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Dynamic Changes of Phytohormone Concentrations during *Prunella vulgaris* L. Development and Impact of Rosmarinic Acid Accumulation through Exogenous ABA Application

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Abstract

Background: Plant hormones not only participate in regulating the growth and development process of plants, but also play an important regulatory role in the synthesis of secondary metabolites. *Prunella vulgaris* L. (*P. vulgaris*) is a perennial herb that has a long history for use as a kind of medicinal and edible plant. In order to understand the relationship between plant hormones and *Prunella vulgaris* L. (*P. vulgaris*) development. **Methods:** The quantification of indoleacetic acid (IAA), salicylic acid (SA), jasmonates (JAs), abscisic acid (ABA), 1-aminocyclopropane-1-carboxylic acid (ACC) and cytokinins (CKs) of *P. vulgaris* during development was performed by a liquid chromatography-mass spectrometry (LC-MS). Furthermore, the effect of exogenous ABA on rosmarinic acid (RA) accumulation was verified. **Results:** Nuts formation was related to the concentrations of IAA, ABA and ACC in the green-fruit stage, and IAA and ABA levels increased rapidly in this stage. High IAA concentrations can promote an increase in ACC. SA, JAs, ethylene (ET) and ABA were related to the defence response of plants to pathogens. SA concentrations increased sharply after the turning stage, while the concentration of JAs, which are antagonistic to SA, was low. The decrease in ABA concentration after this period may be related to the antagonistic effect of IAA or SA. The CKs trans-zeatin (tZ) and trans-zeatin riboside (tZR) promoted the growth and development of early *P. vulgaris*, and their concentrations decreased in the late period, leading to withering and senescence of *P. vulgaris*. The CKs cis-zeatin (cZ) and N6-(2-isopentenyl) adenine (iP) were presumed to be present in nuts. After elicitation with 10 μg/mL ABA, an increase of RA content was observed ($p < 0.05$). **Conclusion:** This study can provide an improved basis for elucidating the growth and development mechanism of *P. vulgaris* in the future, as well as a better understand of the effects of ABA application on RA accumulations.

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1 Introduction

Prunella vulgaris L. (*P. vulgaris*) is a perennial herbaceous plant species in the Labiatae family, which withers after the summer solstice, and has been used in China for thousands of years [1]. The secondary metabolites of *P. vulgaris* include mainly triterpenoids, phenolic acids, flavonoids, volatile oils, and polysaccharides [2]. Many studies have shown that these secondary metabolites have a wide range of biological activities, including anti-viral, anti-inflammatory, anti-tumour, anti-hypertensive and anti-hyperlipidaemic functions [3-5].

Plant hormones are organic compounds with physiological activity produced by plants. These kinds of compounds are indispensable for plant biological activity and occur in small quantities, but they are of great importance, especially in the regulation of various plant growth and development processes and environmental responses of plants and in the regulation of the accumulation of plant secondary metabolites. According to their molecular structures and physiological functions, plant hormones are usually divided into ethylene (ET), indoleacetic acid (IAA), cytokinins (CKs), gibberellins (GAs), abscisic acid (ABA), jasmonates (Jas), salicy acid (SA) and brassinosteroids (BRs). ET can promote the ripening of fruits, and its biosynthesis is related to IAA [6]. In addition, fruit ripening is regulated by the transcription factor ABA-stress-ripening (ASR), and ASR gene expression is affected not only by sucrose and ABA but also by JAs and IAA [7]. Moreover, IAA not only plays an important role in the ripening process of fruits during the early development and morphology of plants but also is relevant. Plants are often attacked by a wide range of microbial pathogens in their environment. Plant hormones such as SA, JAs, ABA and ET play a central role in the biological stress signalling associated with pathogen infection, mainly through synergy and antagonism, to regulate the protective response of plants to biotic and abiotic stress [8]. Studies have shown that CKs are purine-based plant growth regulators in plants whose signals can delay leaf senescence, promote bud branching, and specifically affect the growth of plant buds and roots [9]. Through the analysis of CK concentrations in plant tissues at different stages, the relationships between CK levels and the senescence process of various tissues and plant species have been revealed.

RA is a major component of phenolic acids in *P. vulgaris* and controls the quality of this medicinal plant according to National Chinese Pharmacopeia [10]. ABA could strongly enhance rosmarinic acid production [11].

In this study, we used reaction monitoring/multiple reaction monitoring (SRM/MRM) to quantitatively reveal the dynamic changes of phytohormone concentrations during *P. vulgaris* development. Furthermore, the effect of exogenous ABA on RA accumulation was verified.

2 Materials and methods

2.1 Plant materials and growth conditions

P. vulgaris plants were selected from the Pharmaceutical Plantation Garden of Hunan University of Traditional Chinese Medicine located in Shangsha, China (28 ° 08 ′ N and 112 ° 54 ′ E), which is approximately 88 m above sea level. The site has a subtropical monsoon climate. The annual precipitation is 1361.6 mm. The annual mean temperature is 17.2 °C, and the annual sunshine duration is 1529.3 h.

The samples were collected from 5 different developmental stages: the heading stage (stage 1), the flowering stage (stage 2), the green-fruit stage (stage 3), the turning stage (stage 4) and the ripening stage (stage 5) (Table 1).

Table 1 Sample information of *P. vulgaris*.

Stage	Morphology	Collection date
1	The spikes grow from the apex of the stem, the tip of the ear is pointed and tail-shaped, and the veining is obvious. It is composed of several round calyx and bracts. Each round of bracts oppose 2 pieces and form a fan.	April 15 - May 3
2	Prototype of the bracts petals, some low-end bracts lilac flowers grow from the tip 3 to the tip.	May 4 - May 11
3	Spikes apex open, corolla off, there are 4 small green nuts.	May 12 - May 20
4	Bracts gradually from green to red-brown from the bottom upward, but remain green cobs.	May 21 - Jun 1
5	Complete conversion of the entire ear bracts reddish brown, brown nutlets inner spikes.	Jun 2 - Jun 16

2.2 Method validation

The samples were mixed in equal amounts to prepare QC samples, and QC samples were used at each interval for a certain number of experimental samples in the sample queue to detect and evaluate the stability and repeatability of the system. Twelve plant hormones at ten concentrations and fixed amounts of internal standards ($^2H_5$-IAA, $^2H_4$-SA, $^2H_6$-iP, cis-OPDA, $^2H_4$-ACC, $^2H_5$-tzR, $^2H_6$-ABA, $^2H_4$-tZ and $^2H_6$-iPR, purchased from Olchemim Ltd., Olomouc, Czech Republic) were prepared to establish calibration curves by plotting the peak area ratio against the concentrations of plant hormone derivatives with quartic measurements. The limits of quantitation (LOQs) and limits of detection (LODs) were determined at concentrations for which the signal-to-noise (S/N) ratios were 10 and 3, respectively. Calibration curves were generated from the MRM signals obtained from standard solutions based on the ratio of the chromatographic peak area for each analyte to that of the corresponding internal standard.

2.3 Sample preparation

Plant materials were frozen in liquid nitrogen and stored at -80 °C. The samples were ground in liquid nitrogen, and 80 ± 5 mg was then placed in a 2 mL centrifuge tube. Fifty microlitres of internal standard solution was then added, followed by 1 mL of acetonitrile aqueous solution (1% FA). The mixture was then vortexed for 2 min (Vortexer: QL-866). Afterward, the samples were then extracted at 4 ° C in the dark for 12 h, after which they were centrifuged at 14000 g for 10 min (refrigerated centrifuge: Xiangyi, H1650-W). In total, 800 μL of the supernatant was removed and dried under a stream of nitrogen and then added to 200 μ L reconstituted aqueous acetonitrile (1:1, v/v). The samples were then centrifuged at 14000 g for 10 min, after which the supernatant was removed for analysis.

2.4 UHPLC-ESI-MS/MS analysis

Plant hormone analysis was performed via an ultra-high-performance liquid chromatography (UHPLC) system (Waters I-Class LC) [12]. Separation of the plant hormones was performed on an ACQUITY UPLC BEH C18 column (1.7 μm, 2.1 mm × 100 mm, Waters) at a flow rate of 400 μL/min at a 45 °C oven temperature. Mobile phases A and B consisted of 0.05% formic acid in water and 0.05% FA in acetonitrile, respectively. A gradient of 0-10 min of 2% to 98% B, 10-10.1 min of 98% to 2% B, and 11.1-13

min of 2% B was adopted. The injection volume was 4 μL. A 5500 QTRAP mass spectrometer (AB SCIEX) was used for mass spectrometry analysis in positive/negative ion mode. The 5500 QTRAP ESI ionization source conditions were as follows: source temperature, 500 ° C; ion source gas 1 (gas 1), 45; ion source gas 2 (gas 2), 45; curtain gas (CUR), 30; and ion spray voltage floating (ISVF), -4500 V. MRM was employed in the ion pair for the detection of plant hormones.

2.5 ABA treatment

The experiment was modified on the basis of literature [13]. *P. vulgaris* at heading stage with similar growth performance, such as similar leaf sizes and cluster sizes, was chosen to randomly groups, including the control group, ABA low concentration group (1 μg/mL), ABA middle concentration group (5 μg/mL), and ABA high concentration group (10 μg/mL). *P. vulgaris* per pot was distributed in a randomized complete block design with 3 blocks for 3 biological replicates. First, the initial contents of RA in the leaves of *P. vulgaris* from each group were determined. Then, the positive and negative sides of leaves were evenly and gently smeared with different ABA concentrations. ABA treatment was applied one time per day with five days. Leaves were randomly sampled to determine the increasing rate of RA content on the sixth day. The formula for calculating the increasing rate of RA content was as bellow: The increase rate of RA content = (final content of RA - initial content of RA) × 100%/initial content of RA.

2.6 Statistical analysis

SPSS 25.0 software was used for statistical analysis, and comparisons between groups were performed by one-way variance analysis. The results are expressed as average ± standard deviation, and $p < 0.05$ was considered statistically significant.

3 Results

3.1 Method validation

QC samples were used to inspect the stability of the detection. The RSD% of each test object was less than 30%, indicating that the data were stable and reliable (Figure 1).

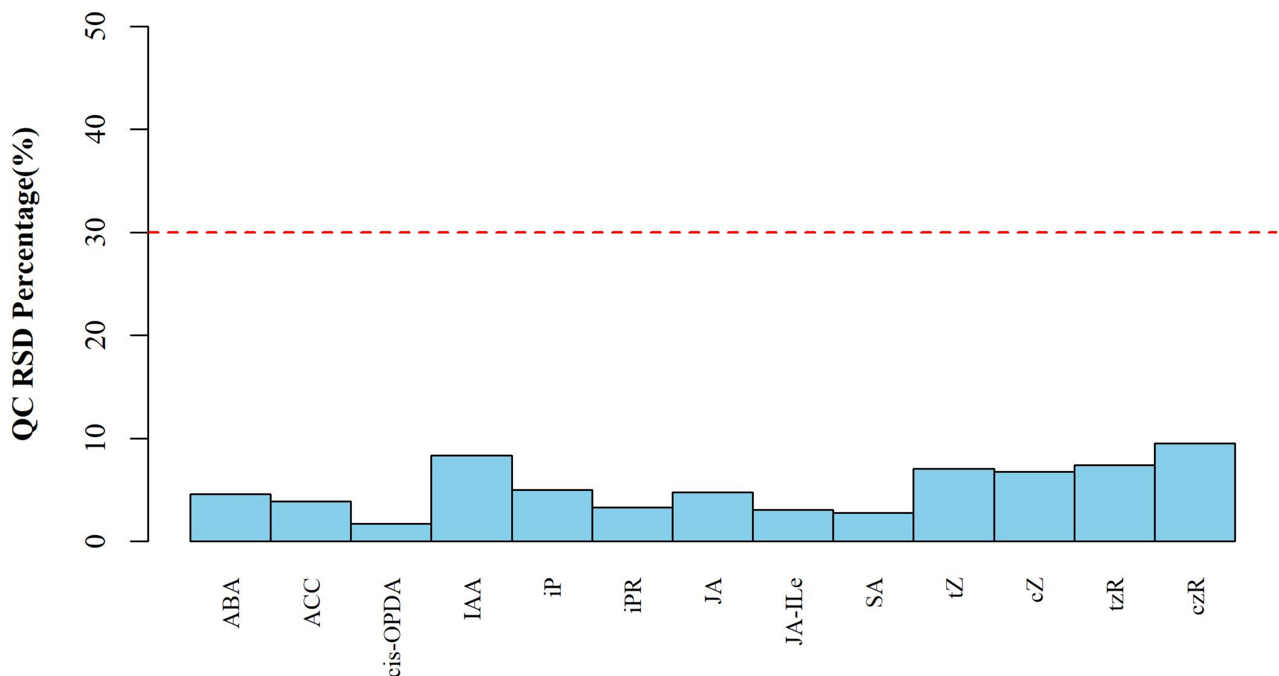

Figure 1 RSD distribution of QC.

Calibration curves were constructed on the basis of the peak area ratios of the analytes to the internal standards versus concentrations of the analyte standards. Calibration curves for the analysis of IAA, SA, iP, cis-OPDA, ACC, tzR, ABA, tZ, czR, JA-Ile, iPR, JAs and cZ were constructed by plotting the peak intensity ratio against the known analyte concentrations. Table 2 lists a summary of the calibration curves, correlation coefficients (R2 values), linear ranges, quantification limits (LOQs), and detection limits (LODs) of the 13 plant hormones. All 13 correlation coefficients were higher than 0.99. The LODs for the different plant hormones were in the range of 0.11-4.8 ng/mL, and the limits of quantification ranged from 1 to 10 ng/mL.

Table 2 Quantitation results of the 13 plant hormones under optimized UPLC-MS/MS conditions.

Phytohormone standard	Calibration curve	Correlation coefficient (R^2)	Linear range (ng/mL)	LOQ (ng/mL)	LOD (ng/mL)
IAA	y = 9.40331e-4x - 7.66121e-4	0.99919	2.5-100	2.5	0.46
SA	y = 0.02974x + 0.10929	0.99993	2.5-500	2.5	0.68
iP	y = 0.01311x - 0.00553	0.99919	1-500	1	0.25
cis-OPDA	y = 3187.47294x - 703.03646	0.99978	1-1000	1	0.25
ACC	y = 0.00488x + 0.02077	0.99948	10-500	10	4.8
tzR	y = 0.03845x - 0.01370	0.99958	1-500	1	0.18
ABA	y = 0.02667x + 0.15605	0.99988	1-500	1	0.25
tZ	y = 0.06376x - 0.00140	0.99965	1-100	1	0.25
czR	y = 0.03322x + 2.71542e-4	0.99916	2.5-500	2.5	0.50
JA-ILe	y = 5.44523e4x - 879.26344	0.99998	1-500	1	0.30
iPR	y = 5.44523e4 x - 879.26344	0.99923	1-50	1	0.22
JA	y = 4370.02946x + 23620.81526	0.99946	1-1000	1	0.43
cZ	y = 0.06489x + 0.00445	0.99714	1-100	1	0.11

3.2 Temporal distribution of plant hormones

During the development of *P. vulgaris* L., the changes in IAA concentration in the dissected spikes became apparent. From stage 1 to 3, the IAA concentration increased gradually from 14.19 ng/g to 869.12 ng/g, and there were significant differences ($p < 0.05$). Although it was maintained at a delicate dynamic balance between stage 3 and stage 4, the IAA concentration increased significantly from stage 4 to stage 5 and reached a peak value of 1766.33 ng/g ($p < 0.05$), and the level increased from stage 1 to stage 5 by 123.48-fold (Figure 2). The SA concentration from stage 1 to stage 2 increased slowly from 152.63 ng/g to 266.54 ng/g. Starting at the point at which the spikes turned brown, the SA concentration increased to 1228.53 ng/g ($p < 0.01$) until all the spikes were brownish red in colour, and the SA concentration peaked at 1843.17 ng/g (Figure 2). The increasing trend of cis-OPDA before stage 2 was not obvious. The cis-OPDA concentration increased significantly from 127.78 ng/g to 261.65 ng/g during stage 2 to stage 3 ($p < 0.05$). After stage 3, the upward trend slowed, and the change was not significant ($p > 0.05$). In addition, JA-Ile and JAs were also detected in the spikes of *P. vulgaris* L. during the five different growth

periods, but no obvious trend was found (Figure 2). The ACC concentration peaked at 23.26 ng/g, which occurred before stage 3. As the spikes began to turn colour, the ACC concentration decreased to 11.56 ng/g ($p < 0.01$), but after stage 5, the ACC concentration did not change significantly (Figure 2). The ABA concentration was 10.00 ng/g at stage 1. It then remained low at stage 2, while it increased to 158.56 ng/g at stage 3 ($p < 0.05$). The ABA concentration decreased after stage 3, but the change was not significant (Figure 2). Six endogenous CKs, trans-zeatin (tZ), trans-zeatin riboside (tZR), cis-zeatin (cZ), cis-zeatin riboside (cZR), N6-(2-isopentenyl) adenine (iP), and N6-(2-isopentenyl) adenosine (iPR), were identified in the spikes of *P. vulgaris* L., whereas DHZ was not detected. The concentration of tzR was relatively stable before stage 3, with no significant change. However, its concentration decreased rapidly from 1.14 ng/g to 0.44 ng/g during stage 3 to stage 4 ($p < 0.01$). At stage 1, the concentrations of tZ and cZR were as high as 2.2 ng/g and 7.46 ng/g, respectively, but the concentrations decreased significantly at stage 2 to 0.96 ng/g and 4.96 ng/g, respectively ($p < 0.05$). From stage 2 to stage 3, the tZ concentration increased to 2.06 ng/g, and the czR concentration peaked at 13.31 ng/g ($p < 0.01$). The cZ concentration was below quantifiable values before stage 2, but its concentration was quantifiable thereafter, ranging from 0.06 ng/g to 3.41 ng/g from stage 3 to stage 5, respectively. The iP concentration remained at a low state before stage 2 but gradually increased from 0.33 ng/g at stage 2 to 1.17 ng/g at stage 5. The iPR concentration decreased from 0.54 ng/g to 0.31 ng/g during stage 1 to stage 2, respectively ($p < 0.05$) (Figure 2).

3.3 Effect of ABA on the accumulation of RA

Secondary metabolites production in plants is regulated by several factors including environmental variables and plant growth regulators [14]. RA is an important secondary metabolite, commonly found in species of the Lamiaceae and Boraginaceae family. The biosynthesis of RA has been vastly investigated for two reasons: (1) RA has been shown to be a useful compound in the field of medicine, and as a food additive, (2) accumulated RA contributes to the defense against microbes [15].

ABA is a stress hormone that coordinates the complex networks of stress responses and its content is rapidly changed in response to stresses [15]. The ABA has been recognized as a stress hormone coordinating the complex networks of stress responses [16]. Several recent studies have showed that, changes in the amount of endogenous ABA may play an important role in the accumulation of RA and related phenolic compounds [17-23].

The effects of application of ABA (0, 1, 5, 10 μg/mL) to shoot leaves of *P. vulgaris* on RA content was evaluated (Figure 3). Five days after elicitation with ABA, an increase of RA content was observed, which was significantly enhanced with 10 μg/mL ABA ($p < 0.05$).

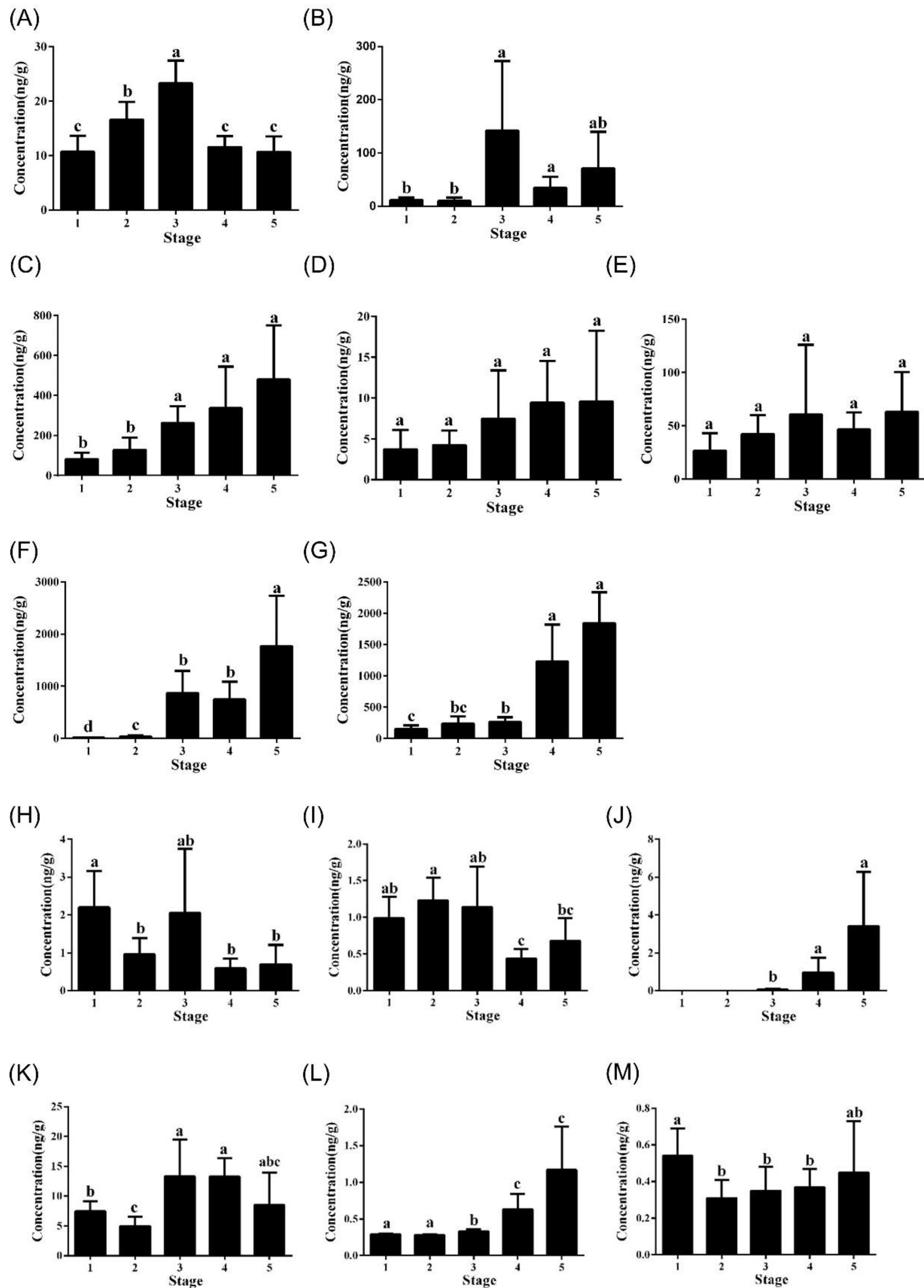

Figure 2 The concentrations of plant hormones in *P. vulgaris* during the development stages. (A) ACC: 1-aminocyclopropane-1-carboxylic acid; (B) ABA: abscisic acid; (C) cis-OPDA: cis(+)-12-oxophytodienoic acid; (D) JA-ILe: jasmonic acid-isoleucine; (E) JA: jasmonates; (F) IAA: indoleacetic acid; (G) SA: salicy acid; (H) tZ: trans-zeatin; (I) tZR: trans-zeatin riboside; (J) cZ: cis-zeatin; (K) cZR: cis-zeatin riboside; (L) iP: N6-(2-isopentenyl) adenine; (M) iPR: N6-(2-isopentenyl) adenosine. Columns with different lowercase letters were significantly different (p < 0.05, least significant difference test). Values are expressed as average ± standard deviation, n = 6.

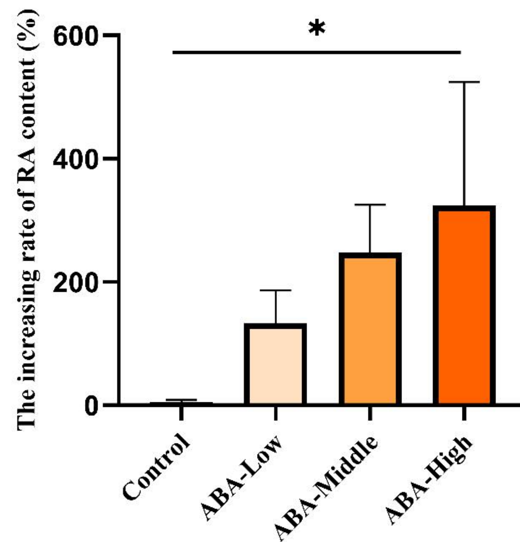

Figure 3 Effect of different concentrations of ABA on the increasing rate of RA content (average ± standard deviation, n = 3, compared with control, * $p < 0.05$).

4 Discussion

4.1 Analysis of changes in IAA concentrations

IAA participates in the morphological changes of *P. vulgaris* L. throughout its growth process. From stage 2 to stage 3, the IAA concentration increased rapidly, which is presumably related to *P. vulgaris* fruit formation in the spikes. As the spikes matured, the IAA concentration reached its highest value. IAA has been reported to be found mainly in rice spikes [24]. A high IAA accumulation in spikes is associated with auxin biosynthesis, metabolic processes, polar auxin transport, and auxin-mediated signalling [25]. Moreover, it has been shown that high concentrations of IAA can promote ethylene biosynthesis by inducing ACC synthase (ACS) to stimulate the synthesis of ACC, thus promoting fruit ripening [6]. This may be the reason for the increase in IAA concentration after fruit formation in the green-fruit stage. At the same time, it also promotes a rapid increase in concentrations of ACC, the direct precursor of ethylene. ACC, the direct precursor of ET, is subsequently used in large quantities for ethylene synthesis. Overall, auxin is considered to be an essential plant growth promoter and a determinant of plant morphogenesis [26,27].

4.2 Analysis of changes in SA concentrations

SA is an important mediator of the plant defence response to pathogens; this hormone can activate pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI), mediates systemic-acquired resistance (SAR) and is a key defence signalling molecule against biotrophs [28]. JAs are produced during the defence response against necrotrophic pathogens [29]. In general, these two hormone pathways function antagonistically. SA usually antagonizes JA signalling through processes that depend on Non-expressor of pathogenesis-related genes 1 (NPR1), suppressor of SA insensitivity 2 (SSI2), WRKY transcription factors and MPK4. Although antagonism between SA and JA is bidirectional, the main regulatory activity appears to be repression of JA signalling via SA-dependent cues. Microarray studies using different SA- and JA-signalling mutants have identified considerably more JA-dependent genes repressed by SA signalling than SA-dependent genes repressed by JA signalling [30].

In our study, the SA concentration was relatively low

before the turning stage, and as the spikes turned brown, the SA concentration increased rapidly. Studies have shown that SA collects at sites of infection during the defence process of plants [31]. It is speculated that, as *P. vulgaris* spikes begin to brown, pathogens invade the site of infection, the immune function *P. vulgaris* is induced, and the SA concentration dramatically increases in the spikes. Our study also found that, at stage 5, the JA concentration in the spikes did not change significantly and were maintained at low levels.

4.3 Analysis of changes in ABA concentrations

ABA is considered a negative regulator of disease resistance. This is due to the negative activity of ABA interference on the regulatory activity of SA, JA and ET in response to biological stress, as well as additional ABA on common components of stress signals [32]. ABA may indirectly affect SA signalling through its influence on JA signalling, and vice versa. Cross-talk between SA and ABA is bidirectional [30]. Moreover, high concentrations of ABA inhibit the biosynthesis of ET [33]. In this study, the ABA concentration reached its peak during the green-fruit stage. On the one hand, ABA is related to the formation of fruits, and ABA can promote fruit development; on the other hand, the increase in ABA concentration is associated with susceptibility to infection during that time [34,35]. After the spikes begin to turn colour, their ABA concentration decreases rapidly, which may be related to the antagonistic effect of the sharp increase in SA concentration after infection. At this stage, the ACC concentration decreases rapidly, and ET begins to be synthesized to promote fruit maturity.

The ABA concentration decreased rapidly and may also be related to high levels of IAA. IAA inhibits the expression of the ABA biosynthesis gene NCED and the ABA receptor gene PYR but promotes the expression of the ABA degradation gene CYP707A, which leads to a reduction in ABA content. ABA also inhibits IAA accumulation genes, auxin transporter genes (FaPIN), auxin synthesis pathway genes and flavin monooxygenase genes (FaYUCCA). This may explain why the IAA concentration does not increase but instead decreases during the turning stage [36].

4.4 Analysis of changes in CK concentrations

CKs interact with light signals involved in numerous physiological processes, such as the induction of flowering and leaf expansion [37]. In addition, CKs can slow these senescence-accompanying changes. Among these CKs, tZ clearly had the highest antisenescence activity, while cZ and iP were considered CKs with low or no antisenescence effects [38]. We found that tZ, tZR and iPR presented high contents in the early stage. For example, tZ had the highest concentration in the heading stage, and the tZR concentration remained at a high state before the green-fruit stage. This may be related to the growth and differentiation of the cambium. Moreover, plant leaf senescence is directly related to decreases in CK concentration and activity [39]. Leaf senescence is the last step in leaf development and is usually accompanied by a change in colour from green to brown or yellow [40]. Yellowing not only depends on age but also can be caused by many other factors, such as harvest and environmental stress [41]. During the late stage of *P. vulgaris* growth, concentrations of both tZ and tZR decrease, which may be related to fruit ripening and spike senescence. The concentration of the two is inversely proportional to the ripening process of *P. vulgaris*. Studies have shown that the effects of CKs are often affected by interactions with other hormones, such as auxin [42]. IAA inhibits the biosynthesis of tZ through the isopentenyladenosine-5′-monophosphate ip nucleotide-independent pathway [43]. The degradation of CKs is affected by downregulating the expression of CKX2, CKX4 and CKX7 genes and upregulating that of CKX1 and CKX6 [44]. Increased CK levels lead to a rapid increase in

auxin biosynthesis in seedlings, developing roots and leaf tissues [45,46]. The concentrations of cZ, cZR and iP increased significantly in the green-fruit stage, and the concentrations of cZ and iP increased from the green-fruit stage to the ripening stage. It is speculated that these hormones are present mainly in gradually maturing fruit.

4.5 Effects of hormone levels on secondary metabolites

The secondary metabolites of *P. vulgaris* have many pharmacological activities. Plant hormones can affect the synthesis and accumulation of secondary metabolites by activating or inhibiting the activity of corresponding transcription factors that regulate the expression of key enzyme genes related to plant secondary metabolism. Among these hormones, IAA, ABA, SA and CKs can affect the shikimate/phenylpropanoid pathways, which are involved in the biochemical synthesis of phenolic compounds (Figure 4). IAA can induce the accumulation of flavonoids by regulating the activities of L-phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) [47]. In addition, studies have shown that auxin can induce the biosynthesis of rutin [48]. ABA, SA and CKs can increase the content of phenols and flavonoids by enhancing the activity of PAL or inducing its expression. SA promotes the accumulation of soluble phenols and lignin, and CKs promote the accumulation of anthocyanins [19,49,50]. In addition, cytokinin-SHY2 signalling can regulate the content of flavonoids in the roots [51]. R2R3-MYB, bHLH, and WD40 proteins in the JA signalling pathway are involved in regulating the biosynthesis of anthocyanin. The synthesis of most plant anthocyanins occurs via the R2R3-MYB transcription factor or the MYB-bHLH-WD40 complex (MBW). Among these transcription factors, MYBL2 and CAPRICE (CPC) of the R2R3-MYB complex negatively regulate the biosynthesis of anthocyanins by hindering the formation of the MBW complex, inhibiting the transcriptional activation activity of the complex, and therefore inhibiting the expression of anthocyanin synthesis genes [52].

Studies have shown that ABA and JAs can promote the biosynthesis of terpenoids. The WRKY and bHLH family transcription factors in the JA signalling pathway are involved in the regulation of the biosynthesis of tanshinone, salvianolic acid and paclitaxel. These transcription factors negatively regulate the biosynthesis of tanshinone and salvianolic acid and positively regulate Taxol biosynthesis [53-56] (Figure 4).

Auxin can quickly downregulate the expression of genes encoding essential indole alkaloid biosynthesis enzymes (the strictosidine synthase (SSS) gene and the tryptophan decarboxylase (TDC) gene) [57]. All the transcription factors of the bHLH, AP2/ERF and WRKY families in the JA signalling pathway are involved in the regulation of the biosynthesis of monoterpenoid indole alkaloids (MIAs), vinblastine, a variety of nicotine alkaloids, isoquinoline alkaloids and berberine [58]. Moreover, SA has a cumulative effect on alkaloids but has an inhibitory effect on the synthesis of alkaloids induced by JA [59] (Figure 4).

In addition, indole-3-acetaldoxime (IAOx) represents the junction between the core pathway of glucosinolates (GLSs) and the auxin synthesis pathway. IAOx plays a role in the regulating branch points in the dynamic balance between the indole glucosinolate core pathway and the auxin synthesis pathway. JAs, SA, ET and ABA can induce GLS biosynthesis genes and increase GLS concentrations [60]. ABA can induce the biosynthesis of isoriccardin C, marchantin C and riccardin F, which are classified as bis(phenylbenzyl) bisbibenzyls [61] (Figure 4).

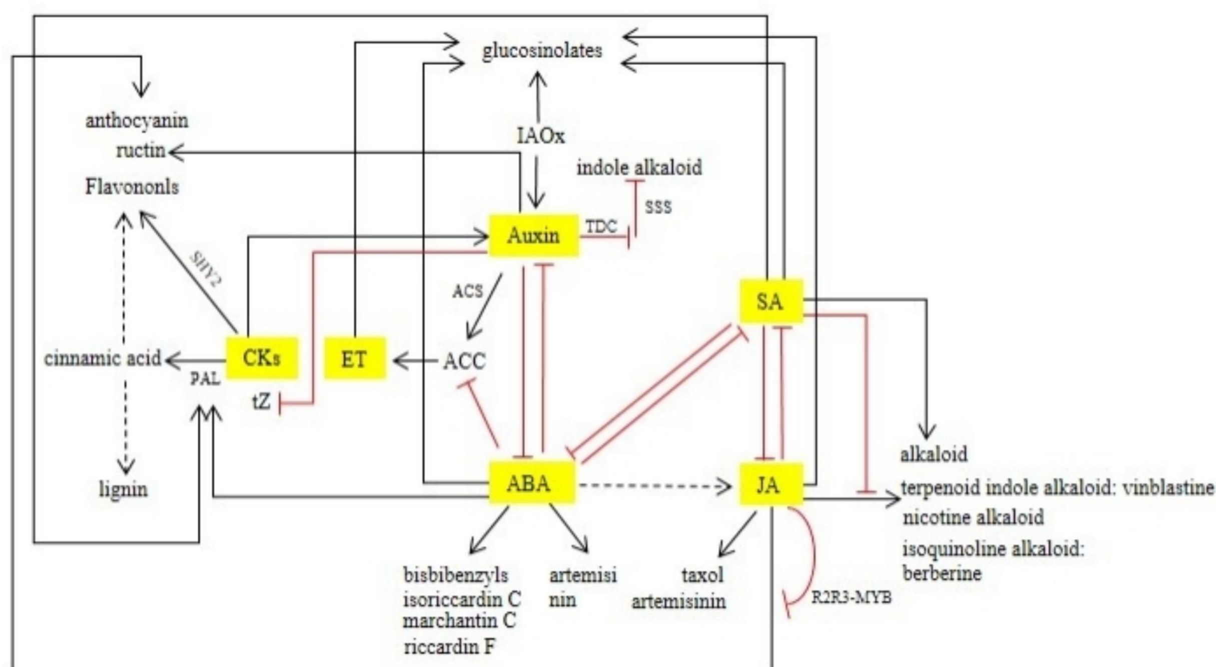

Figure 4 Summary of the results of this study. Interactions of plant hormones and their effects on secondary metabolites. → Forward regulation; —| Reverse regulation.

According to the results of this study, ABA treatment of 10 μg/mL has increased RA content significantly as compared to non-treated group. Similar results have recently been reported by other researchers in *Melissa officinalis* [14] and *Dracocephalum moldavica* L. [62].

ABA-induced increase in RA content could be in part due to the role of ABA in enhancing PAL activity and over-expression of genes involved in RA biosynthesis pathways [63,64]. Cui et al. reported that the activities of PAL and TAT and consequently accumulationof the phenolic compounds including RA, salvianolic acid and caffffeic acid are signifificantly increased by ABA treatment [65]. Shen et al. found that ABA up-regulates the expressions of PAL, TAT and RA synthase (RAS) genes [64]. Weiss and Ori found that PAL activity is suppressed with exogenous ABA treatment [66]. PAL activity in dragonhead leaves is increased by exogenous ABA treatment of 5–40 μM. Increased PAL activity with an increase in ABA levels was also reported in *Fragaria ananassa* [67], *Cynomorium songaricum* [65] and *Microdochium nivale* [19].

5 Conclusion

In this study, the MRM method was used to quantitatively analyse various plant endogenous hormones in *P. vulgaris* during development, revealing the dynamic changes in hormones and the relationships between hormones and the phenotypic changes, growth and development of *P. vulgaris*. We confirmed that exogenous ABA could significantly promote the accumulation of RA in *P. vulgaris*. The regulatory activity of plant hormones on the accumulation of secondary metabolites can serve as a basis for elucidating the growth and development mechanism of *P. vulgaris* in the future and provides a reference for studying the molecular mechanism through which plant hormones regulate secondary metabolites.

Acknowledgements

Not applicable.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Author Contributions

Q.S., Z.Z., Y.Lin, Y.Li, and J.X. performed the experiments. Q.S. and Z.Z. analyzed the data, wrote this manuscript. B.X., L.L. and P.W. revised this manuscript. L.L. designed and supervised this research. All authors approved the final version of this article, including the author list.

Ethics Approval and Consent to Participate

No ethical approval was required for this article.

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Availability of Data and Materials

Data will be made available on request.

Supplementary Materials

Not applicable.

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