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Review

Effect of exogenous indole-3-butanoic acid (IBA) application on the morphology, hormone status, and gene expression of developing lateral roots in *Malus hupehensis*

Jiang-Ping Mao, Dong Zhang, Xin Zhang, Ke Li, Zhen Liu, Yuan Meng, Chao Lei, Ming-Yu Han*

Department of Horticulture College, Northwest Agriculture & Forestry University, Yangling 712100, China

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ABSTRACT

Malus hupehensis seedlings were treated with exogenous applications of IBA (0.1 mg L^{-1} and 0.5 mg L^{-1}) in order to investigate the mechanism by which IBA promotes and effects lateral root (LR) development. Root morphology, plant height, plant crown diameter, and endogenous levels of hormone in LRs were evaluated. Additionally, patterns of differential gene expression related to root development were examined by RT-qPCR in IBA-treated and untreated roots during LR development. Results indicated that IBA treatment promoted both root and shoot growth, and that 0.5 mg L^{-1} IBA had a more obvious effect on growth promotion than 0.1 mg L^{-1} IBA. IBA treatment also induced an increase in the levels of endogenous auxin (IAA), zeatin-riboside (ZR), and the ratios of IAA/ABA and ABA/GA_{1+3}, however, reduced gibberellin (GA_{1+3}) and abscisic acid (ABA) levels. In addition, there was also a significant upregulation in the expression of *MdYUCCA4*, *MdPIN1*, *MdPIN2* and *MdAUX1* in response to the IBA treatment; thus leading to an increase in auxin levels. Although *MdIAA23* was induced, the expression of *MdIAA5* and *MdIAA14*, a negative regulator of *MdARF7* and *MdARF19*, were downregulated in IBA-treated apple seedlings. This resulted in elevated expression of *MdARRO1*, *MdGATA1* and *MdSCR1*, which was associated with an increase in lateral root development. Additionally, higher levels of endogenous auxin elevated the expression of *MdWOX11*, and thus the expression of *MdWOX5*, *MdLBD16 and MdLBD29*; collectively resulting in enhanced expression of the cell cycle related gene: *MdCYCD3;1*. This pattern of expression was again associated with a promotion in lateral root development. In summary, changes in gene expression and hormone levels resulted in an increased number of LRs and other root growth parameters in IBA-treated plants.

1. Introduction

Lateral roots (LRs) in apple and other plants are critical for supporting plant growth, providing water and nutrients necessary for maintaining the health of trees. In addition to LRs, roots with a diameter < 2 mm are defined as fine roots functioning in absorption, and the regulation of hormone synthesis and translocation in trees (Wells and Eissenstat, 2001). The development of LRs in apple consists of three stages: initiation, root primordia development, and emergence (De Smet, 2012; Lee et al., 2015). LRs originate from pericycle cells with a xylem pole, and are referred to founder cells (Dubrovsky et al., 2001). Once activated by a specific signal, founder cells begin to undergo cell division and form LR primordium, ultimately resulting in the formation of LRs (Malamy and Benfey, 1997).

Apple is one of the most commercially important tree fruits in the world. Since roots are so essential for the uptake of nutrients and water, understanding root development in apple rootstocks is important for the production of the highest quality apple trees (Wang et al., 2016). Root growth in apple rootstock is highly correlated with growth of the aerial portions of the tree. *Malus hupehensis* is regarded as a superior rootstock in China due to its strong resistance to abiotic stress. *M. hupehensis* is also apomictic which results in very uniform phenotypes and genotypes. Collectively, these features (beneficial effects on tree growth, and genetic uniformity) make *M. hupehensis* an excellent model system for root studies in apple.

Presently, research on molecular biology of rooting has primarily focused on *Arabidopsis thaliana* (Bhalerao et al., 2002; Fukaki et al., 2006; Hewitt and Watson, 2009; Hewitt, 1952; Hewitt and Hucklesby, 1966; Hewitt et al., 2005; Hewitt et al., 2010; Lavenus et al., 2013), and only a few reports related rooting in apple have been published. Auxin

* Corresponding author at: College of Horticulture, Northwest A & F University, Yangling 712100, Shaanxi, China.
E-mail addresses: mjp588@163.com (J.-P. Mao), afant@nwsuaf.edu.cn (D. Zhang), 495426841@qq.com (X. Zhang), keli505@163.com (K. Li), 453035830@qq.com (Z. Liu), 1228672957@qq.com (Y. Meng), 1406864754@qq.com (C. Lei), hanmy@nwsuaf.edu.cn (M.-Y. Han).

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promotes LR initiation in *Arabidopsis* (Himanen, 2002; Marhavý et al., 2013), and its accumulation alone appears to be sufficient for LR initiation (Grunewald et al., 2012; Shkolnikinbar and Barzvi, 2010). Although the general mechanism by which auxin positively regulates LR development in apple has been reported (Gao et al., 2008), more details are required in order to determine the specific mechanism by which IAA positively regulates LR development in apple. Currently, little is known about how IAA and other hormones interact to regulate LR development. Cytokinin (CK), abscisic acid (ABA), and gibberellin (GA) are known to negatively regulate LR formation (Chen et al., 2006; Gou et al., 2010; Huang, 2002; Lohar et al., 2004), but how these hormones change after treatment with IBA; remains to be elucidated in *Malus hupehensis*.

Information on how genes related to IAA signaling pathways change during LR development when an IBA treatment is administered is still lacking. Previous studies have reported that coordinated regulation of auxin influx and efflux carriers encoding genes (*AUX1* and *PINs*) in the cells where LR formation occurs optimise auxin supply to support LR initiation (Lewis et al., 2011; Marhavý et al., 2013; Staff, 2014). Both *YUCCA4* and *YUCCA6* also appear to play an essential role in auxin biosynthesis and plant development (Cheng et al., 2006). Thus, it is plausible that *AUX1*, *PIN1*, *PIN2*, and *YUCCA4* may also have important roles in LR development in apple. Regarding the auxin signalling pathway, degradation of auxin resistant/auxin (AUX/IAA) proteins promote auxin response factors (ARFs), which in turn activate transcription of auxin response genes (Gray et al., 2001a; Orman-Ligeza et al., 2013). Interestingly, *IAA14* interacts with *ARF7* and *ARF19* (Fukaki et al., 2005), suggesting that auxin stimulates LR initiation in a cell autonomous manner through the SLR/IAA14–ARF7–ARF19 signalling module in *Arabidopsis*. The molecular mechanisms underlying auxin induced root development and signal transduction in apple, however, still needs to be elucidated.

Recent studies in *Arabidopsis* have reported that auxin can induce the expression of *WOX11*, *WOX5*, *LBD16*, *LBD29* and *CYCD3;2* during root development (Hu and Xu, 2016; Liu et al., 2014; Okushima et al., 2007; Pi et al., 2015; Vanneste et al., 2005). Whether or not these genes up-regulated expression during root development after treatment with IBA in apple remains to be seen. *WOX5* and *LBD29* have also been reported to maintain stem cell renewal through the regulation of cell cycle genes (Feng et al., 2012; Forzani et al., 2014; Mao et al., 2017). Therefore, we hypothesize that *MdWOX5, MdLBD16,* and *MdLBD29* promote lateral root development by upregulating the expression of cell cycle related genes, which in turn may play a critical role in lateral root formation. In apple, the molecular mechanisms underlying IAA signal transduction and the regulation of LR development still remain to be elucidated. Although key regulators of lateral root development (SCARECROW; *SCR*) and *GATA23* have been identified in *Arabidopsis* (Rybel et al., 2010; Tian et al., 2014), the functional role of homologous genes in apple is still unknown. *ARRO-1*, has also been previously reported to function in adventitious root formation (Smolka et al., 2009), thus it is plausible that *ARRO-1* may also regulate LR development in apple.

In the present study, the effect of an exogenous application of IBA on hydroponically-grown seedlings of *Malus hupehensis* was examined. The objectives of the experiment is that investigate the mechanism by which IBA promotes and effects LR development and the functional role of the genes which rely on IAA mediated pathway during the development of LRs in *Malus hupehensis*. Therefore, root morphology, plant height, plant crown diameter, and endogenous levels of Cytokinin (ZR), IAA, ABA, and GA_{1+3}, and the ratios of IAA/ABA, ABA/GA_{1+3} during LR development were evaluated. The levels of endogenous hormones, and the expression of a number of select genes reported to be involved in auxin, cell cycle and root development, were monitored during the course of this study. Based on the resulting data, we conclude that auxin regulates LR development via auxin synthesis, transport, and signal transduction-related genes; ultimately resulting in the upregulation of genes that regulate LR development. Collectively, the results of the study provide a theoretical basis for understanding IAA regulation of LR development in apple.

2. Materials and methods

2.1. Plant material, growth conditions, and IBA-treatment

Malus hupehensis seeds were planted in an experimental orchard located at the Northwest Agriculture and Forestry University in Yangling (108°04′E, 34°16′N), China on March 15, 2016. When seedlings reached an 8–10 leaf stage, pulling seedlings out of ground and placing seedlings with no sign of insect or disease damage were transplanted to greenhouse conditions, under hydroponic solution. The plants were maintained under 24 h cycle of 12 h light at 25 ± 1 °C, followed by 12 h dark at 15 ± 1 °C. Relative humidity was approximately 70–80% and the photosynthetic photon flux density (PPFD) during the light cycle was about 800 μmol m^{-2} s^{-1}. Seedlings were cultured hydroponically in 1/2 Hoagland nutrient solution (composition is listed in Supplemental Table S1) for two weeks to allow them to recover from the transplant shock. The 1/2 Hoagland nutrient solution was exchanged weekly. The hydroponic device consisted of an open-topped plastic container (50 cm × 35 cm × 15 cm). The entire container was covered with black plastic and a polystyrene foam board with holes for the seedlings was placed on the top of the container. The stem of each seedling was positioned in each hole by surrounding it with a sponge. Each container contained 30 seedlings and the solution in each container was aerated by an air pump. A total of 90 seedlings were separately treated with 0.1 mg L^{-1} or 0.5 mg L^{-1} IBA daily for 28 d beginning on June 15, 2016, IBA was added to the hydroponic solution. IBA was purchased from Voerson Biological Reagent Co., Ltd. (Xian, China). An additional 90 seedlings, which served as controls, were cultured in 1/2 Hoagland solution without the addition of IBA.

2.2. Measurement of morphological parameters

Prior to the IBA treatment, an EPSON EXPRESSION 10,000 xl type scanner (LA l600 scanner, Canada) was used to obtain images of roots. The resolution of the scanner was set to 400 dpi, and the scanned image was analysed using WinRHIZO Pro root analysis software (WinRHIZO 2003 b, Canada). Morphological parameters, including root length, root surface area, and root volume were calculated from the images using the described software. In addition, the number of lateral roots, seedling height, and stem diameter were also measured. Plant height was measured from the top of the fiber board to the tip of the stem with a ruler. A Vernier calliper was used in two directions to measure the crown diameter and their average was recorded as the crown diameter. After the application of 0.1 mg L^{-1} or 0.5 mg L^{-1} IBA (Mustafa and Khan, 2016; Zhang and Zhou, 2003), the same indices were measured at 0, 7, 14, 21, and 28 d in both the treated and untreated seedlings. A total of 18 plants were measured and sampled at each time point. The collected samples were immediately immersed in liquid nitrogen and stored at −80 °C until subsequent use for the analysis of hormone levels and gene expression.

2.3. Extraction and measurement of IAA, ABA, GA_{1+3}, and ZR in lateral roots of seedlings

Hormones were extracted and purified using the procedure described by (Dobrev and Kamínek, 2002). Every hormone was measured at each time point in three biological replicates. Hormone levels were measured using a high performance liquid chromatography system (Waters 2489 UV/visible detector, Waters, USA). An external standard was used for quantitative analysis. IAA, ABA, GA_{1+3}, and ZR standards were purchased from Sigma (USA).