Adventitious Root Formation in Apples

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Adventitious root (AR) formation is required for the vegetative propagation of economically important horticultural crops, such as apples. Asexual propagation is commonly utilized for breeding programs because of its short life cycle, true-to-typeness, and high efficiency. The lack of AR formation from stem segments is a barrier to segment survival.

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1. Multiple Hormonal Pathways Mediate Adventitious Rooting

After separation from the donor plants, stem cuttings critically modify hormone homeostasis in the detached shoots [1]. Different growth regulators were examined to increase the rooting ability of stem cuttings. In the last few decades, it has been conducted on the formation of ARs in apples. Different endogenous hormones play a key role in forming ARs in apples. Auxin, as a master controller, promotes AR induction and initiation stages while inhibiting the AR emergence stage. Auxin crosstalk with melatonin (MT) also promotes the AR induction stage. Cytokinin (CK) works antagonistically to auxin. Gibberellic acid 3 (GA_3) also plays a negative role in the initial stages. Moreover, ethylene (ET) and jasmonic acid (JA) can inhibit the induction stage, and their roles in other stages are unclear. Abscisic acid (ABA) is a negative regulator of AR formation at all stages.

1.1. Auxin: A Master Regulator for ARs

Auxin is involved in various physiological events, including vascular differentiation, cell expansion, lateral roots (LRs), and ARs. More auxin is needed to induce ARs, but this is not necessary for the AR emergence stage [2]. Auxin treatment was shown to boost assimilate translocation from the leaf and sugar content at the root growth site [3]. Indole-3-acetic acid (IAA) is perhaps the most prevalent natural auxin, but Indole-3-butyric acid (IBA) is the most frequently utilized exogenous auxin for increasing ARs in most species, particularly in difficult-to-root genotypes. Recently, it was conducted on M 116 apple clonal rootstock to identify the effects of different concentrations of IBA and naphthaleneacetic acid (NAA) (IBA: T1, 1500; T2, 2000; T3, 2500; T4, 3000; T5, 3500; T6, 4000 ppm; NAA: T7, 500; T8, 1000; T9, 1500 ppm) compared with T10, control plants during adventitious rooting under mist chamber conditions. It was suggested that increasing auxin levels correlated positively with rooting success. IBA treatments significantly improved rooting. T5 had the highest rooting percentage (57.12%), AR numbers per cutting (7.33), AR length (34.85 cm), and AR diameter (5.27 mm) when compared to other treatments and control [4]. Furthermore, the Jork9 was also treated with auxins (IAA, IBA, and NAA). The cultures were kept in darkness during the initial time of rooting treatment. During this phase, the AR initials were made, then cultures were shifted

to light. NAA and IAA or IBA treatments obtained the lowest ARs (8) and the highest ARs (15), respectively. The maximal AR numbers were recorded throughout a broad range of IAA levels (10–100 µM), although at only one level of IBA or NAA (10 µM and 3 µM, respectively) [1]. It was indicated that the duration of exposure to auxins mainly controlled the AR formation. The cuttings are not highly responsive to auxin within the first 24 h after being collected. It is believed that during this lag time, cells dedifferentiate and become capable of responding to the rhizogenic signal, auxin. The cells that give rise to the AR primordia are often seen between the vascular bundles and store starch over the first 24 h. The rhizogenic activity of auxin in the induction stage then commits previously activated cells to create AR primordia for up to 72 h. During this time, auxin pulses stimulate the highest AR numbers. Auxin is no longer necessary after 96 h, and auxin levels advantageous for establishing root meristemoids are restrictive during this period [5].

IBA promotes AR formation in M.9 and M.26 apple rootstocks, but higher AR formation was seen in M.26, which is due to the high amount of free IAA in the stem basal part compared to M.9. Furthermore, the conjugated IAA level was higher in M.9 as compared to M.26, suggesting that the difference between AR responses in both rootstocks may be associated with free IAA content in stem basal parts [6]. During the whole process of adventitious rooting, endogenous auxin, or applied externally, plays a critical function at each step. In the AR initial phases, a high level of endogenous auxin is generally associated with a high rooting rate [7]. When the auxin is applied exogenously to increase root formation, it influences the endogenous auxin concentration, which generally reaches a maximum after wounding [8]. Still, the auxin peak was not detected [9]. The endogenous level of auxin induces the AR primordia formation, and the quantity of primordia formation is raised together with the elevation of the IAA level [10]. In apples, IBA was found to be highly important for inducing adventitious rooting in M.26 apple rootstock. The other hormones seem to be indirectly involved in the control of ARs by their interaction with the auxin, where the endogenous level of IAA was observed to be higher at the early stage and induces primordia. Then, the level of IAA decreases towards a later stage [11]. These variations in the IAA levels suggest that auxin plays a critical function at the early stages and may not be crucial after the AR primordia have been established. IBA-treated cuttings of *Malus prunifolia var. ringo* produced more ARs than cuttings treated with the auxin transport inhibitor NPA, which completely inhibited AR formation. This shows that IBA plays a role in AR formation [12]. In M9-T337 apple stem cutting, IBA inhibited AR elongation at later stages of development by decreasing cell length and by decreasing the expression of genes related to cell elongation [13]. Moreover, transcriptome sequencing situations on IBA-treated T337 cuttings showed that auxin, both endogenous and exogenous, regulates AR formation through homologous signaling pathways to some degree. AR formation is largely controlled via the auxin signaling pathway. Furthermore, various hormone, wounding, as well as sugar signaling pathways work together with the auxin signaling pathway to regulate adventitious rooting in T337 [14]. In M.9 apple rootstock, IAA stimulates the process of AR formation by the upregulation of PIN-FORMED (PIN). Auxin promotes the multiplication and extension of AR founder cells via starch grain hydrolysis, resulting in endomembrane system multiplication, lenticel dehiscence, and AR emergence. This effect was inhibited by the NPA application [15]. Nevertheless, in stem cuttings of Malus species, the IBA promotes more root formation than the IAA. At the same time, it was changed only at deficient IAA levels, implying that the IBA was operational or controlled IAA activity [16].

Recently, Meng and colleagues [17] applied 1 mg/L IBA to different apple rootstocks with different rooting abilities, including MP, SH6, T337, and M.26, to detect the effect of IBA on AR formation compared with control plants. It was showed that MP was easy to root and developed ARs in both groups. However, M.26 produced some ARs in IBA-treated groups. However, T337 and SH6 have poorly developed ARs in both groups. Moreover, microRNA160 (miR160) mediates AR formation; therefore, five members of miR160 were found in the apple with two target genes: auxin response factor 16 (*ARF16*) and *ARF17*. MiR160 was highly expressed in SH6 and T337 and lower in MP and M.26 in both groups. Following that, miR160a was cloned from M.26 and overexpressed in tobacco plants, causing severe defects in AR formation, which were released by IBA application to transgenic plants [17]. A comprehensive proteomic analysis was conducted on the role of IBA (1 mg/L) in T337 during AR formation. It was showed that AR rate and length were increased after IBA treatment compared with control cuttings. IBA treatment increases the content of IAA, ABA, and (brassinolide) BR and decreases ZR, GA, and JA content. The expression profiles of differentially expressed proteins were strictly related to phytohormone signaling, protein homeostasis, carbohydrate uptake and energy synthesis, reactive oxygen and nitric oxide signaling, as well as biological processes associated with cell wall remodeling. These are all the most important AR formation processes by IBA application [18].

Concerning auxin signaling pathways, the degradation of AUX/IAA stimulated *ARF* transcripts, which in turn stimulated the auxin-responsive gene expressions [19]. Numerous genetic investigations have shown the contribution of *ARFs* in plant developments. In Arabidopsis, *ARF7* and *ARF19* show specific and dynamic gene expression profiles throughout embryogenesis, seedling development, and rooting [20][21]. Single mutants *arf7* and *arf19* limit the formation of ARs and LRs, but double mutants are initiated to produce significantly fewer roots [21][22]. They are considered key players in the formation of ARs in apples, where they are more expressed at important time points [23]. Several miRNAs are key players in root growth, including miR160, which is considered necessary for the development of root tips and gravity sensing via the participation of their targets, such as *ARF10* and *ARF16* [24], which regulate auxin-responsive gene expression through binding with auxin response elements in the promoters. In addition, callus initiation was repressed by miR160 from pericycle-like cells but activated by *AFR10* [25]. Furthermore, smaller and gravitropic roots and tumor-like apexes were seen by the over-expression of miR160c [24]. This phenotype was related to unrestrained mitosis and a lack of columella cell differentiation in the root apical meristem (RAM) [24]. A very similar phenomenon was also perceived in double mutants of *arf10* and *arf16*, where miR160 and their target genes might be necessary to limit cell divisions in root tips and stimulate cell extension and differentiation. Moreover, the contribution of miR160 and *ARFs* in response to auxin signals during AR formation might play a critical role in regulating apple ARs [26]. In contrast with miR160, miR167, also an auxin-related miRNA whose target genes are *ARF6* and *ARF8*, is an adverse controller for adventitious rooting. Furthermore, *ARF8* and *ARF17* play opponents in auxin homeostasis in Arabidopsis [27][28].

On the other hand, miR167 in rice seemed to be a key controller of AR formation [29]. According to Arabidopsis, in which miR390 was regulated by auxin via the roles of *ARF2*, *ARF3*, and *ARF4*, conversely, miR390 expression was restricted by *ARF4* at the base of root primordia [30]. Moreover, AR development was positively regulated by miR390a in apple, which shows its regulatory ability over *ARFs*. MiR390 and miR160 are important in forming ARs. The *ARFs* (target genes) also act as a crucial controller in auxin signal transduction during the process of

adventitious rooting in apple rootstocks [26]. Auxin-induced adventitious rooting largely depends on miR156 high expressions in *Malus xiaojinensis* via *MxSPL26*, independent of *PINs* and *ARFs* [31]. Conversely, it does not affect *Eucalyptus grandis* [32]. So, rooting regulated by miR156 might be species-specific. However, miR156 can be an essential factor for AR induction but not a determining element in woody plant species. Moreover, in apple cuttings, wounding is knotty with the induction of adventitious rooting [26].

The auxin gradient in root tips is maintained by PIN auxin polar transport, which stimulates root growth [33]. In apples, the *PIN* family consists of eight members. All were differentially expressed at different stages of AR formation. For example, *PIN8* and *PIN10* were upregulated and largely associated with the induction stage. Upregulation of all *PINs* was detected towards the initiation stage, whereas *PIN4*, *PIN5*, and *PIN8* were all upregulated and largely associated with the emergence stage, suggesting their regulatory roles in adventitious rooting [15]. Furthermore, CK repressed *PIN1* expression during adventitious rooting to regulate polar auxin transport [34]. The AUXIN RESISTANCE1 (*AUX1*) gene is recognized as an auxin influx carrier [35]. Moreover, the aux1 mutant developed few roots and showed reduced root gravitropism; it reduced IAA accumulation in the roots and acted as an auxin influx transporter to stimulate root growth via allocating IAA among root and shoot organs [36].

In Arabidopsis, LATERAL ORGAN BOUNDARIES-DOMAIN (LBD) *LBD16* and *LBD29* are positively controlled by *ARF7* and *ARF19* and induce rooting [22]. *ARF7* and *ARF19* stimulate *LBD16* and *LBD19,* which, in turn, activate adventitious rooting in apples [23]. The Wuschel-related homeobox gene *WOX11* was identified as a key regulator of Arabidopsis root development and apple ARs [37][38]. Short root *(SHR*) also plays a positive function in forming the LR stem cell niche as well as AR's meristem [39]. *WOX5* stimulates *WOX11* to endorse the beginning of root primordia and organogenesis [40], and *WOX5* is considered the initial indicator for adventitious rooting [41]. Furthermore, *WOX5*, *WOX11*, and *SHR* are all auxin-inducible and contribute positively to integrating numerous signals related to root induction [42][43]. Furthermore, in Arabidopsis, Liu and colleagues found that the auxins induced *WOX11* during the initial stage of cell fate transition, and *WOX11* increased the transcript of *LBD16* and *LBD29* during adventitious rooting [43]. Subsequently, harmonizing transcript abundance of these genes could promote rooting by stimulating cell cycle-related (*CYCD1;1* and *CYCP4;1*) gene expression in apples [44][23].

1.2. Cytokinin: A Required Inhibitor

CK is an essential hormone that plays many vital roles in regulating the cell cycle and several developmental processes. Root growth requires CK signaling and perception [45]. It has linked CK with the inhibition of AR formation [46][47][48][49]. Exogenous treatment of CK inhibited the formation of ARs, and auxin is generally used to initiate ARs in different plant species [50][51][52]. In AR induction, the hypothesis of an inhibitory effect for high CK contents and a high CK to auxin ratio has garnered significant scientific evidence, based on the finding that low CK to auxin ratios favor root regeneration in explants. The crosstalk of CK-auxin also played several functions in controlling the size of root meristems, with auxin and CK presenting opposing positions in controlling root growth [53]. The low CK concentrations promoted AR initiation. M.26 apple cuttings were treated exogenously with 2 mg/L

6-benzyl adenine (6-BA) to 3 day and 7 day old cuttings by transferring them from IBA containing medium to 6-BA medium. Stem anatomy showed that 6-BA treatment limits primordia formation in 3 d-treated cuttings but not in 7 day treated cuttings. The endogenous IAA concentration and the IAA/CK and IAA/ABA ratios were seen to be lower in 3 d-treated cuttings than in 7 d-treated cuttings. The high abundance of CK in 3-d treated cuttings increased the expression levels of CK signaling pathway genes, *RR1, RR2, RR3*, and *AHK4*, which prevented the genes related to auxin synthesis and transport, *PIN1, PIN2, PIN3, AUX1, YUCCA1*, and *YUCCA10*, resulting in a decline in inner auxin content. Thus, reduced auxin content inhibits the expression levels of auxin signaling pathway genes *IAA23, ARF6, ARF7, ARF8*, and *ARF19*. Furthermore, the transcripts of the cell cycle and AR development-related genes were also reduced, which all have a negative effect on forming AR primordia (initiation stage). Contrarily, reduced CK content in 7 day treated cuttings reduces the negative effect on auxin content, increasing the gene expression recognized to stimulate the formation of AR primordia. These results showed that 3–7 day is the period of primordia formation. The decline in AR formation in the 3 day treated group is due to suspending the AR primordial formation stage in apples [44].

Di Zhao and colleagues [54] found the interaction between CK and auxin during AR formation in wild apple (*Malus sieversii Roem*). Overexpression of *MsGH3.5* (encoding IAA amido synthetase) developed fewer ARs than the empty vector by significantly decreasing the amount of free IAA and elevating specific IAA amino acid conjugates. This feature is similar to GH3's involvement in auxin balance, which involves conjugating the concentration of free active IAA to amino acids. CK content was increased and altered the gene expression involved in CK biosynthesis, absorption, and signaling in overexpressed plants. Moreover, external CK treatment promoted *GH3.5* expression via the action of RR1a (the CK type-B response regulator that regulates the CK main response). RR1a induced the *GH3.5* transcript via binding to its promoter, connecting auxin and CK signaling. Overexpressed *MsRR1* plants also showed some ARs, consistent with *MsRR1a*'s control of *MsGH3.5* expression. Taken together, *MsGH3.5* influences adventitious rooting by changing auxin and CK content and their sophisticated signaling pathways [54]. However, CK may have a stimulatory effect in the first few hours after cutting excision by activating the cell cycle earlier [55]. It was showed that CK is essential in the initial phase of adventitious rooting in the Jork 9 apple rootstock. Lovastatin and simvastatin are CK-synthesis inhibitors and inhibit AR formation. This negative effect was partially released by adding zeatin, confirming its role in forming ARs. Still, it stimulates cell divisions at low concentrations just after wounding [56].

1.3. Ethylene: A Positive or Negative Regulator for ARs

ET plays a crucial function in regulating ARs in many species. Many experiments were conducted to identify their roles in root formation. The findings of these experiments were extremely flexible for specific species. ET behaved as activators or inhibitors and did not influence the formation of ARs. Because of earlier findings, auxin affects ET synthesis [57]. Subsequently, many efforts have been made to identify how auxin interacts with ET during AR formation. High ET level tissues amplified the responsiveness of root developing tissues in response to endogenous IAA. The role of ET was known in M9-T337 apple stem cutting during AR formation. The $AgNO_3$ (ethylene inhibitor) reduced the appearance of ET and promoted the AR's emergence and development. However, the ET precursor, 1 aminocyclopropane-1-carboxylic acid (ACC), was added to the MS medium, where it

may convert into ET, inhibiting AR emergence and decreasing AR length in M9-T337 [13]. Harbage and Stimart [58] found that ET was not involved in AR formation in apple micro-cuttings of Gala and Triple Red Delicious. It was found that IBA-induced ET formation was reduced by aminoethoxyvinylglycine (AVG), although the AR number continued to be IBA-dependent. ACC restored the inhibitory effect of IBA+AVG on rooting, while ACC separately had little impact on the AR number. Unlike 2,3,5-triiodobenzoic acid (TIBA) and N-1-naphthylphthalamic acid (NPA), which impede polar auxin transport, ET production is increased without increasing the AR number [58]. ET inhibits or promotes the process of root development depending on the stage of the process. It has a stimulatory role at the initial stage but reflects an inhibitory role at later stages of ARs' development [5]. Root development can also be inhibited by ET, mainly by limiting cell expansion, but this does not affect root meristem activation [59].

1.4. Abscisic Acid: A Negative Regulator for ARs

The ABA mainly responds to various environmental factors, including water and salt stress, controlling root architecture and regulating root elongation by controlling cell division and elongation [60]. Root cell division is limited to meristems, a region with a quiescent center (QC) bordered by stem cells, which further split to form cells that differentiate into several root tissues, along with the formation of stem cells, which keep on isolating and allow for indeterminate root growth [61][62]. In comparison, high ABA content is well known to inhibit root formation and development.

Consequently, in Arabidopsis, the ABA regulated quiescence and inhibited differentiation in the root meristem, hence continuing the stem cell population [63]. ABA controlled root growth by regulating the dividing cell population in the root tips. Furthermore, ABA also held root elongation by controlling cell length, an intense effect on the development of the root. However, cell division continuously is crucial for root growth. ABA has been found to limit LR and AR proliferation in tobacco, apple, and Arabidopsis [64][65][66]. High ABA content in Jonathan apple microcuttings is related to root inhibition. It is thought that high ABA is the key factor involved in the inhibition of ARs in apples [67]. Recently, Qingzhen 1 apple plants were exogenously treated with 5 µM ABA, where root development was significantly inhibited compared to control plants by significantly increasing the endogenous content of ABA and by decreasing IAA, ZR, and JA concentrations. ABA treatment upregulates the expressions of ABA-related genes (*ABF3, ABI1, AREB2*, and *CYP707A2*) and downregulates the expressions of auxin-related genes (*ARF19, YUCCA8, PIN1, PIN2, PIN3,* and *YUCCA3*), root development, and cell cycle (*WOX5, WOX11, CYCD1;1*, and *CYCD3;1*) [68]. AR proliferation was strongly repressed by the treatment of high nitrate and polyethylene glycol (PEG) by crosstalk with endogenous ABA, whose content was considerably higher than control cutting at all sampling points, suggesting that high endogenous ABA content is harmful for AR formation in apples [69][70].

1.5. Jasmonic Acid: A Positive or Negative Regulator for ARs

JA, usually related to mechanical wounding, provides defense against plant pathogens. In the last decade, it has focused primarily on JA's role in plant development and exposed JA as a critical hormone. It participates in different developmental mechanisms: hypocotyl elongation, primary root (PR) elongation, LR and AR formation, and flower

development [71]. Recently, JA seemed higher in B9 apple rootstock cuttings when treated with high nitrate and inhibited AR formation by JA signaling pathways [69]. The endogenous concentration of Methyl jasmonate (MeJA) was higher in GL-3 apple plants when exposed to drought and inhibits AR formation [70]. In many plant species, wounding can increase the jasmonates [72]. JA is promptly induced after cutting or in the case of injury. It accumulates at the place of injury, bringing responses that defend plants from pathogens' attacks. At the base of petunia stem cuttings, JA rapidly accumulates during the induction stage of adventitious rooting. It appears to serve as a positive regulator for the formation of ARs [73]. Based on these findings, Lischewski and colleagues [74] tested JA's role in forming ARs in petunia stem cuttings. They found some ARs are formed due to the downregulation of allene oxide synthase, which represses a critical aspect in the synthesis of cis-12-oxo-phytodienoic acid [74]. However, the same writers demonstrated that when petunia cuttings were treated continuously with exogenous JA, they formed significantly fewer ARs than the control cuttings [74]. The findings mentioned above are in dispute with those suggesting that JA could be a positive controller for ARs initiation and that JA's continuous treatment inhibited the ARs initiation stage. It has revealed that JA is an essential controller for the formation of ARs, possibly acting as an inhibitor at the AR initiation stage, while their functions may be more complicated and plant species sensitive. JA severely inhibited cell differentiation and expansion [75][76]; however, this information is very limited in identifying the action mode of JA at the particular stage of ARs formation and development.

1.6. Melatonin: A Positive Regulator

MT is thought to be necessary for the creation and growth of ARs in apples. [37]. In plants, MT served as a vital regulatory signal [77] and was essential for root formation, stress response, explants, and shoots [78][79][80]. It has indicated that the exogenous treatment of MT stimulated AR formation in cuttings of *Malus prunifolia*, where MT mainly affects the AR induction stage by IAA homeostasis. *WOX11* was induced by MT, and apple plants overexpressing *MdWOX11* developed more ARs than the GL-3 WT plant, suggesting that *MdWOX11* promotes ARs by MT signaling [37]. It has been advocated that exogenous MT at low concentrations could increase the endogenous content of IAA, and it is supposed that this stimulating MT effect on growth and development might be triggered by this rise in IAA levels [81]. On the other hand, it has suggested that the effect of MT on root formation and differentiation is IAA independent [82]. The IAA content was increased at the AR induction stage after the application of MT; however, it was reduced at the AR initiation stage and emergence stage in apple [37].

1.7. Gibberellic acid and brassinosteroids: a positive or negative regulator for ARs

The specific roles of GA and BR are still largely unknown in the regulation of ARs. The effect of GA_3 was known in M9 cv. Jork stem discs. First, the discs were cultured in darkness for 24 h on a root-inducing medium containing 24.6 µM IBA. Afterward, the discs were shifted to light exposure and cultured on a hormone-free medium and a medium containing 10 µM GA_3 for different time points of adventitious rooting. The results suggest that GA_3 treatments limit AR formation from the initial to final stages of AR formation [83]. Moreover, the concentration of GA_3 was significantly increased at the initiation of ARs, indicating that GA_3 plays an important role in forming apple ARs [12]. However, some shreds of evidence show that BR participated in AR formation. The availability of BRs triggers dual effects on the formation of ARs: the enhancing effect at low levels and the inhibitory effect at high

levels [84]. Their high concentration inhibited AR formation in apple rootstock [12]. However, the above information is not enough to identify the specific role of GA and BR in forming ARs.

2. Role of Phenolic Compound in the Regulation of ARs

The involvement of phenolic compounds in AR formation has been well established for a long time [85][86][5]. These were shown to either synergize or inhibit the activity of auxin [87]. Phenolic compounds keep plants from oxidative stress [88]. Besides shielding auxins from oxidation, phenolic compounds have been associated with them in various ways. Flavonoids may also limit auxin transport [89]. Flavonoids either interact with PIN2 or have an impact on the extent of PIN proteins [90]. The role of phenolic compounds was tested on Jork 9 stem slices during AR formation. The results suggested that all orthodiphenols, paradiphenols, and triphenols investigated with IAA enhanced adventitious rooting from stem slices. The most effective treatment was ferulic acid (FA) (a methylated orthodiphenol), which raised the number of ARs from 0.9 to 5.8. There was little or no improvement with NAA following the inclusion of phenolics. FA and phloroglucinol (a triphenol) were known in-depth. Based on their effects on the IAA dose–response curve and the duration of their activity, both acted as antioxidants, preventing IAA decarboxylation and oxidative stress in the tissue [85]. Auxin degradation includes oxidative decarboxylation via peroxidases, but since phenolics influence peroxidase activity, auxin catabolism at the cuttings' base may be prevented [85]. After wounding, there is an elevation in JA, auxin, and phenolic compounds at the cutting base during the AR induction stage, with a decrease in peroxidase activity. However, peroxidase activity increases to a peak during the initiation stage, and the auxin concentration decreases [5][73].

3. Role of Sugars in the Regulation of ARs

Sugars are both energy sources and signaling molecules that control plant growth. The detailed sugar metabolism focusing on the anatomical changes in apple rootstock Jork 9 were conducted by Jasik and De Klerk [91]. As highlighted in the initial developmental stage, a large number of starch grains were found in the cells that started AR primordium formation at the stem base, and thus the percentage of plastids grew dramatically, with the starch grains occupying a major share of all apparent plastids at the same stage. The utilization of sugars produced by the hydrolysis of all of these starch grains provides energy for adventitious rooting, which is associated with an increase in the number of cambial mitochondria, dictyosomes, and nuclei to the visible detriment of vacuolar expansion. Moreover, Pawlicki and colleagues [92] used different combinations and concentrations of carbohydrates and auxin to investigate AR differentiation. They found that sucrose (29–50 mM) stimulated adventitious rooting but also supported callus formation. In contrast, sorbitol, which is required in large quantities for optimal adventitious rooting, was not highly supportive of callus formation. Furthermore, the combinations of sucrose and mannitol (59/29 mM) or glucose and sorbitol (117/59 mM) ensued in 100% AR formation, with more than 6 ARs per disc. Then, in the presence of the sucrose-mannitol combination, the discs were treated with 49.2 µM of IBA for 540 min, obtaining an adventitious rooting of 100%.

The number of ARs was affected by sucrose concentration, although the effect was minor across a wide range of sucrose concentrations (1–9%). There was also a synergy between sucrose and auxin, which facilitated adventitious rooting. When slices were cultivated in a sucrose-free medium for 0–2 days, root formation was decreased. However, 2 days of cultivation minus sucrose had no impact or even a minor enhancer effect on later days, suggesting that sucrose is needed as a source of energy and a building block during adventitious rooting in apples [93]. The cuttings of M7 apple rootstock were treated with IBA and found to contain glucose, sorbitol, fructose, and inositol. In the initial 10 days of AR formation, the content of soluble saccharides improved significantly, and this was associated with the rapid cell divisions at the stem base in this stage. Specifically, the amount of fructose content in the stem basal part was related to rooting potential. At the AR primordia differentiation and AR emergence stages, the concentration of soluble saccharides in the cuttings had dropped to its initial level, indicating that soluble saccharides are crucial for the initial stages but not essential for later stages of AR formation in apples [94].

It was conducted on T337, where the IBA treatment induces the expression of sucrose synthase4 (SUS4), sucrose phosphate synthase4 (SPS4), and polyol/monosaccharide transporter (PMT5) at the induction stage [14]. The application of KCl to B9 apple rootstock also upregulates the expression of various starch and sucrose metabolism-related genes [95]. These results show that IBA and KCl had some crosstalk with sugars during the AR induction stage, and sugar was transported into the stem basal parts by the activities of several genes, providing adequate energy and signal activity for ARs to begin.

4. Role of Polyamines in the Regulation of ARs

Polyamines (PAs), including putrescine, spermidine, and spermidine, are a class of organic compounds with two or more main amino acid groups found abundantly in plant cells. Additionally, PAs are important in controlling DNA duplication and cell proliferation, senescence, stress responses, and morphogenesis. They also play a key function in forming and developing root architecture [66]. Exogenous application of spermidine on *Malus prunifolia var. ringo* stem cuttings increased AR formation by interacting with IAA. Spermidine application upregulated the expression levels of spermidine-related genes (*SAMDC1, PAO*, and *SPDS6*) and auxin biosynthesis-related genes (*IAA7, IAA14*, and *IAA23*) during AR formation. In contrast, *WOX11* upregulated the expression levels of *LBD16* and *LBD29*, which prompted the transcripts of genes related to the cell cycle (*CYCD1;1* and *CYCP4;1*) [31]. Nonetheless, they have been shown to be inhibitors in a number of species, including poplar [68] and walnut [70]. PAs, in conjunction with auxin, modulate cell division and root primordia initiation during the induction stage [71]. Nonetheless, the relationship between PAs and auxin for the rooting process is still lacking.

5. Role of Nutrients in the Life of ARs

Mineral nutrients are necessary for plant metabolism. AR formation and nutrients are closely related.

5.1. Role of Nitrogen in the Formation of ARs

Nitrogen is regarded as an important macronutrient that is needed for plant development and higher yields [96]. Nitrogen assimilation in soil occurs in two ways, such as organic and inorganic [97]. Nitrate is a vital nitrogen source that also functions as a signaling molecule for controlling flowering time, AR, and LR formation, as well as prompting auxin-related gene expressions [98][99][100]. Nitrate levels in the soil are relatively low due to its high solubility, leaching capabilities, and fast absorption by bacteria and fungus [101]. In higher plants, such as apples, there are two types of nitrate transport mechanisms: low-affinity transport systems (LATS) and high-affinity transport systems (HATS), which are responsible for uptake, distribution, and storage of nitrate [102]. The nitrate supply is immediately and strongly detected by the plant cells. Following this, the nitrate signaling system changes the relative expression levels of several gene sets to control cell and organ metabolism. Furthermore, the availability of nitrate has a significant effect on AR formation. In general, the external concentration of nitrate generated binary effects on the formation of AR depending on their concentrations, with an activated impact at low levels; however, a limiting effect at high levels was seen in Arabidopsis [103]. A similar phenomenon was also seen, where different nitrate concentrations (9.4 mM/L, 18.8 mM/L, 28.1 mM/L, 46.9 mM/L, and 84.5 mM/L) were exogenously treated to B9 apple rootstock stem cuttings during adventitious rooting, and 28.1 mM/L was found to be the most favorable nitrate level for adventitious rooting, and 46.9 mM/L and 84.5 mM/L were found to be inhibitors [98]. High nitrate inhibits AR in apples by elevating the endogenous levels of ABA, ZR, JA, BR, and GA_3, which may create a hormonal imbalance in the plant. In addition, the high ratios of IAA/ABA and IAA/ZR promote ARs under nitrate treatments. Furthermore, transcriptome analysis showed that hormone signaling pathway-related genes were upregulated, and root development and cell cycle-related pathways were repressed by the application of high nitrate [69]. Moreover, auxin and ABA signaling miRNAs (miR390a, miR160a, miR167, miR169a, and miR394) were activated, and miRNAs related to cell fate transformation, expansion, and enlargement (miR166, miR171, miR319, miR156, and miR396) were repressed by high nitrate [104]. It was explained that the mechanism of a different set of genes how 28.1 mM/L treatment promotes AR formation in B9 compared with 46.9 mM/L (inhibiting treatment). The results showed that treatment with 28.1 mM/L noticeably upregulated the relative expression levels of nitrate related genes (*NRT1.1, NRT2.1, NIA1*, and *ANR1*) and auxin biosynthesis (*IAA14* and *IAA23*), which enhances the AR development-related gene expression (*WOX11, ARRO1*, and *SHR*) and collectively induces the expression of cell cycle related genes (*CYCD1;1*, *CYCD3;1*, and *CYCP4;1*) in comparison with 46.9 mM/L nitrate treatment [105]. *NRT2.1* a high affinity nitrate transporter showed the highest response to nitrate availability, indicating that *NRT2.1* may play a key role in forming AR in apples, and the overexpression of *MdNRT2.1* gene in tobacco produced superior roots compared to WT plants.

Ammonium, such as nitrate, played an important role in root development in apple and other crops [106][107][108]. The effects of nitrate and ammonium were known in apples, where significant differences were detected in root morphology within a week of application. The roots were thin and long in response to nitrate treatment, although they were thick and short in response to ammonium application, with prominent enlarged areas behind the tip. Furthermore, nitrate-treated roots were nearly devoid of root hairs, but those treated with ammonium were entirely covered in thick, long root hairs, and the root hair cylinder diameter in the ammonium treated was around three times that of the nitrate treated [107]. Hilo and colleagues [108] found that ammonium-treated cuttings (without nitrate) had a higher total nitrogen content, which was indicated by enhanced glutamine and asparagine levels. It

was pointed to faster ammonium assimilation in the stem base, which could have resulted in a lower expression level of N-regulated genes such as the ammonium transporter *AMT1* [108]. Moreover, the effects of ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3) were also known in three apple scion cultivars [106]. The percentage ARs of Gala and Royal Gala rose dramatically when the level of NH_4NO_3 in the medium was reduced from full strength to 1/4 strength, but not in Jonagold. A further decrease in NH_4NO_3 concentration from 1/4 strength to zero considerably decreased the rate of ARs in Gala but not in Royal Gala. However, Jonagold rooted optimally in the absence of NH_4NO_3. Moreover, without NH_4NO_3, adventitious rooting for all three cultivars was as high as 100% when KNO_3 was given at full strength. These results suggest that the effect of NH_4NO_3 was cultivar-specific, but KNO_3 treatment at full strength promoted ARs in all cultivars.

5.2. Role of Potassium in the Formation of ARs

Potassium (K) is an indispensable macronutrient, and it is the most abundant cation absorbed by higher plants. It comprises more than 10% of the plant's total dry weight [109]. Its decline to below 10 g/kg of dry weight leads to serious growth issues in various plant species. Indeed, despite not being an important element of any structural and functional molecules, it is involved in various key physiological processes, such as metabolism and plant growth and development [110], flowering [111], improved fruit quality [112], and AR formation in apples and other plants [95][113]. In addition, under K deficiency in soil, plant root growth is weakened. As a result, the yields and outcomes are often limited [114]. The uptake and translocation of K is mediated by several K channels and transporters [109][115].

The underlying physiological and molecular mechanisms regulating AR by KCl application were known in B9 apple rootstock. KCl-treated cuttings produced a significantly higher number of ARs and increased AR length than control cuttings. At most time points during AR formation, KCl promoted several hormone levels, including IAA, ZR, JA, and GA, and decreased the ratios of ABA/JA, ABA/ZR, and ABA/IAA. Moreover, transcriptome analysis revealed that KCl promoted ARs through the auxin signaling pathway and sugar metabolism and by increasing the genes related to AR development and cell cycle [95]. Z.R. Zhao and colleagues found the promotive effect of K on the formation of ARs in cucumber cotyledons and mung bean hypocotyls, as well as in kidney beans [113]. It was indicated that the K application promotes adventitious rooting in apples and other crops.

The effect of nitrate and potassium treatments on the various endogenous hormones during adventitious rooting in apples is shown in **Figure 1**.

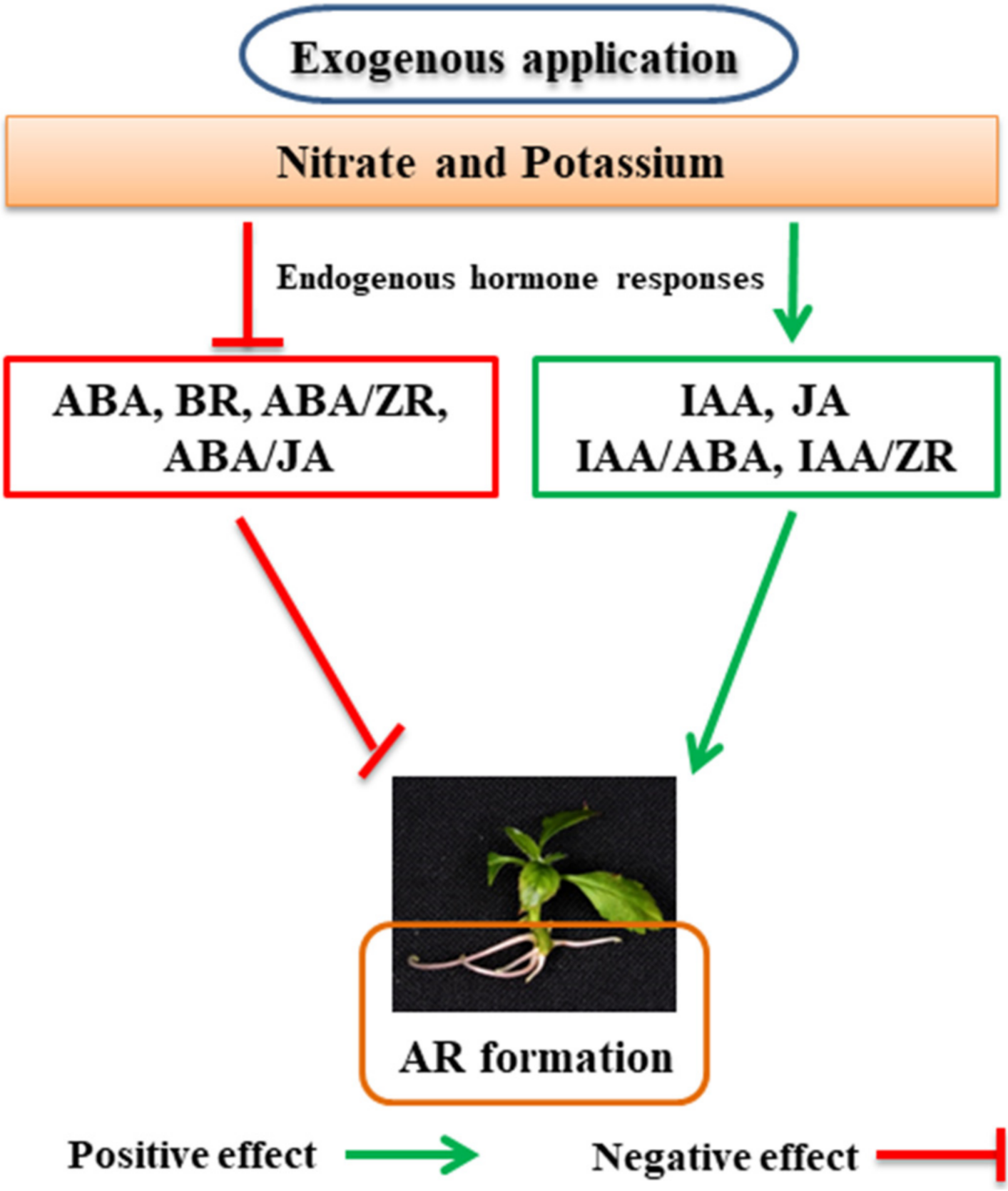

Figure 1. A suggested schematic diagram of how exogenous application of nitrate and potassium regulates different endogenous hormones during the formation of adventitious roots (ARs) in apples. Indole-3-acetic acid (IAA), zeatin ribosome (ZR), jasmonic acid (JA), abscisic acid (ABA), and brassinosteroid (BR). Ratios of hormone

content, such as ABA/ZR, ABA/JA, IAA/ZR, and IAA/ABA. The green color shows a positive effect, and the red indicates a negative effect.

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