochondrial ATP production in the root due to inhibited cytochrome C oxidase activity, leads to cytosolic acidification as a consequence of enhanced carbon metabolism to lactic acid, and thereby severely limits growth, development, and survival (Drew 1997; Ellis and others 1999;

Branco-Price and others 2005). In our present study, the two *Malus* species showed negative growth responses to root-zone hypoxia stress, but with significant differences in intensity between the genotypes that originated from different habitats. *M. hupehensis* is able to cope with hypoxia stress better than *M. sieversii*. *M. hupehensis* growth hardly differed between the normoxic and hypoxic conditions. In contrast, all the measured growth parameters were significantly reduced in *M. sieversii* under hypoxia stress when compared to the control. These differences in growth characteristics may be indicative of different abilities between the two *Malus* species to adapt to hypoxia, which might be associated with their origins (Table 1).

Plant hormones play a critical role in regulating plant developmental processes and counteracting biotic and abiotic stresses (Robert-Seilaniantz and others 2007). In some plants, tolerance to stress has been linked to morphological adaptations apparently regulated by specific hormones (Hwang and VanToai 1991; Xiong and others 2002; Arbona and Gómez-Cadenas 2008). In this study, the use of two *Malus* species with different abilities to tolerate hypoxia has allowed the discrimination between common and specific hormonal responses. The results indicate that exposure to root hypoxia altered endogenous hormonal levels in leaves and roots during the experiment.

There are many reports that ABA is involved in regulating plant growth and environmental stresses (Azuma and others 1995; Voesenek and others 2003; Mahouachi and others 2007). Our results showed that ABA levels rapidly increased in leaves and roots of both stressed *Malus* species, although not at the same time. ABA in leaves and roots increased more in *M. sieversii* throughout the period. Although just a slight increase was observed in leaves and roots of *M. hupehensis*, there were no changes in leaf and root contents at the end of the experimental period. These data might suggest that ABA plays an important role in signaling networks involved in responses to hypoxia tolerance. In other reports, a similar ABA increase under stresses has been described (Olivella and others 2000; Arbona and Gómez-Cadenas 2008; van Zanten and others 2009). In addition, Ellis and others (1999) observed that ABA application was able to substitute for hypoxic pretreatment in roots. Similar results were obtained by Hwang and VanToai (1991), who found that ABA induced anoxia tolerance in maize root tips. These investigators also reported that ABA-induced anaerobic tolerance was inhibited by cycloheximide, supporting the role of ABA as an early mediator between stress perception and the induction of physiological responses.

The essential role that IAA plays in plant growth and development has already been reported (Olivella and others 2000; Mahouachi and others 2007). A rapid and transient increase in IAA was observed in leaves and roots of both *Malus* species. However, a biphasic scheme in leaf and root IAA accumulation was observed, with two transient accumulations in *M. sieversii.* This result is in agreement with reports on the differences in IAA in citrus plants with differing flood tolerances (Arbona and Gómez-Cadenas 2008). Our observations suggest an essential involvement of IAA in the physiological responses of roots to hypoxia stress, probably related to the induction and plant growth that could contribute to coping with hypoxia stress.

GA has been known to regulate various aspects of plant development, including seed germination, stem elongation, and fruit development (Jackson 1990; Garcia-Martinez and Gil 2002; van Zanten and others 2009). In addition, the exogenous application of GA_3 increased tolerance of rice to submergence (Fukao and others 2006). Modulation of GA levels and the regulation of GA-mediated signaling components are important for plant developmental patterns. Hypoxia stress treatment significantly increased leaf GAs content in both *M. sieversii* and *M. hupehensis*, but with differences in the patterns of accumulation. Root GAs content in the two *Malus* species showed significantly different changes in response to hypoxia stress, suggesting GAs in stressed plants may act as a negative message to slow plant growth (Jackson 1990).

Recently, MJ, JA, and their related compounds designated as jasmonates have been demonstrated to play an important role in the signal transduction pathway in response to stresses, resulting in significant physiological phenomena (Koiwa and others 1997; Wasternack 2007; Balbi and Devoto 2008). Our results showed MJ levels transiently increased in leaves and roots in *M. sieversii*. In contrast, MJ levels in leaves and roots were decreased in *M. hupehensis* under hypoxia stress. Yet the role of MJ in regulating growth under hypoxia stress is less well known. Alternatively, it is possible that the role of MJ is related to the hypoxia tolerance.

However, the roles of other hormones such as ZR, DHZR, and IPA in plant responses to stress remain unclear. Previous results pointed to CTKs as the most probable indices of aging and maturation, and more emphasis was placed upon this type of hormone (Valdés and others 2004). We compared ZR, DHZR, and IPA contents in both *Malus* species under normoxic and hypoxic conditions. ZR and DHZR in the two species showed significantly different responses compared with controls. This allows us to establish that the role of CTKs is related to hypoxia tolerance. In addition, hypoxia stress significantly reduced IPA contents in roots of *M. sieversii* at the end of the experimental period. This could be due to the oxygen requirement of the enzymes responsible for IPA biosynthesis.

It is difficult to assign the regulation of a process to a unique hormone. Some hormones affect the biosynthesis of others, and also hormones act through complex signaling

pathways in which there is crosstalk among them. Several studies have examined the role of plant hormones and their interplay in regulating fermentation and acclimation of plants to hypoxia stress (Bailey-Serres and Chang 2005). Kato-Noguchi (2000) found that alcohol dehydrogenase activity in alfalfa seedlings was induced by auxin, ABA, and CTKs at different concentrations but not by GA under normal growth conditions. Ethylene has also been shown to act either alone (Pierik and others 2007) or in coordination with IAA in the stimulation of shoot elongation under flooding (Voesenek and others 2003). Our data have clearly indicated that not only ABA and IAA, but also CTKs, played a key role in the regulation of plant growth.

Comparison of growth and biomass accumulation of the two *Malus* species revealed important differences. Hypoxia stress inhibited growth of both *Malus* seedlings, but with significant differences in intensity between the two genotypes that originate from the different habitats. It is clear that *M. hupehensis* is more tolerant of hypoxia than *M. sieversii.* Based on variations in endogenous hormonal levels in leaves and roots in both *Malus* species that differ in their ability to tolerate hypoxia, we conclude that not a single hormone but multiple hormones and their interplay are responsible for hypoxia tolerance. The results contribute to the understanding of the intricate set of connections between plant hormones that regulate physiological responses to stress.

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