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PHYTOHORMONES INTERPLAY ON ROOTING AND GROWTH OF MARUBAKAIDO (*Malus prunifolia* Borkh. var. ringo Asami) APPLE ROOTSTOCKS

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Graduate School of Horticulture

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千葉大学学位申請論文

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Abstract

Adventitious root (AR) formation is complex process which involves various hormonal signals at interplay. In this study, changes in gene transcription during AR formation in hardwood cuttings of Marubakaido apple (*Malus prunifolia* borkh. var. ringo asami) rootstock were examined by RNA-seq analysis. In line with above, 11 genes related to root development were identified as candidate genes for modulation of adventitious root formation. Among them, the induction of *ABC transporter G family member*-, *NO VEIN*-, and *Auxin transporter-like protein 2*-like genes, which are known to be associated with cellular auxin distribution and maintenance of root meristem niche, before the appearance of first visible ARs in *Marubakaido* as revealed by quantitative RT-PCR. In addition, we conducted comprehensive co-expression network and MAPMAN analysis and found a relationship between adventitious root formation and phytohormones such as cytokinin and abscisic acid (ABA). Finally, we also found the genes encoding receptor of plant peptide hormone, such as *Barely any meristem 3*-like gene (a receptor of plant peptide hormone CLAVATA45) and *phloem intercalated with xylem*-like gene (a receptor of the plant peptide hormone known as tracheary element differentiation inhibitory factor or TDIF), construed as the association of plant peptide hormone with adventitious root formation in *Marubakaido* apple rootstock. CLAVATA and TDIF are known plant peptide hormones related to root stem cell and vascular differentiation, respectively. Therefore, we conclude that the process of adventitious root formation such as the maintenance of stem cell and vascular differentiation in *Marubakaido* apple rootstock was controlled by complex plant hormone signaling, including auxin, cytokinin, ABA, and plant peptide hormone.

In the second study, we examined the dwarfing mechanism in apples, in one-year-old *Marubakaido* (invigorating) apple rootstock stools foliar-sprayed with 860 mg L^{-1} of paclobutrazol (PBZ) as a single application or without. M.9 apple rootstock (dwarf) was used as a positive control. The phytohormones were estimated in the shoot bark and sub-apical shoot and gene expression in the apices of terminal shoots. Evident responses to PBZ were observed a fortnight after treatment, as the shoot and internode lengths were suppressed significantly. Endogenous indole-3-acetic acid (IAA) increased in the PBZ

treatment, and the polar auxin transporter genes *MdPIN1* and *MdLAX1* and the biosynthesis gene *MdYUCCA10a* were upregulated along with the *MdARF2* gene. Additionally, PBZ increased the abscisic acid (ABA) concentration and the biosynthesis-related gene *MdNCED1* but repressed the degradation gene *MdCYP707A1*. The ABA transporter gene *MdAITb-like* was upregulated by PBZ. The concentrations of the gibberellins (GAs) GA_1 and GA_4 decreased in the PBZ-treated rootstocks. The GA transporter gene *MdNFP3.1-like* and the signaling gene *MdGID1b-like* were strongly downregulated by PBZ, whereas the catabolic gene *MdGA2OX2* was upregulated. PBZ treatment significantly reduced *trans*-zeatin (*t*Z) levels and downregulated the cytokinin biosynthesis gene *MdIPT6* but upregulated the *MdCKX7* degradation gene. Additionally, PBZ upregulated the cytokinin-related transporter genes *MdPUP7-like* and *MdPUP9-like*. Collectively, our results show that the physiological and molecular effect of PBZ52 was observed within two weeks, and this was indicated by the modulation of phytohormonal levels as well as transporter and other gene expression in Marubakaido apple rootstocks.

List of abbreviations

2-Oxo IAA, 2-Oxo indole-3-acetic acid

ABCB, ATP- binding cassette B family

ABCG, ATP- binding cassette G family

AFBs, Auxin binding F-box

AHKs, Arabidopsis histidine kinases

AHPs, Arabidopsis histidine phosphotransfer proteins

ARRs, Arabidopsis response regulators

AUX, Auxin influx transporter

AUX1/LAX, Auxin resistant/ like aux1 proteins

CYP735A, Cytochrome P459

DELLA, GA INSENSITIVE, REPRESSOR OF GA1-3, RGA-LIKE1 (RGL1), RGL2, and RGL3

DPA, Dihydrophaseic acid

ENT, Equilibrative nucleoside transporter

GA20OX, GA20 oxidase

GA29OX, GA29 oxidase

GA34OX, GA34 oxidase

GA3OX, GA3 oxidase

GID2, GIBBERELLIN-INSENSITIVE DWARF2

LOG, LONELY GUY

NFP, Nitrate transporter proteins (NRT1/PTR-Family)

PA, Phaseic acid

PM, Plasma membrane protein

PPCCs, Protein phosphatases 2Cs

PYL, PYR-like

PYR, Pyrabactin resistance

RCAR, Regulatory components of ABA receptor

SCF, Skp1-cullin-F box

SLY, SLEEPY

SNE, SNEEZY

SnRK2s, SUCROSE NON-FERMENTING-1-RELATED KINASES 2

SWEET, Sugars Will Eventually Be Exported Transporters

TIR, Transport inhibitory response

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CHAPTER 1

INTRODUCTION

1.1 General introduction

In modern agriculture, horticulture, and forestry, vegetative propagation by use of cuttings is such an appropriate mode of propagation and economically viable. In woody fruit trees such as apples, adventitious root formation remains the most critical factor during vegetative propagation of rootstocks (Webster, 1995). Adventitious root formation is an inherent genetic trait regulated by both environmental factors such as temperature, light and relative humidity, and endogenous factors for instance phytohormones, sugars, minerals, and other molecules. Hardwood cuttings once removed from the parent plant generally undergo various anatomical changes together with alterations in metabolic activity and gene expression during wound response and subsequent rooting initiation. Numerous authors have reported that adventitious root formation is a process which comprises of a series of independent phases associated with molecular, physiological and biochemical events (Kevers *et al.,* 1997; De Klerk *et al.,* 1999, Naija *et al.,* 2008; Pop *et al.,* 2011).

According to Li *et al.* (2009), Pacurar *et al.* (2014) and Guan *et al*. (2015), adventitious root formation consists of three different phases which include; Induction stage which is the earliest period without any visible histological events but involves molecular and biochemical events; the second is initiation stage characterized with the start of histological events like root primordial organization and occurrence of small cells with large nuclei with dense cytoplasm, the third is the expression/elongation/extension which encompasses formation of typical dome shaped structures, intra-stem growth and emergence of root primordia and each phase tightly regulated physiologically and genetically (Villacorta-Martín *et al.,* 2015). Incidentally, each phase of adventitious rooting has its own phytohormone requirements, hence this underscores their role during the rooting process (Weisman *et al*., 1988; Sorin *et al*., 2005; Rigal *et al*., 2012). To improve the rooting performance of Marubakaido apple rootstocks, identification of

molecular phytohormone mechanisms at interplay which regulate adventitious rooting is required.

On the other hand, in commercial apple (*Malus domestica* Borkh.) cultivation, tree height and planting density are critical. Consequently, the use of dwarfing rootstocks is widespread in the industry. Dwarfing rootstocks influence tree architecture, growth vigor, management practices and economic yield. Dwarfing rootstocks have been implicated in rootstock-induced growth restriction through the imbalance of carbohydrates, reduced cell growth and metabolism (Foster *et al.*, 2017), and contain higher proportion of bark and a reduced number of xylem cells with reduced diameters compared to invigorating rootstocks (Soumelido *et al*., 1994).

Relatedly, paclobutrazol (PBZ) has been found to have a major effect on plant height reduction, as well as the regulation of phytohormones levels (Edgerton, 1986; Zhu *et al.*, 2004; Fan *et al*., 2018). The growth inhibitory response of PBZ is attributed to the interference of gibberellin (GA) biosynthesis by repressing the oxidation of *ent*-kaurene to *ent*-kauronoic acid through inactivating cytochrome P450-dependent oxygenase (Miki *et al*., 1990). Notwithstanding, plant hormones are considered an essential component of regulating growth and development, and coordination of their biosynthesis, transport, degradation and regulation of signaling components is crucial in maintaining homeostasis in plant tissues (Sauer *et al*., 2013; Rademacher, 1997; Lange and Lange, 2006; Foster *et al*., 2017). However, a detailed molecular understanding of PBZ-mediated dwarfing as a result of the regulation of phytohormone biosynthesis, transport, inactivation, and signaling has been largely unexplored in apple rootstocks.

1.2 Objectives

1) To clarify the involvement of auxin, cytokinin, abscisic acid (ABA), and plant peptide hormone related genes during adventitious root formation in Marubakaido hardwood apple rootstock stem cuttings.
2) To investigate PBZ-mediated dwarfing in Marubakaido apple rootstock dwarfing through modulation of endogenous phytohormone, transporter genes as well as other gene expression.

1.3 Scope of studies

1) Comparison of the transcriptome profiles obtained before adventitious root formation and at the start of adventitious root formation by RNA-sequencing (RNA-seq) analysis in 2016/2017 season.
2) Gene expression patterns analysis throughout the process of adventitious root formation by reverse transcription quantitative PCR (RT-qPCR) using hardwood cuttings from the subsequent growing season 2017/2018.
3) Analysis of endogenous phytohormones; auxin, ABA, GA_1, GA_4, and *trans*-zeatin after PBZ application.
4) Analysis of genes related to biosynthesis, transport, inactivation, and signaling of auxin, ABA, GA_1, GA_4, and zeatin phytohormones.

CHAPTER 2

LITERATURE REVIEW

2.1 Apple rootstocks

Apple (*Malus domestica* Borhk.) is the most widely cultivated fruit tree and originates from central Asia (FAOSTAT, 2018). Apple cultivars are difficult to root and do not come from seed but are vegetatively propagated through cuttings; consequently, leading existence of a graft chimera of rootstock and scion. Rootstocks have been used as a means of propagating fruit trees for more than 2000 years to maintain the genotypes of desirable traits (Roach, 1985). Rootstocks have functions such as fixation and support, absorption, secretion and synthesis. Additionally, rootstocks have significant effects on the growth and development of scions, yield and fruit quality, and the resistance to abiotic and biotic stresses (Marini and Fazio, 2018). Apple rootstocks mainly comprise of seedling rootstocks (including rootstocks from wild, semi-wild, and cultivated apples and other genera), apomictic seedling rootstocks, and cloning-propagated rootstocks. However, it was not until 80 years ago, that most trees in commercial apple orchards around the world were propagated using seedling rootstocks. Apple trees from seedling rootstocks were easy to produce, inexpensive, freestanding and performed well on a wide range of soil types in most climates.

2.1.1 Origin and history of apple rootstocks in the world

At the onset of the 20th century, the first apple rootstock trial was established in the East Malling Station, UK. The scion cultivar ‘Lane’s Prince Albert’ was grafted on to 16 clonal rootstocks. Over the years of research, a set of rootstocks were identified capable of inducing a wide range of characters on to the scion and this allowed researchers to use dwarfing rootstocks as a tool to study various aspects of apple tree growth and physiology (Hatton, 1935). The nomenclature for EM rootstocks changed to M, and Arabic numerals were replaced with Roman numerals during 1970s. For instance, over the years Type IX changed to EM, to M IX and is currently M.9. In the 1950s to

1970s, the Malling stocks were heat-treated to eliminate viruses, with the designation East Malling-Long Ashton (EMLA), so the virus-free M.9 is designated M.9 EMLA.

After classification of the known rootstocks, a joint breeding program followed which was initiated by M.B crane of the John Innes Horticultural Institute at Merton and H.M. Tydeman at East Malling (Preston, 1956). The common problem of the woolly apple aphid (WAA; *Eriosoma lanigerum*), which was a common problem in North America and especially in New Zealand was tried solving by breeding the Malling-Merton (MM) rootstocks for resistance against the apple aphid. The East Malling clones were crossed with the WAA-resistant 'Northern Spy' and evaluated for WAA-resistance and propagation characteristics (Le Pelley, 1927). The 'Northern Spy' was crossed with susceptible cultivars and produced resistant cultivars. Of the resulting 3500 seedlings, 15 were selected and numbered Malling-Merton 101-115, which were later renamed MM.101-115. Other countries also crossed the M series with their native material and created various rootstocks, for example MAC series and CG series (USA), O3 (Canada), B series (the former Soviet Union), P series (Poland), and GM256 (China).

2.1.2 Origin and history of apple rootstocks in Japan

Ever since the introduction of apple to Japan in1872, various Rosacea plants had been examined for their essential role as apple rootstocks. The *Malus* species were exclusively chosen for practical use, specifically those native to Japan from old time, such as *M. prunifolia* var. *ringo* Asami, *M. Sieboldii* Rehd., *M. Sieboldii* var. *arborescens* Rehd., *M. baccata* var. *mandsltrica* Schneid., and others. From all the native species evaluated, Marubakaido (*M. prunifolia* var. ringo Asami) rootstock has been the most important standard rootstock, though being vigorous; it has a high rooting ability and adaptability to a wide range of soils (Lee *et al*., 1982; Sadamori and Murakami, 1952), resistance to drought and excessive moisture, no burrknot production, resistance to woolly apple aphid and collar rot, and its characters induce early fruiting and high productivity (Tsuchiya, 1979).

Around 1965, studies on high density planting using dwarfing rootstock, such as M.9, M.26, and MM. 106, commenced in Japan with the aim of replacing the medium

density Marubakaido rootstock (Fukuda, 1988). In 1986, approximately 20% of the apple orchards adopted the high-density planting with dwarfing rootstocks. However, because of the problems associated with the commonly used rootstock M.9 for example; difficulty in rooting ability, poor anchorage, susceptibility to low temperature injury and crown gall disease (Barritt, 1995; Webster, 1993; Howard, 1989); strategies for its efficient utilization as part of the rootstock is Japan had to be sought after. In Japan, the interstock system has been adopted for propagation, and this involves grafting dwarfing rootstocks on rooted cuttings of Marubakaido due to ease of rooting. The M.9, M.26 have been propagated by the interstock system as the main dwarfing rootstock in Japan (Koike and Tsukahara, 1987; Koike *et al*., 1993).

Thereafter, at Nagano Fruit Tree Experiment Station, M.9Nagano an apple chlorotic leaf spot virus (ACLSV) free clone was developed by heat treatment of original M.9 (Koike *et al*., 1993). A controlled cross of Marubakaido (Seishi) x M.9 was made in 1972 at the Morioka Branch, Fruit Tree Research Station, now reorganized as the Apple Research Center, NIFTS. Over years of screening and several field trials in experimental stations in each apple growing district in Japan, JM series apple rootstocks were released in 1996 and 1997 (Soejima *et al.*, 1998). JM.5 (very dwarfing), JM.1, JM.7, JM.8 (dwarfing like M.9 EMLA) and JM.2 (semi-dwarfing) are the recognized series in Japan. JM.7 has excellent rooting ability by hardwood cutting, resistance to crown rot (Phytophthora sp.), woolly apple aphid, apple stem pitting, and apple stem grooving virus and these characteristics have attracted growers' attention.

2.1.2 Propagation of apple rootstocks

The commonly used method for commercial and experimental propagation of homogenous horticultural plants and forest trees is a sexual vegetative propagation when apomictic seed is not available (Davis and Haissig, 1994). Utilization of cuttings for propagation is considered the most efficient and cost-effective method to produce large quantities of homogenous plants among the most common vegetative propagation methods (budding, grafting, layering, and cuttings) while simultaneously maintaining desirable genetic traits (Rout *et al*., 2006; Druege *et al*., 2019). In apple fruit trees, the

mode of propagation in different rootstocks is mainly asexual or vegetative propagation through hardwood cuttings and tissue culture (Wertheim and Webster, 2003).

Despite considerable worldwide effort over the past 50 years, regeneration of trees from hardwood cuttings from many economically important fruits, such as apple remains a difficult process. For instance, the commonly used dwarfing rootstocks such as M.9 and M.26 are difficult to root, thus making the fruit tree regeneration challenging to fulfil the desires of the growers. However, the Japanese native apple rootstock has a better rooting ability compared to the Malling series (Tsuchiya, 1979). Generally, apple propagation through cuttings depends on the ability to form new adventitious roots. Therefore, understanding on early adventitious root formation, especially physiological and transcriptomic regulation is paramount.

2.1.3 Adventitious root (AR) formation in Apples

2.1.3.1 Morphological and Anatomical changes during AR formation in apple

Studies of changes in the apple stem tissues have pointed out appearance of larger nuclei (nucleus swelling) and dense cytoplasm in cambial cells and adjacent phloem as the first histological evidence during adventitious root formation (Zhou *et al*., 1992; Naija *et al*., 2008; Guan *et al*., 2020). This is followed by occurrence of cell division in or near the cambium zone. Naija *et al.* (2008), reported that the first cell divisions took place in the phloem region near the cambium in MM 106 apple rootstocks. Additionally, during this stage rapid starch grain depletion is observed upon initiation of adventitious root formation in apple cuttings (Guan *et al*., 2020). The last stage of root formation comprises of the development of primordia into organized roots, where root primordia protrude among other tissues and roots emerge from cutting surface (Naija *et al*., 2008). According to anatomical studies in *Arabidopsis thaliana*, founder cell division results in the formation of AR primordia (ARP), which then develop into new roots (Della Rovere *et al*., 2013). In cuttings of M.9 apple rootstocks, Guan *et al*. (2020) observed noticeable lenticel enlargement at 72 h, and new ARs emerging from the lenticels were easily observed as small protrusions at 168 h (Fig. 1F, G). It is worth noting that the process of AR formation is controlled by many factors such as; juvenility; ontology;

species/genotype; various environmental conditions, such as extreme temperatures and salt stress; the content of H_2O_2, NO, and Ca^{2+}; and endogenous and exogenous applications of plant hormones (Dawood *et al*., 2016).

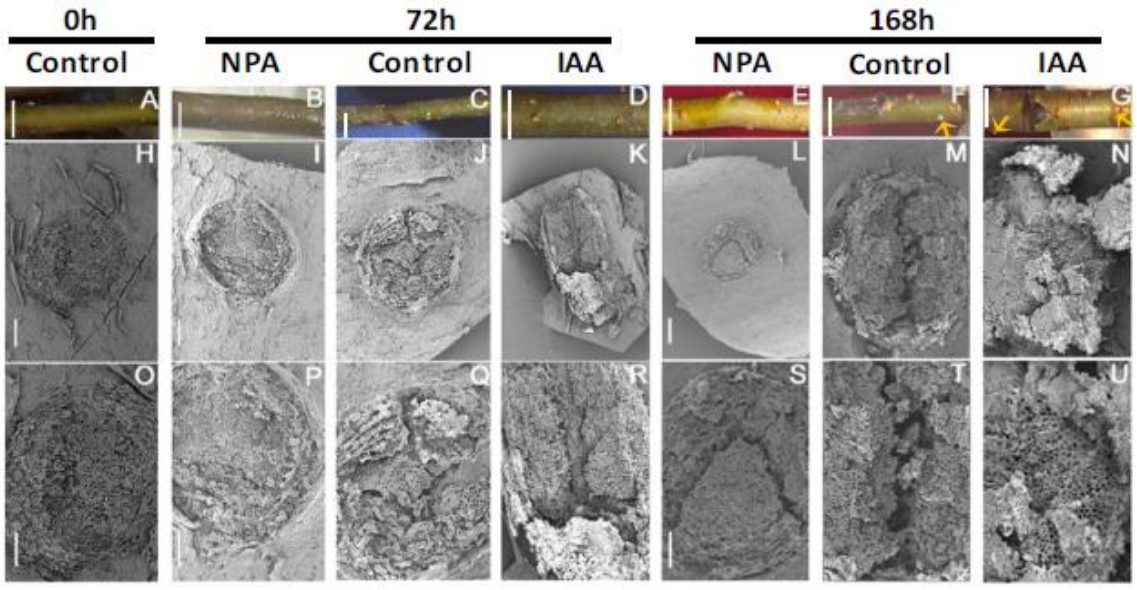


Fig. 1 Morphological changes in lenticels during adventitious roots upon *N*-1-naphthylphthalamic acid (NPA) and IAA treatments. Apple cuttings were cultured in Hoagland's solution and sampled every 24 hours (h) from 0 to 168 h. Three distinctive development phases were observed at 0, 72, and 168 h. A–G Physical appearance of submerged cuttings. Arrows point to the origination of new ARs. H–U SEM micrographs of lenticels at different AR developmental phases; Bars: A–G = 0.4 cm, H–U = 100 μm. Bars I = J = K; L =M= N; P= Q = R; and S = T = U. (Source: Guan *et al*., 2020).

Generally, in woody perennial plants depending on the species, two mechanisms of adventitious root formation exist; first, the AR founder cells initiate in the stem but remain dormant until the induction of AR formation by environmental conditions (Guan *et al*., 2015) and second, AR founder cells initiate de novo from cells, such as phloem or xylem parenchyma cells, within or adjacent to vascular tissues, such as cells of the interfascicular cambium or the phloem/ cambium junction (De Klerk *et al*., 1995; Naija *et al*., 2008; Guan *et al*., 2015). In plants, ARs can initiate from internodes, callus formed at

the base of cuttings, or the hypocotyl of herbaceous plants (Lovell and White, 1986; Li *et al.*, 2009). Recent studies by Guan *et al*. (2020) revealed that in apple, ARs emerge from the lenticels, in which large intracellular spaces allow for gaseous exchange in the stems. Furthermore, at the initial stage (at 0 h), the cells underlying the lenticels in the interfascicular cambium adjacent to vascular tissues had no observable meristematic activity, whereas the founder cells had already undergone numerous cell divisions 72–168 h post-cutting (Fig. 2). Hence, this justified AR formation in M.9 apple rootstock cuttings occurs via the second mechanism.

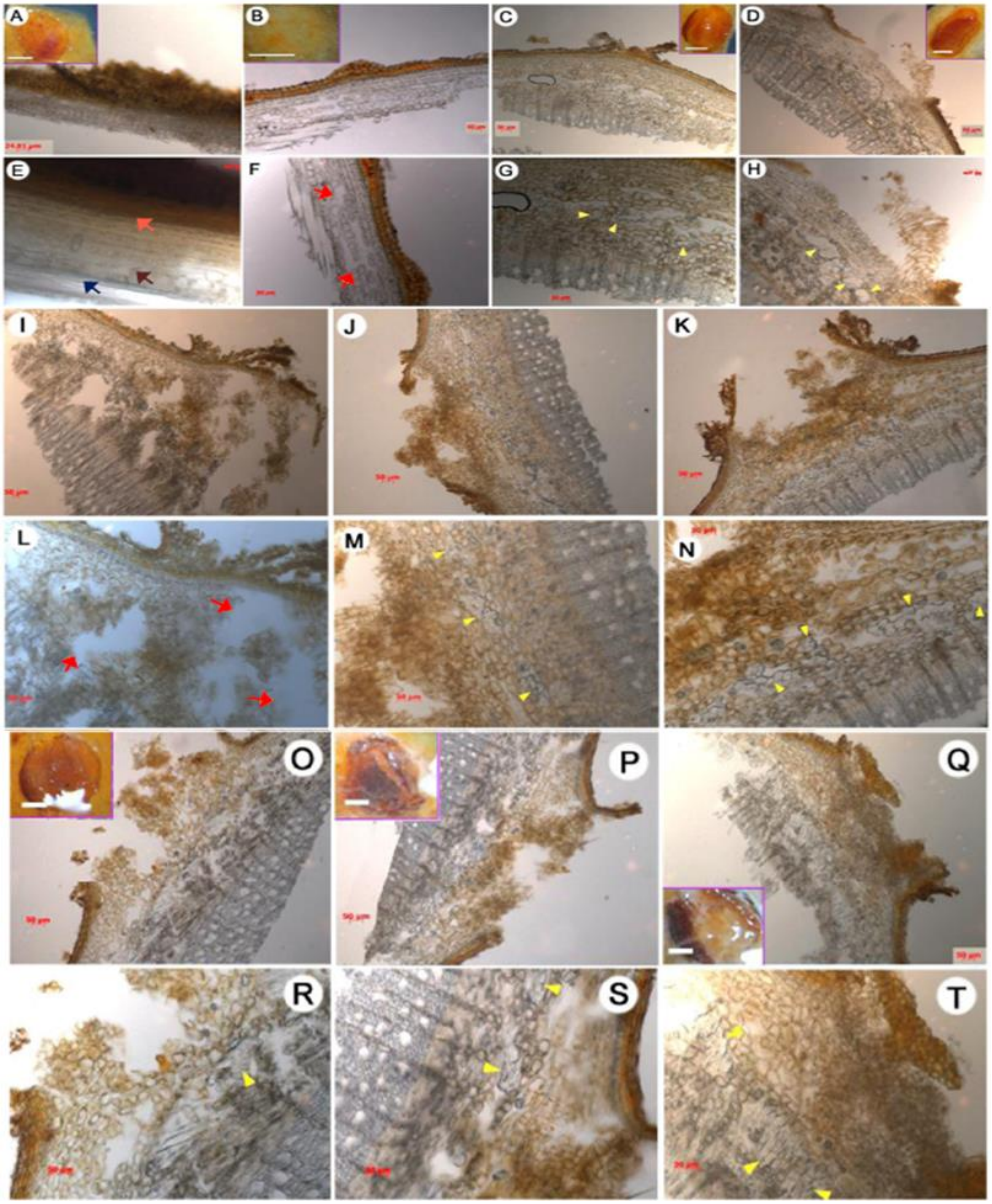


Fig. 2 Anatomy of lenticels during the rooting of apple cuttings. Each lenticel was observed under a stereomicroscope at 0 h (A, E), 72 h (B–D, F–H), 120 h (I–N), and 168 h (O–T). The treatments are as follows: control (A, E, C, G, J, M, P, S), NPA (B, F, I, L, O, R), and IAA (D, H, K, N, Q, T). Scale bars, 50 μm. Inset scale bars, A–D = O–Q = 0.5 mm. Arrows: yellow, proliferated founder cells; pink, epidermis; brown, parenchyma cells located in the interfascicular cambium; dark blue, vascular tissues; red, cavities. (Source: Guan *et al*., 2020).

2.1.3.2 Phases involved in AR formation in apple

The process of AR formation is divided into three distinct phases; induction, initiation, and extension, which ultimately lead to new visible root systems. In apple rootstocks, the process as well involves three phases mentioned earlier characterized by cellular changes illustrated by anatomical observations: induction (0 –72 h), initiation (72 –120 h), and extension (120 –168 h) (Guan *et al*., 2020 Fig. 2).

Jasik and De Klerk (1997) demonstrated that establishment of primordium initial cells in cambium adjacent to cortex and pith begins cellular differentiation. Using M.9 apple rootstock cuttings treated with IAA, NPA and untreated control, Guan *et al*. (2020) showed that in the untreated control and IAA- treated cuttings, the induction phase was at 72 h and this was characterized by increased founder cells with dense cytoplasm and shallow nuclei (Fig. 2G, H), as well as hydrolysis of starch grains. On the other hand, several starch grains remained in the chloroplasts of NPA-treated cuttings at 72 h, and this suggested that NPA inhibited starch hydrolysis during AR formation; meanwhile, IAA enhanced the conversion of starch into 'cash energy' required for AR induction. In the basal part of a stem cutting, starch grains are associated with accumulation of carbohydrates and auxin (Agullo-Anton *et al*., 2014).

AR initiation starts with the division and elongation of many clustered founder cells that filled the cavity opened by lenticel splitting in M.9 apple rootstock cuttings (Fig 2M, N) (Guan *et al*., 2020). To the contrary, NPA treated apple cuttings observed severely impaired founder cells division (Fig. 2l). Primordial founder cell division occurred at this phase upon induction of AR formation. Generally, the initiation phase is divided into five steps of primordium development as described by Itoh *et al.* (2005). The first step involves establishment of epidermis-endodermis, central cylinder and root cap initials. Division of the AR primordium initial cells anticlinically and periclinically occurs to form primordial tissues. The second step is the differentiation of the AR epidermis and endodermis via periclinal divisions, root cap anticlinal divisions, and periclinal divisions in the central cylinder. During the third step, endordermal differentiation and periclinal divisions form cortical layers. In the fourth step, establishment of fundamental organization where root cap initial cells form the columella by periclinal division and the large meta-xylem vessel in the central region of the stele

occurs. The last step involves cell elongation and vacuolation occur in the basal region of the stele cells, and finally the cortex and the root apex emerge from the stem epidermis.

The extension phase is characterized by high mitotic activity which corresponds with increased percentage of elongated cells. IAA treatment of cuttings has been implicated to cause early occurrence of elongated AR fonder cells in apple (Guan *et al*., 2020). In this phase, ARs continue intra-stem growth through the epidermis and emerge as a result of visible programmed cell death (PCD) (Itoh *et al*., 2005; Steffens and Sauter, 2009). Ethylene controls induction of cell death during AR emergence which is dependent on epidermal cell identity (Steffens *et al*., 2012). Additionally, ethylene regulates AR formation during water logging and stimulates the PCD process during the extension phase of AR formation.

2.2 Phytohormones and molecular physiology during AR formation

2.2.1 Auxin

Auxin is considered a major endogenous hormone that plays a crucial role during the process of AR formation (Wiesman *et al*., 1988) and changes in endogenous auxin concentrations are associated with the physiological stages of rooting (Heloir *et al*., 1996). IAA, NAA, and IBA has been reported to hasten AR formation in hardwood cuttings with IBA having a superior positive effect compared to the other two phytohormones (Wei at al., 2014). Generally, during the process of AR formation, free endogenous IAA levels portray a transient increase in the induction phase, pass through a minimum at the initiation step and again increase in the extension phase (Bellamine *et al*., 1998).

A detailed understanding of AR formation has been greatly accelerated by RNA-seq analysis including expression of different genes in excised tissues obtained at different developmental stages (Wei *et al*., 2014; Liu *et al*., 2009). In *Pinus taeda* stem cuttings, the capacity of auxin to trigger gene expression has been suggested as an early and critical point in AR competence (Greenwood *et al*., 2001). Transcriptome analyses of tea cuttings and mung seedlings in response to IBA portrayed existence of several IBA-regulated genes associated with adventitious rooting, including genes encoding proteins involved in auxin signaling, and cellular influx and efflux (Wei *et al*., 2014; Li *et al*., 2016). Consequently, polar auxin transport (PAT) is orchestrated through action of PIN-

FORMED (PIN) efflux carriers, the AUXIN RESISTANT 1/LIKE AUX1 (AUX/LAX) influx carriers, and MULTIDRUG RESISTANCE/P-GLYCOPROTEIN (PGP) class of ATP-Binding Cassette (ABC) (PGP/ABCB) transporters, has been demonstrated to be essential during AR formation process in *Arabidopsis* seedlings (Noh *et al*., 2001; Geisler *et al*., 2005; Mravec *et al*., 2008).

Molecular studies have shown that during long-distance transport of IBA, transporters such as ABCG36 and ABCG37 appear to efflux IBA but not AUX1, PIN2, PIN7, ABCB1 (ATP BINDING CASSETTE TYPE B 1) which only transport IAA (Strader and Bartel, 2009). This study result suggested an independent transport system for movement of inactive precursor to its specific site of action and points to differential roles of IAA and IBA in promotion of AR formation. Incidentally, Růžička *et al.* (2010) reported that ABCG37 auxin-efflux carrier mediates root auxin homeostasis and development in the root tip of Arabidopsis.

It has been previously demonstrated that cytokinin downregulates the expression of PIN auxin efflux carriers in the root meristem thus modulates the distribution of auxin (Zhang *et al*., 2011; Ruzicka *et al*., 2009). Zhang *et al*. (2013) showed that the direct repression of *LAX2* expression in *Arabidopsis* partially accounted for the negative effect of cytokinin on the auxin maximum in the quiescent center (QC), and thus regulated the mitotic activity at the QC in the root apical meristem.

NO VEIN (*NOV*) gene encodes a nuclear factor required for cotyledon outgrowth and separation, leaf vascular development, and stem cell maintenance in the root apical meristem (RAM) (Tsugeki *et al*., 2009). *NOV* genetically interacts with GNOM, a gene encoding an Auxin Response Factor (ARF) GDP/GTP exchange factor that plays a role in proper localization of PIN proteins (Kleine-Vehn *et al.*, 2008), and is essential for expression of Auxin-efflux carrier, PIN2, 3, 4, and 7 which modulate cell vascular differentiation and lateral root formation in *Arabidopsis* root (Tsugeki *et al*., 2009).

2.2.2 Abscisic acid (ABA)

The phytohormone abscisic acid (ABA) plays a major role in regulating root growth. The mechanism by which ABA influences root growth is complex, as it involves interactions and signaling through other phytohormones. Růžička *et al.* (2007) and

Swarup *et al.* (2007) suggested that ethylene limits root growth by its effect on auxin, with auxin acting as the primary inhibitor of root growth. High levels of ABA suppress ethylene synthesis, which in turn reduces auxin transport and biosynthesis in the root tip, removing this primary inhibitor of root growth, and thereby promoting root growth. Recently, McAdam *et al.* (2016), confirmed that ABA acted as a shoot derived signal which had a profound influence over root growth in well-watered plants.

Additionally, ABA functions as a promoter of postemergence of lateral roots by enhancing auxin-dependent transcription through the action of the ABA receptor PYR1-like8 (PYL8) (Zhao e*t al*., 2014). PYL8 has been demonstrated to interact directly with the transcription factor MYB73 during lateral root formation. Taken together, the abovementioned reports suggest ABA participates in root formation.

2.2.3 Cytokinin

At the induction phase during AR formation, cytokinin has an inhibitory effect but can play a promotive effect in the first 24 h, when it starts to drive cell cycle movement, resulting in mitotic processes (De Klerk, 2002). In *E. globulus*, Corrêa *et al*. (2005) reported that kinetin inhibited AR formation if present during the induction phase.

Conversely, in *Populus* the cytokinin type-B response regulator *PtRR13*, a transcription factor that acts as positive regulator in the cytokinin signaling pathway, has been demonstrated to negatively regulate AR formation; but inactivation of *PtRR13* upon cutting severance due to the removal of root sources of cytokinin, alleviates AR formation inhibition by allowing basipetally transported auxin to accumulate at the cutting base, thereby promoting AR formation (Ramírez-Carvajal *et al*., 2009). Relatedly, in radish, Hoang *et al.* (2020) reported the involvement of some stress-responsive genes like *ERF105* and *WRKY33* in root secondary growth. However, further studies are required to clearly elucidate the role of these genes in AR formation in hardwood cuttings.

Furthermore, induction of increased transcript levels of genes homologous to *SCARECROW* (*SCR*) (SCR-like or SCL) rooting-competent cuttings of *Pinus radiata* and *Castanea sativa* species was observed within the first 24 h of root induction process when cell reorganization takes place, before establishment of AR primordium (Sanchez *et al*.,

2007). During the establishment of root meristem in *Arabidopsis*, Heidstra *et al*. (2004) reported that SCR which is a member of GRAS family of transcription factors (TFs) controlled cell division, differentiation, and cell homeostasis.

2.2.3 Plant peptide hormone

Peptide signaling in plants likely occupies a pivotal role because receptor and ligand genes are relatively abundant in their genomes (De Smet *et al*., 2009). Membrane integral receptor kinases (RKs) are necessary for the plant intercellular signaling network as they perceive these secreted peptides and allow plants to respond to various external and internal cues to regulate their growth and development. The leucine-rich repeat receptor kinases (LRR-RKs) family perceive different peptide ligands, hormones, and can control multiple cellular processes such as cell division, proliferation, differentiation, stem cell balance (Torii, 2004).

A few examples of ligand-receptor pairs acting in a specific role during plant developmental context have been identified. A typical example is the leucine-rich repeat receptor-like kinase (LRR-RLK) CLAVATA 1 (CLV1), whose activity is modulated non–cell autonomously by its dodecapeptide ligand CLV3 to maintain stem cell niche homeostasis in the shoot meristem. The CLV1 and CLV3 belong to larger clades of respective homologs, the CLV-like LRR-RLKs and the CLV3/Endosperm SURROUNDING REGION (CLE) peptides (Clark *et al*., 1997; Ogawa *et al*., 2008). Notwithstanding, *Arabidopsis* CRINKLY 4 (ACR4)–CLE40 and plant peptide-containing sulfated tyrosine (PSY1) have been reported to participate as signals in controlling root stem cell system homeostasis (Stahl *et al*., 2009). PSY1 has been reported to be required for the activity of ROOT MERISTEM GROWTH FACTOR1, another peptide hormone controlling root growth (Matsuzaki *et al*., 2010).

Also, Depuydt *et al.* (2013) demonstrated that CLV-like LRR-RLK BARELY ANY MERISTEM 3 (BAM3) and its putative ligand CLE45 are involved in guiding progression of protophloem development in the *Arabidopsis* primary root meristem, which determines the meristem's postembryonic growth capacity. Another receptor kinase PHLOEM INTERCALATED WITH XYLEM (PXY) has been demonstrated to play a crucial role in maintaining polarity within vascular meristems (Fisher and Turner,

2007). Additionally, Hohmann *et al*. (2017) demonstrated that the TRACHEARY ELEMENT DIFFERENTIATION FACTOR RECEPTOR/PHLOEM INTERCALATED WITH XYLEM (TDR/PXY) sense small, post translationally modified CLE peptide hormones to maintain plant cell populations in the shoot and in the root. In general, the detailed role of peptide hormones during adventitious root formation process in hardwood cuttings is still at its infancy, thus calling for further studies.

2.3 Paclobutrazol as a growth retardant

Paclobutrazol [(2RS, 3RS)-1-(4-chlorophenyl)-4, 4-dimethyl-2-(1H-1, 2, 4-trizol-1-yl)-pentan-3-ol] (PBZ), is one of the members of triazole family having a growth retarding property (Fig. 2.1). PBZ has been reported to inhibit vegetative growth in a wide variety of fruit crops such as apple (Greene, 1991), pear (Asin *et al*., 2007) avocado (Wolstenholme *et al*., 1990) and mango (Upetria *et al*., 2013). Also, PBZ application to fruit crops has been considered important in improving productivity through enhanced flower induction and limits competition between fruit production and vegetative production (Meilan, 1997; Rademacher, 2018). The mode of application of PBZ to fruit trees is by soil drench, trunk soil-line pour, trunk injection and foliar spay (Liyembani and Taylor, 1989).

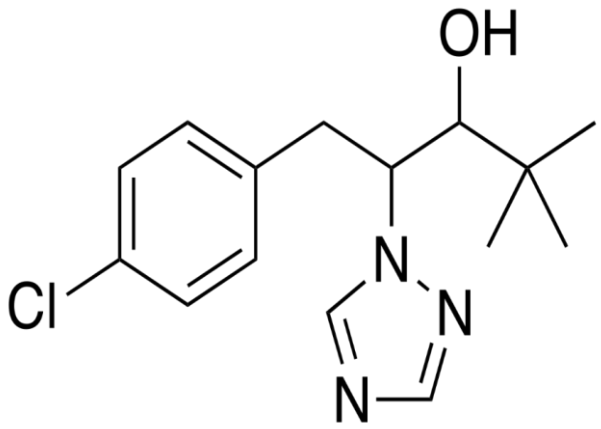


Fig. 2.1 Molecular structure of paclobutrazol (MW 293.79 g/mol; Chemical formula—C15H20ClN3O)

2.3.1 Paclobutrazol regulation of plant growth through phytohormone modulation

The growth restrictive effect of PBZ is mediated by modulating the levels of endogenous phytohormones such as gibberellins (GAs), abscisic acid (ABA), auxin (indole-3-acetic acid (IAA)), and cytokinins (Fletcher and Hofstra, 2000).

Paclobutrazol interferes with gibberellin biosynthesis by inhibiting the oxidation of ent-kaurene to ent-kauronic acid through inactivating cytochrome P450-dependent oxygenase (Zhu *et al*., 2004; Fig. 4). Generally, GAs and auxins act synergistically in cell and shoot elongation (Ross *et al*., 2003). Previous investigations have implicated PBZ to suppress endogenous GAs and auxin concentrations in plants. GAs and auxin reductions were considered primary mechanism by which PBZ retards vegetative growth in mango tree (Upreti *et al*., 2013). In one-year-old olive plants (*Olea europea* cv. Arbosana), endogenous GAs as well as auxin levels were significantly suppressed by PBZ application and this resulted in growth retardation (Ajmi *et al*., 2020).

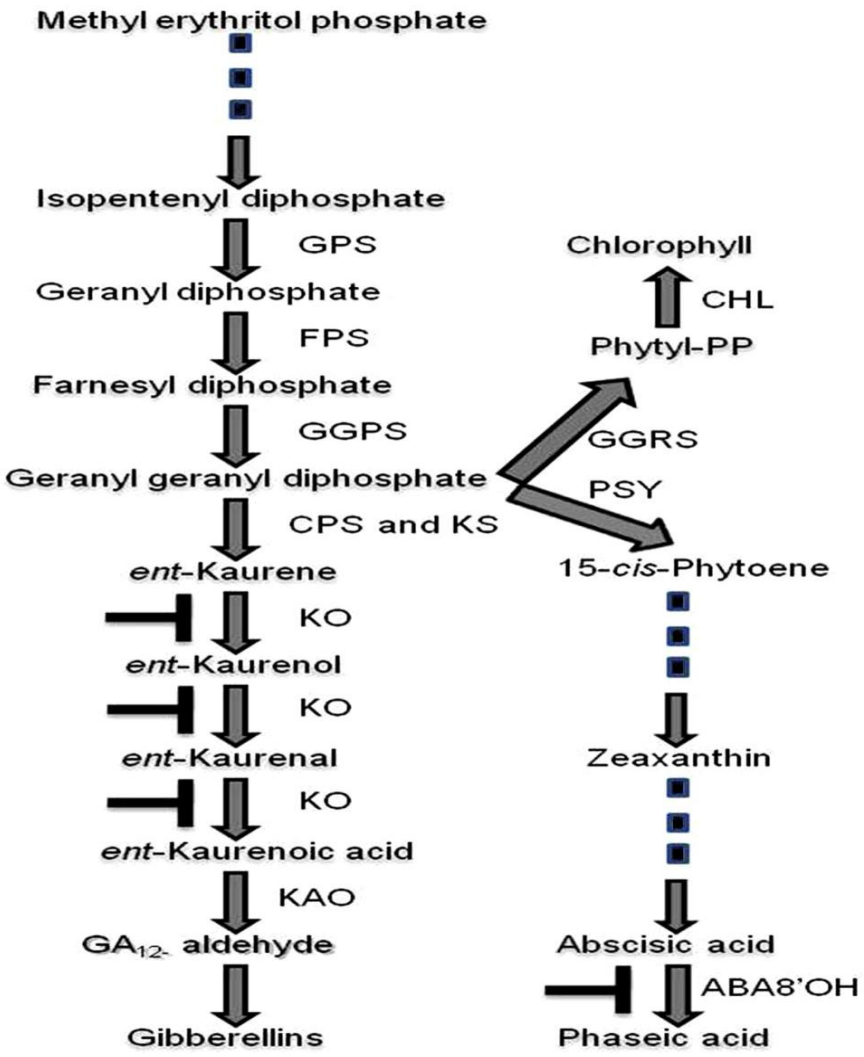


Fig. 2.2 Terpenoid pathway for biosynthesis of gibberellins, abscisic acid, phytol, and steroids, and path for degradation of abscisic acid. Steps blocked by paclobutrazol indicated with ⊣. Geranyl diphosphate synthase (GPS), Farnesyl diphosphate synthase (FPS), Geranyl geranyl diphosphate synthase (GGPS), *ent*-copalyl-diphosphate synthase (CPS), *ent*-kaurene synthase (KS), *ent*-kaurene oxidase (KO), *ent*-kaurenoic acid oxidase (KAO), Geranyl geranyl reductase (GGRS), Chlorophyll synthase (CHL) and Phytoene synthase (PSY) are the enzymes involved in the terpenoid pathway. ABA 8'- hydroxylase (ABA 8'OH) involved in the enzymatic degradation of ABA into Phaseic acid. KO, KAO and ABA 8'OH are the enzymes inhibited upon PBZ application (Source; Soumya *et al*., 2017).

Paclobutrazol is well documented to affect the biosynthesis of abscisic acid synthesized via the terpenoid pathway (Fig. 2.2) and regulates the synthesis of other endogenous phytohormones such as cytokinin and auxin. Paclubotrazol enhances the production of ABA just like it promotes the production of phytol. Rademacher (1997), demonstrated that when the synthesis of GAs is blocked, more precursors in the terpenoid pathway are accumulated and shunted to promote the synthesis of ABA. Paclobutrazol has been reported to inhibit ABA catabolism thus enhancing its concentrations (Marshall *et al*., 2000). Aly and Latif (2011) found that Endogenous ABA levels were increased in the leaves of wheat (*Triticum aestivum* L.) because of PBZ application. Additionally, it has also been shown that PBZ application increases ABA and cytokinin (zeatin (Z), zeatin riboside (ZR), and dihydro zeatin riboside (DHZR)) in mango trees (Upreti *et al*., 2013). Furthermore, Fletcher *et al*. (2000) suggested that triazoles stimulate cytokinin synthesis. Paclobutrazol considerably increased zeatin and zeatin riboside concentrations in young apple trees (Zhu *et al*., 2004). However, some studies have reported opposite trends with regards to the effect of PBZ on endogenous cytokinin in plants and this requires further studies clarify the discrepancy.

Murti *et al*. (2001) suggested that since plant growth and development is controlled by an interplay among endogenous phytohormones and that concentrations of one hormone may influence others; the growth restrictive effect of PBZ may be clarified by changes of different phytohormones unlike a single hormone. Hence, assessment of interplay of various phytohormones is required to clarify PBZ-mediated dwarfing during plant growth.

2.4 Phytohormones involvement in plant growth

Plant hormones are considered an essential component of regulating growth and development, and coordination of their biosynthesis, transport, degradation, and regulation of signaling components is crucial in maintaining homeostasis in plant tissues (Sauer *et al*., 2013; Foster *et al*., 2017).

2.4.1 Gibberellins (GAs)

Gibberellins (GAs) are phytohormones involved in several processes in plant growth and development. The growth responses to GAs include seed germination (Kassen *et al*., 1989), shoot elongation (Eriksson *et al*., 2006), leaf expansion (Serrani *et al*., 2007), root elongation (Gou *et al*., 2010), and flowering (Zhang *et al*., 2019). Both GA biosynthesis and catabolism are required to maintain GA homeostasis in plant tissues. It is well documented that suppression of biosynthesis (feedback regulation) is mediated by decreased expression of *GA20OX* and *GA3OX*, GA biosynthetic genes, while increased catabolism (Feedforward regulation) occurs as a result of upregulation of *GA2OX*, GA catabolic genes (Thomas *et al*., 1999; Yamaguchi *et al*., 2000).

According to the previously stated literature, the binding of GA to GID1-type receptors promotes interaction between GID1 and a DELLA protein leading to the destruction of DELLA and lifting of its inhibition GA responses (Eckardt, 2007). NPF transporter family members have reportedly been involved in GA transport across the plasma membrane (Corratge-Faillie and Lacombe, 2017; Park *et al*., 2017). GA transporting activity of NFP3.1 has been examined in *Xenopus oocytes* using GA-FIs and GAs coupled with high performance liquid chromatography- mass spectrophotometer (LC-MS) analyses; the observation was that NFP3.1 imports GA_4 and to a lesser extent GA_1 and GA_3 in a pH-dependent manner (Tal *et al*., 2016). Recently, Zhang *et al*. (2019) also reported expression of two GA transporter-like genes related to NPF3.1 in apple.

2.4.2 Auxin (Indole-3-acetic acid (IAA))

Responses to local indole-3-acetic acid (IAA) concentration is dependent on local IAA concentrations because of combination of biosynthesis, degradation, and transport (Grones and Friml, 2015). In Arabidopsis, overexpression of *YUCCA* genes leads to auxin overproduction while loss-of-function *yucca* mutants display developmental defects (Kim *et al*., 2007). Auxin transporter proteins located on the plasma membrane provide active movement of IAA in a directional cell-to-cell movement called polar auxin transport (Grones and Friml, 2015) mediated by three classes of proteins: the

AUX1/LAX family, the PIN-FORMED (PIN) family and P-glycoprotein ABC subfamily proteins (Petrasek and Friml, 2009).

Overexpression of *ZmPIN1a* efflux transporter genes in maize transgenic lines markedly reduced height via transport of more auxins from the shoot apex to the root meristem (Li *et al*., 2018). Increased polar auxin transport with elevated endogenous auxin levels in the basal end of citrus epicotyl cuttings occurred to be inhibitory for in vitro shoot organogenesis (Hu *et al*., 2017). Also, Auxin response factors (ARFs) are considered components that confer specificity to auxin response through selection of target genes as transcription factors. ARFs regulate auxin-mediated transcriptional activation/repression (Li *et al*., 2016). Relatedly, it is documented that *ARF2* is a general repressor of cell division and organ growth (Schruff *et al*., 2005), including cell elongation (Li *et al*., 2004).

2.4.3 Abscisic acid (ABA)

The biosynthesis of abscisic acid (ABA) is mainly regulated by the rate limiting step enzyme *9-cis*-epoxycarotenoid dioxygenase *NCED* (Lefebvre *et al*., 2006) while degradation is catalyzed by cytochrome P450 monooxygenase *CYP707A* (Kushiro *et al*., 2004). Growth cessation in poplar apical buds was observed after ABA levels peaked and this was concomitant with induction of genes related to ABA biosynthesis (*ABA1, NCED3, and ABA2*) (Ruttink *et al*., 2007). Meanwhile, to transfer ABA across the plasma membrane from the sites of synthesis to sites of action ABA transporters are required (Kang *et al*., 2015; Kumori *et al*., 2010).

Several members of the NITRATE TRANSPORT1/PEPTIDE TRANSPORT FAMILY (NFP) have been implicated to show ABA transport activity; for example, loss-of-function mutants of NITRATE TRANSPORT1/PEPTIDE TRANSPORT FAMILY (NPF) 4.6/ NRT1.2/ABA-IMPORTING TRANSPORTER (AIT) 1 (*NFP4.6/AIT1*) became less sensitive to exogenous ABA application, thus revealing a decreased inhibition of seed germination (Kanno *et al*., 2012).

2.4.4 Cytokinin

Cytokinins accumulate in the above ground plant organs during plant growth and development; however, the rate of importation of roots-synthesized cytokinin, locally biosynthesized cytokinin, and local catabolism is of relevance (Kudo *et al*., 2010). Miyawaki *et al*. (2006) reported that the production of bioactive cytokinin through isopentenylation of adenosine phosphate by isopentenyl transferases (IPT) is the key step in cytokinin biosynthesis. Nine *IPT*s are encoded in *Arabidopsis* genome, of which *IPT1*, *IPT3-8* specifically use ATP and ADP as isoprenoid acceptors (Kakimoto, 2001). Moreover, retarded growth and decreased levels of N^6-(Δ^2-isopentenyl) adenine (iP), *t*-Zeatin and derivatives was displayed by the quadruple mutant *ipt1,3,5,7*. In relation to translocation, the root-borne *t*-zeatin type and shoot-borne iP type cytokinin move through the xylem and phloem acropetally and basipetally respectively (Hirose *et al*., 2008; Kudo *et al*., 2010).

Cytokinin membrane transporters have been recognized and implicated in transmembrane transport and intercellular translocation (Ko *et al*., 2014; Hirose *et al*., 2008; Zürcher *et al*., 2016). Among the transporters reported, several members of purine permease (*PUP*) family have been suggested to be involved in cytokinin signal perception (Liu *et al*., 2019). The *AtPUP1*, *AtPUP2*, and *AtPUP14* have showed direct cytokinin uptake activity in yeast expression system. The *AtPUP14* for instance, is suggested to be a protein mainly located at the plasma membrane which imports *t*-Zeatin into cells thus depleting apoplastic cytokinin pools and inhibits cytokinin signal perception by membrane-localized receptors (Zürcher *et al*., 2016; Zürcher and Muller, 2016).

The irreversible inactivation of cytokinin is catalyzed by cytokinin oxidase/dehydrogenase (CKX) enzymes (Galuszka *et al*., 2007). Among the seven *CKX* family members (*CKX1- CKX7*) in Arabidopsis whose products are contained in the vacuole and extracellular space, *CKX7* is the only member represented in the cytosol (Köllmer *et al*., 2014). Werner *et al*. (2006) observed stout phenotypes in Arabidopsis seedlings that were overexpressing *CKXs* genes and had reduced active cytokinin levels. In apple dwarfing rootstocks, *CKX7* genes were upregulated (Foster *et al*., 2017). But in rice repression of *OsCKX2* genes increased cytokinin levels (Ashikari *et al*., 2005). The

expression pattern of *CKX* genes from different species mentioned above connotes that they are specifically regulated, and it may infer that *CKX*s are central regulators controlling the pools of active cytokinin (Werner *et al*. 2006).

CHAPTER 3

Association of auxin, cytokinin, abscisic acid, and plant peptide response genes during adventitious root formation in Marubakaido (*Malus prunifolia* Borkh. var. ringo Asami) apple rootstock

3.1 Introduction

Plant regeneration is an important trait in crop propagation in modern agriculture. In woody fruit tree species, including apple (*Malus* spp.), propagation by hardwood cuttings is a widely accepted technique for the vegetative propagation of rootstock cultivar (Christensen et al., 1980; Webster, 1995). *De novo* root organogenesis, in which adventitious roots are formed from hardwood cuttings, occurs in three overlapping phases: (1) dedifferentiation of previously committed cells; (2) formation of internal root meristem; and (3) growth of root primordia and elongation of root from stem (Ribeiro *et al.*, 2016). Altamura (1996) reported the emergence of adventitious root not only in callus on basal cut surface but also in bud and lenticel portions. The adventitious root from bud and lenticel portions is formed by small round bodies named root primordia in the cortex above buds. Actually, M.9 and M.26 apples, which are rootstocks, did not produce adventitious roots by hardwood cuttings in the open field and did not appear to form root primordia in both the callus on cut surface and the cortex above buds (Fukuda *et al.*, 1988). Therefore, in apple, the existence of root primordia is considered to correspond with successful adventitious root formation.

The phytohormone auxin is well studied as a critical exogenous factor in the induction of adventitious root formation in various woody plants, including apple (de Klerk *et al*., 1999; Rout *et al.*, 1999; Dobránszki and da Silva, 2010). In the last 80 years, synthetic auxins, such as indole-3-butyric acid (IBA), have been used as an industrial enhancer for adventitious rooting of hardwood cuttings. In Marubakaido (*Malus prunifolia* Borkh. var. ringo Asami), which is one of the popular apple rootstocks in Japan and is easily propagated by hardwood cuttings, the improvement of adventitious root formation by the application of IBA on the cut surface of hardwood cuttings was reported (Suriyapananot, 1990). Similarly, the leaf cuttings of *M. xiaojinensis*, a native

apple rootstock in China, acquired rooting competence via IBA treatment (Xiao *et al*., 2014). The molecular background of adventitious root formation induced by auxin-responsive genes, such as *adventitious rootless1*, *auxin response factors*, and micro RNA *miR156*, was investigated by using a transgenic approach in rice, *Arabidopsis*, and apple (*M. xiaojinensis*), respectively (Liu *et al*., 2005; Sorin *et al*., 2005; Gutierrez *et al*., 2009; Xiao *et al*., 2014). On the other hand, in M.9 apple rootstock, although callus development on the basal cut surface was found, no root developed even on IBA treatment (Fukuda *et al*., 1988), suggesting that the effect of auxin on adventitious root formation in apple may be limited.

Recently, another factor modulating adventitious root formation was reported in *Arabidopsis*, *PHABLOSA*, which encodes homeobox superfamily proteins, directly activated by cytokinin signaling and stimulated root development (Ioio *et al*., 2012). Advanced genetic analysis using a next-generation sequencer revealed the complex genetic orchestration of transcription factor families, including *MYB*, *APETALA2*/*ethylene response factor* (*AP2*/*ERF*), and *GRAS* family genes, in *Panax ginseng* (Subramaniyam *et al*., 2014). In white mulberry (*Morus alba*), R2R3-type *MYB* gene was activated in the cortex of hardwood cuttings during adventitious root formation (Du *et al*., 2017). Through the transgenic approach using *Arabidopsis*, Song *et al*. (2010) demonstrated that rice *WRKY23* disturbed lateral root formation and was involved in abscisic acid (ABA) response. Similarly, *PtaERF003*, which belongs to *AP2/ERF* family, had a positive effect on both adventitious and lateral root proliferation in *Populus* (Trupiano *et al*., 2013). Furthermore, quantitative trait locus analysis of adventitious rooting in apple hardwood cuttings indicated that *AP2/ERF* is a potential candidate for the modulation of adventitious root formation (Moriya *et al*., 2015).

In this study, to clarify the genetic background of adventitious root formation in hardwood cuttings of apple, first, we compared the transcriptome profiles obtained before adventitious root formation and at the start of adventitious root formation by RNA-sequencing (RNA-seq) analysis. Then, we analyzed gene expression patterns throughout the process of adventitious root formation by reverse transcription-quantitative PCR (RT-qPCR) using hardwood cuttings from another growing season. As a result, we confirmed

the concomitance of the expression patterns of auxin related genes with adventitious root formation. Moreover, we conducted a comprehensive co-expression network analysis of genes identified by RNA-seq analysis. Finally, we discussed the involvement of cytokinin, ABA, and plant peptide hormone- related genes in adventitious root formation.

3.2 Materials and Methods

3.2.1 Plant material

About a hundred hardwood cuttings were collected from Marubakaido apple rootstocks grown in the Horticulture Farm of the Center for Environment, Health and Field Sciences, Chiba University, Kashiwa, Chiba, Japan (35 °N, 140 °E). Approximately 20-cm-long hardwood cuttings were planted in 500-mL containers filled with 200 mL of distilled water with aeration and the whole set up was maintained at 25 °C in an incubator under cool white fluorescent light with a 16-h photoperiod. The change of distilled water in the containers and the recording of callus formation and rooting ratio were conducted at two- to three-day intervals.

3.2.2 RNA-seq and informatics analysis

Total RNA was isolated from the cut surface portions of hardwood cuttings by using the method described by Henderson and Hammond (2013). Briefly, total RNA was extracted with cetyltrimethylammonium bromide-based buffer and then purified on a silica column. For RNA-seq analysis, since the number of hardwood cuttings were limited, basal cut surface portions of 50 hardwood cuttings were collected only at two data points; that is, when callus formation was observed in 70 % of cuttings (C70) and when the first visible adventitious root formation was noted in 10 % of cuttings (R 10). Single total RNA pools were prepared separately from 50 hardwood cuttings in C70 and that in R10 for RNA-seq analysis. Individual RNA pools of C70 and R10 were provided a paired-end cDNA library construction after a quality check with a Bioanalyzer.

Library construction and sequencing for the Illumina HiSeq 2500 was provided as a custom service of Eurofins Genomics K. K. (Tokyo, Japan). The polyA fraction (mRNA) was isolated from total RNA, followed by its fragmentation. Then double-

stranded (ds) cDNA was reverse transcribed from fragmented mRNA. The ds cDNA fragments were processed for adaptor ligation, size selection (for 200 bp inserts) and amplification to generate strand-specific cDNA libraries. Prepared libraries were subjected to paired-end 2 x 125 bp sequencing on the HiSeq 2500 platform with v4 chemistry. Sequence analysis of the paired-end cDNA library was conducted using an Illumina HiSeq 2500 system by Eurofins Genomics, which were deposited in the Sequence Read Archive under accession number PRJNA503524 (BioProject ID).

Quality control of clean reads was performed by using the FastQC program (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The clean reads were assembled using the Trinity algorithm (https://github.com/trinityrnaseq/trinityrnaseq/wiki) and this was followed by the processing of the assembled reads with Cufflinks package (http://cole-trapnell-lab.github.io/cufflinks/) to identify differentially expressed genes (DEGs) between C70 and R10, which are based on fragments per kilobase of exon per million fragments mapped (FPKM) methods. Then, sequences defined as DEGs were functionally annotated by gene ontology (GO) analysis. Initially, we examined GO analysis using traditional method (Blast2GO); however, the function of most DEGs was unclear. Hence, this prompted us to further conduct plant science-based annotation; TAIR plant ontology (PO; https://www.arabidopsis.org/tools/bulk/po/index.jsp), TAIR GO (https://www.arabidopsis.org/tools/bulk/go/index.jsp) and STRING (https://string-db.org/) software based on the results of BLAST search against *Arabidopsis* genome database TAIR10 (https://www.arabidopsis.org/). In addition, we also conducted the apple genes-based GO analysis by MapMan (https://mapman.gabipd.org/) software based on the results of BLAST search against *Malus* × *domestica* GDDH13 v1.1 Whole Genome database (https://www.rosaceae.org/analysis/242).

3.2.3 RNA extraction and gene expression analysis using RT-qPCR

In this study, RNA-seq analysis was conducted using single total RNA pools in C70 and R10 (one biological replicate each). To exclude the bias of RNA-seq library constriction, the differentially expressed pattern of genes identified as DEGs was analyzed by RT-qPCR analysis. For RT-qPCR, the basal cut surface portions of 25

hardwood cuttings were periodically collected from December to January 2017–2018. From the basal cut surface portions of 7-10 hardwood cuttings of each sampling days, three independent total RNA pools were prepared and cDNAs synthesized with random primer using ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). The specific primers were designed based on the nucleotide sequences identified by RNA-seq *de novo* assembly using the Trinity software (Table S1). Specific primers of *elongation factor 1*, *histone*, and *SAND* genes were selected for the expression analysis of internal controls (Saito *et al*., 2017). Transcript levels of target genes were defined by the $2^{-\Delta\Delta Ct}$ method and normalized to the transcript level of the geometric average of three internal control genes in each sample.

3.3 Results

3.3.1 Morphological changes on cut surface of hardwood cuttings

In 2016–2017 season, hardwood cuttings were obtained on 28 November 2016. On the cut surface of the hardwood cuttings, callus formation on 70 % (C70) and the first visible root formation on 10 % (R10) of cuttings were noted on 12 and 20 December 2016 (14 and 22 days after planting), respectively. In 2017–2018 season, hardwood cuttings were obtained on 5 December 2017 (0 days after planting). The first visible callus was assumed to appear around 15 December 2017 (10 days after planting) and callus formation was observed on 23.3 % of the cut surface of hardwood cuttings (Fig. 3.1). The callus formation gradually increased to 45.5 % on 2 January 2018 (23 days after planting), and this was followed by a sharp increase to 100 % on 17 January 2018 (38 days after planting). The first visible adventitious root formation was noted on 22 January 2018 (43 days after planting); adventitious root formation was observed on 58.3 % of the cut surface of hardwood cuttings on 9 February (60 days after planting).

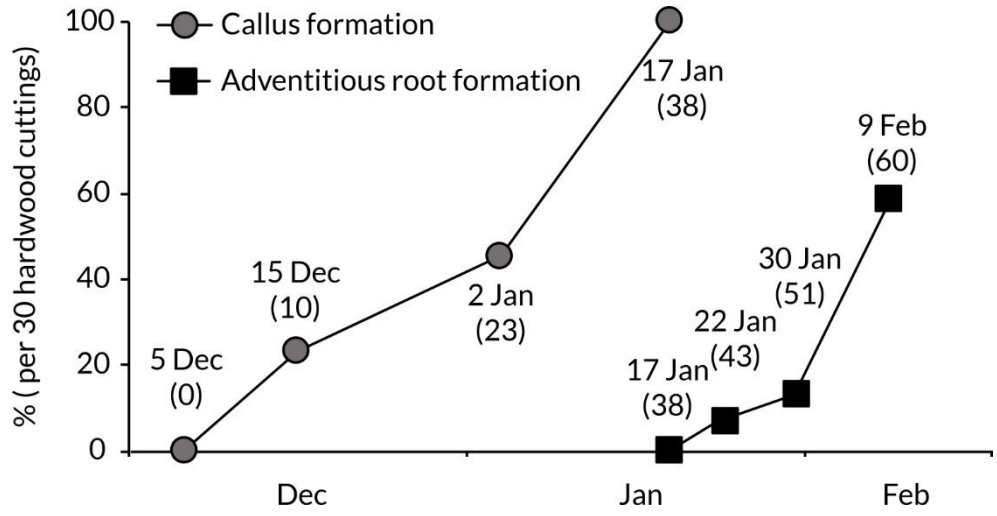


Fig. 3.1 Changes in callus and adventitious root formation ratio of hardwood cuttings in 2017–2018 growing season. Number in parentheses indicates days after planting

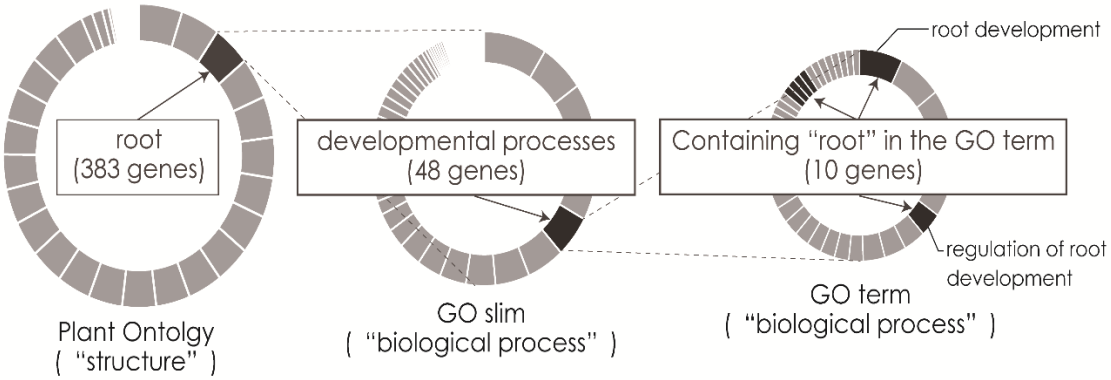
3.3.2 Isolation of genes related to adventitious root formation by GO enrichment analysis

To clarify the genetic factor(s) responsible for adventitious root formation, the DEGs between hardwood cuttings on 12 December 2016 (C70) and those on 20 December (R10) were identified by RNA-seq analysis. RNA-seq generated about 15,871,356 and 18,764,496 reads of C70 and R10 respectively, each 125 nucleotides long, with approximately 2 billion nucleotides from their RNA extract. Low quality reads (quality value <30) and over-represented sequences were removed read alignment. The clean reads were assembled *de novo* using the Trinity algorithm and constructed 146,750 contigs as unigenes. Among them, we found that 730 and 552 genes were up- and down-regulated (*p*-value <0.05 and a log2-fold change greater than +2 or less than -2 in C70 vs. R10).

Functional annotation of the DEGs was firstly started from the analysis of tissue localization of DEGs by using *Arabidopsis* PO. In the result, 383 of DEGs were annotated as root localized genes (Fig. 3.2a). Finally, the functions of these root localized genes were annotated by using GO slim/term classification, which resulted in 6 root morphology related GO terms for the 10 genes (Fig. 3.2b). Among these genes, qRT-

PCR analysis was conducted on *ABC transporter G family member* (*ABCG23*), *NO VEIN* (*NOV*), and *Auxin transporter-like protein 2* (*LAX2*), using hardwood cuttings in 2017–2018 season (Fig. 3.2). The expressions of genes related to root development, *ABCG37*-like and *NOV*-like were relatively similar; up-regulated up to 38 days after planting, and quickly down-regulated in the 43 days after planting (Fig. 3.2c). The expression patterns of root cap development related genes *LAX2*-like, also resembled those of *NOV*; *LAX2*-like expressions peaked on the 38 days after planting, but was maintained relatively high even in 43 days after planting, then downregulated thereafter until the 60 days after planting (Fig. 3.2c, right panel).

(a)



(b)

GO term (GO accession)	*Arabidopsis* gene ID (**gene name** *Malus* gene ID)
root development (GO:0048364)	AT1G32640 (**MYC2**; MD16G1274200), AT1G55020 (**LOX1**; MD04G1204500),
	AT3G53480 (**ABCG37**;MD16G1137900), AT4G13750 (**NOV**; MD04G1012800),
	AT5G09810 (**ACT7**; MD01G1001600), AT5G16780 (**MDF**; MD16G1194300)
regulation of root development (GO:2000280)	AT3G09070 (**unknown**; MD04G1147100), AT5G65790 (**MYB68**; MD12G1143800)
	AT2G37430 (**C2HC-ZF family**; MD04G1119600)
lateral root formation (GO:0010311)	AT1G55020 (**LOX1**; MD04G1204500)
post-embryonic root development (GO:0048528)	AT5G16780 (**DOT2**; MD16G1194300)
root cap development (GO:0048829)	AT2G21050 (**LAX2**; MD10G1121700)
root system development (GO:0022622)	AT3G09070 (**unknown**; MD04G1147100)

(c)

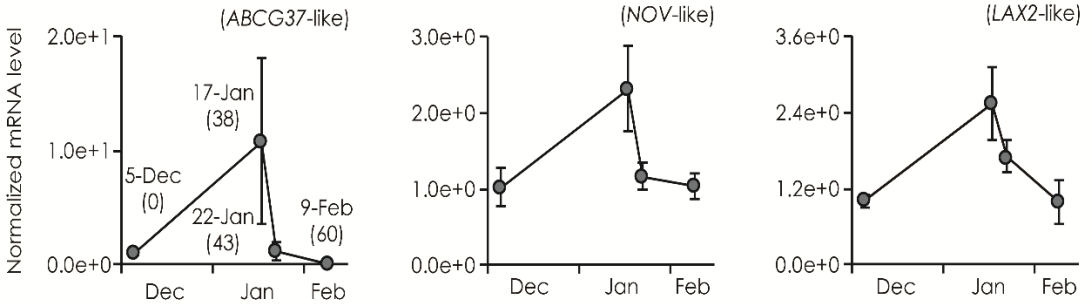


Fig. 3.2 (a) Summary of PO, GO slim, and GO term classification for selecting DEGs relating adventitious root formation. (b) List of DEGs selected by PO, GO slim, and GO term classification, based on annotation by TAIR database. (c) Normalized expression

levels of *ABCG37*-, *NOV*-, and *LAX2*-like genes. Error bars show the standard deviations of three biological replicates. Number in parentheses indicates days after planting.

3.3.3 Comprehensive co-expression network analysis of DEGs

We also analyzed the comprehensive co-expression network of DEGs using STRING database to determine the relationships and/or interactions among DEGs (Fig. 3.3a). We identified one large network (named network-a). To identify the functions of genes belonging to this network, GO analysis was carried out. In this network, genes related to bioactive compounds response such as chitin, abscisic acid, and salicylic acid were found (Fig. 3.3b). In addition, we also evaluated the overrepresentation of GOs by Venn diagram, resulting in the distribution of abscisic acid and salicylic acid responses being assumed to be independent (Fig.3.3c left panel). Subsequently, we analysed the expression of genes related to "response to abscisic acid", the resulting *protein phosphatase 2C family protein* (*AP2C1*) was up-regulated up to the 38 days after planting and down-regulated thereafter until the 60 days after planting (Fig. 3c right panel). The expression level of *MYB73*-like gene was high even before planting and decreased 38 days after planting; however, it was re-upregulated 43 days after planting.

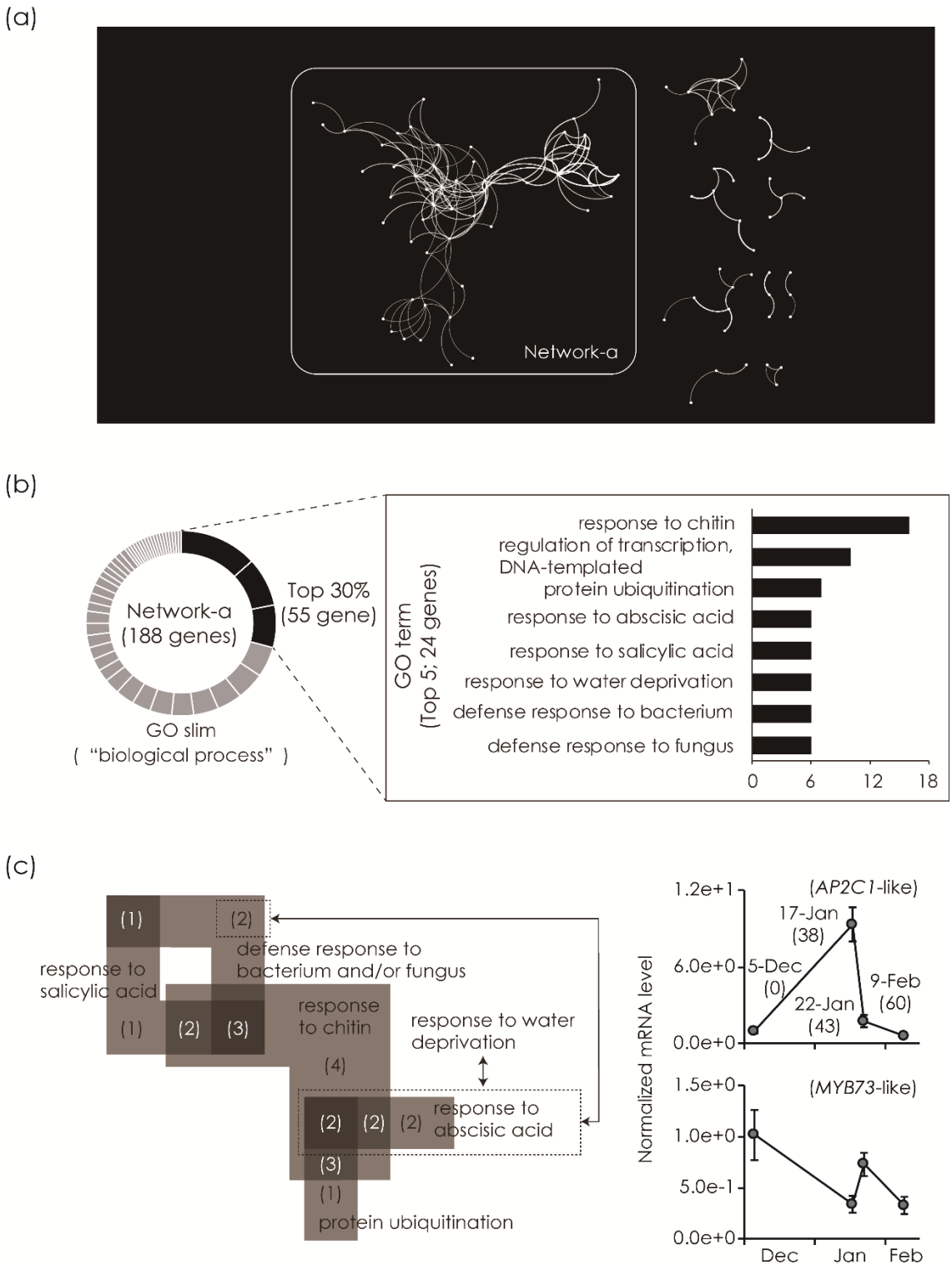


Fig. 3.3 (a) Comprehensive co-expression network of DEGs determined by STRING algorithm. Each DEG is represented by a circle. Thicker lines represent stronger

interaction calculated by using active interaction sources. (b) Summary of GO slim/term of genes belonging to the network-a. Distribution of top 5 hit GO term, which belongs to GO slim occupying up to 30 %, are shown. (c) Analysis of overrepresentation of GOs obtaining “response to chitin”, “protein ubiquitination”, “response to abscisic acid”, “response to salicylic acid”, “defense response to bacterium” and “defense response to fungus” by Venn-diagram (left panel). Number in parentheses indicates the number of genes. Arrow indicated the partial overrepresentation of genes obtaining each GO term. Normalized expression levels of *AP2C*- and *MYB73*-like genes (right panel). Error bars show standard deviations of three biological replicates. Number in parentheses indicates days after planting

3.3.4 Functional annotation of genes encoding transcription factor by MapMan

To specify the detailed functions of DEGs, GO analysis was conducted using MapMan software, and this revealed the functions of DEGs to be concentrated in three categories that is: “RNA”, “protein” and “signaling” (Fig. 3.4a). Most of the genes in “RNA” belonged to “regulation of transcription” such as transcription factor families (Fig. 3.4b). Similarly, the concentration of GOs on “regulation of transcription, DNA-templated” was also found by the comprehensive co-expression network analysis (Fig. 3.3b). Therefore, these two analyses might indicate similar response with another aspect.

The transcription factor families were identified as genes encoding WRKY and APETALA/ethylene-responsive element binding protein (AP2/EREBP, also called AP2/ERF), MYB, and C_2H_2 zinc finger (C_2H_2-ZF) family proteins (Fig. 3.4b). In the DEGs classified into “protein”, most of them were annotated as genes associated with “degradation” and “post translational modification”. The function of DEGs related to “signaling” response was identified as the genes encoding “receptor kinases” and “calcium”.

Changes in the transcriptional levels of *WRKY*- and *AP2/EREBP*-like protein family genes were investigated using the sample in the 2017–2018 growing season (Fig. 3.4b). *WRKY33*-like gene expression was temporary increased up to the 38 days after planting and completely suppressed in 43 days after planting (Fig. 3.4b, middle panel). *ERF109*-like gene expression also peaked 38 days after planting but sharply decreased

thereafter until the 60 days after planting (Fig. 3.4b, right panel). We also analyzed the transcriptional levels of receptor kinases genes, especially leucin rich repeat (LRR) kinase (Fig 3.4c, left panel). *Barely any meristem* (*BAM3*)-like gene expression reached a peak 38 days after planting (Fig. 3.4c middle panel), maintained a relatively similar level 43 days after planting, then decreased thereafter until the 60 days after planting. In contrast, *Phloem intercalated with xylem* (*PXY*)-like gene expression was enhanced up to the 38 days after planting and remained high even at 60 days after planting (Fig. 3.4c, right panel).

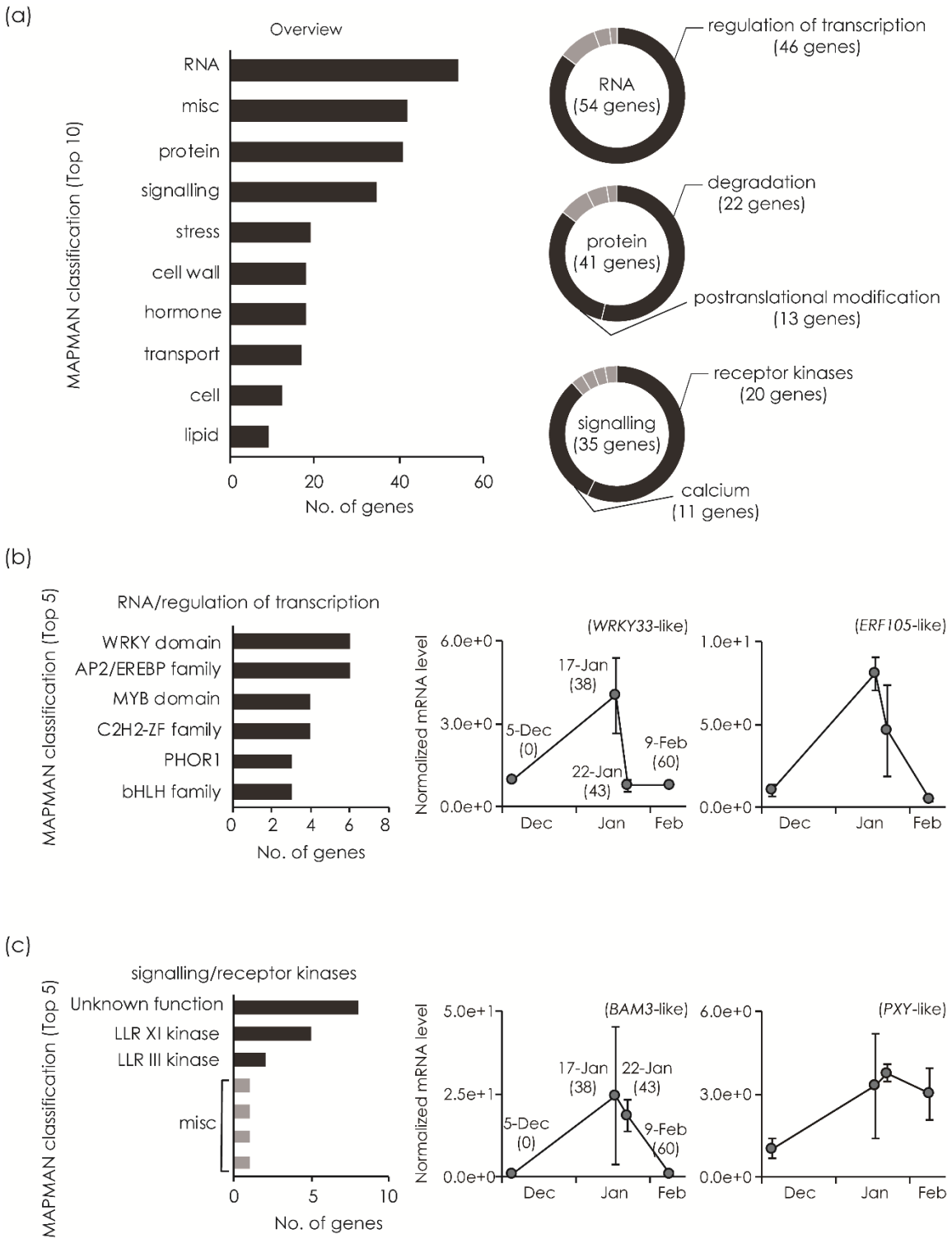


Fig. 3.4 (a) Summary of genes classified into the functional categories "Overview" by MAPMAN analysis (left panel). Detailed MAPMAN ontology of genes classified into

"RNA", "protein", and "signaling" (right panel). (b) Distribution of genes obtained "regulation of transcription" as MapMan ontology. Normalized expression levels of *WRKY33*- and *ERF105*-like genes. Error bars show standard deviations of three biological replicates. Number in parentheses indicates days after planting. (c) Distribution of genes obtained "receptor kinases" as MapMan ontology. Normalized expression levels of *BAM3*- and *PXY*-like genes. Error bars show standard deviations of three biological replicates. Number in parentheses indicates days after planting.

3.4 Discussion

3.4.1 Relationship between auxin-related gene expression and adventitious root formation

Over the last 80 years, synthetic auxins, such as IBA, have been used as an industrial enhancer for adventitious rooting of cuttings in fruit tree species such as peach (Jizhong and Siwei, 1989), grape (Galavi *et al*., 2013), olive (Centeno and Gómez-del-Campo, 2008), and Japanese persimmon (Tetsumura *et al*., 2001). Through the transcriptome analysis of the hardwood cuttings of *Marubakaido*, an easy-to-root apple rootstock, we were able to identify 10 genes as candidates for adventitious root formation (Fig. 3.2b). Among them, *ABCG37*, *NOV*, and *LAX2* were well-studied as auxin-responsive and/or related genes. Moreover, RT-qPCR analysis showed that the induction of *ABCG37*-like gene expression took place before the first visible adventitious root formation (Fig. 3.2c). *ABCG37* was known as the auxin efflux carrier and recently considered to be one of the modulators for auxin distribution and homeostasis in root tip of *Arabidopsis* (Růžička *et al*., 2010). Tsugeki *et al*. (2009) showed that *NOV* was required for cell type-specific expression of auxin-efflux carrier, *PINFORMED2, 3, 4*, and *7*, which modulate cell fate determination such as vascular differentiation and lateral root formation in *Arabidopisis* root. We also analyzed the expression pattern of the auxin influx carrier, *LAX2*-like gene, and found that higher expression of *LAX2*-like was maintained during the first visible adventitious root formation. Zhang *et al*. (2013) demonstrated that *LAX2* was strongly expressed in primary root cap and modulates the specification of quiescent center in *Arabidopsis*. Moreover, *ACTIN7* (*ACT7*) and *MERISTEM-DEFECTIVE* (*MDF*) were also suggested to be involved in auxin inducible

callus formation and auxin responsive root stem meristem maintenance, respectively (Kandasamy *et al*., 2001, Casson *et al*., 2008). These results suggest that auxin homeostasis, such as cellular patterning of auxin, plays an important role in stem cell maintenance following root meristem morphogenesis.

3.4.2 Relationship between other phytohormones and adventitious root formation

In the hardwood cuttings of certain apple rootstocks, such as M.9, no root developed even after IBA treatment (Fukuda *et al*., 1988), suggesting that the relationship between auxin and adventitious root formation is still unclear. To clarify the possible role of other phytohormones in adventitious root formation, we analyzed the comprehensive co-expression network of DEGs. In the result, a large comprehensive co-expression network was found (Fig. 3.3a). It was worth noting that the auxin-related genes (Fig. 3.2b) were completely independent of this network, assuming only an auxin signaling might not be enough postulation for adventitious root formation. Finally, we identified abscisic acid and salicylic acid related genes as DEGs (Fig. 3.3b). A previous study showed the degradation of auxin by salicylic acid treatment and inhibitory effect on adventitious root formation in apple (De Klerk *et al*., 2011). Therefore, although the salicylic acid response was considered to affect internal auxin level, salicylic acid itself might not play a critical role during adventitious root formation. In contrast, the modulation of adventitious root formation by ABA has been demonstrated in apple, especially auxin/ABA ratio, was reported to change competence of adventitious root formation (Noiton *et al*., 1992). The AP2C1, which encodes the clade B of PP2C-type phosphatase is known to enhance ABA signaling (Brock *et al*. 2010). In this study, among six ABA responsive genes, the induction of *AP2C1*-like gene was observed before adventitious root formation (Fig. 3.3c). Furthermore, when the first visible adventitious root formation was observed, the expression of *MYB73*-like was upregulated. We noted the induction of R2R3-type *MYB*, *MYB73*-like gene, which was reported to directly interact with the ABA receptor, PYL8, and regulate lateral root growth in *Arabidopsis* (Zhao *et al*. 2014), suggesting that ABA affected adventitious root formation of apple.

Otherwise, the functional analysis by MAPMAN elucidated that the expression of *WRKY33*- and *ERF105*-like genes was obviously enhanced before adventitious root

formation (Fig 3.4b middle and right panels respectively). Recently, the involvement of *WRKY33*- and *ERF105*-like genes in cytokinin-dependent radial root growth was demonstrated in radish (Jang *et al*., 2015). However, detailed information about the relationship among the *WRKY33*-/*ERF105*-like genes, cytokinin, and adventitious root formation is still insufficient. In contrast, among many kinds of transcription factor families, we also identified *SCARECROW* (*SCR*)-like gene as a DEG (Fig. S1). SCR is a key regulator of stem cell definition and radial patterning (Sabatini *et al*., 2003). Zhang *et al*. (2013) showed the cytokinin responsive expression pattern of *SCR* and the contribution of *SCR* to stem cell niche maintenance in quiescent center of *Arabidopsis*. Taken together, cytokinin signaling may affect the maintenance of root meristem following radial development of root.

3.4.3 Relationship between plant peptide hormone and adventitious root formation

We also identified DEGs annotated as "receptor kinase" by MAPMAN analysis, most of which were functionally unknown (Fig. 3.4c). However, in relation to above, we eventually isolated 5 of the genes encoding peptide hormones receptor such as *BAM3*- and *PXY*-like genes among LRR kinases. Recent studies using transgenic *Arabidopsis* plant revealed that LRR-RKs, including the *BAM3*-like gene, act as receptors of CLEs. BAM3 was known as receptor of CLE45 and the necessity of BAM3/CLE45 module activity level in the acquisition of phloem cell fate determination was pointed out (Rodriguez-Villalon *et al*., 2014). In addition, *Arabidopsis crinkly4* (*ACR4*) and *phytosulfokine receptor*-like genes were found in LRR kinases, which were reported as CLE40 and plant peptide containing sulfated tyrosine 1 (PSY1) receptor, respectively (Fig S1). ACR4/CLE40 module was reported to control the stem cell niche in *Arabidopsis* root meristems (Stahl *et al*., 2009). PSY1 was also known to be required for the activity of ROOT GROWTH FACTOR1, which was another peptide hormone controlling root growth (Matsuzaki *et al*. 2010). So far, it was tempting to speculate the relationship between peptide hormone and adventitious root formation of apple; however, a further study would be needed to verify this matter. Similarly, we also found the induction of *PXY*-like gene (Fig. 3.4c), which was identified as a tracheary element differentiation inhibitory factor (TDIF; CLE41/44), receptor (Hirakawa *et al*., 2008). It

was demonstrated that TDIF signaling regulated xylem differentiation and lateral root development via crosstalk with auxin in *Arabidopsis* (Cho *et al*., 2014). As aforementioned, the relationship between vascular differentiation and auxin-*NOV* signaling was studied and the results strongly supported the relationship between TDIF and vascular development in apple.

In this study, hormone related genes differentially fluctuated during adventitious root formation between two growing seasons and different analytical methods reflected the robustness of relationship between adventitious root formation and hormonal signaling. Therefore, these results might have suggested that adventitious root formation in apple is controlled by complex hormonal signaling, such as auxin, cytokinin, ABA, and plant peptide hormone. To clarify this supposition, a further study focusing on endogenous plant hormones levels will be needed.

CHAPTER 4

Paclobutrazol elevates auxin and abscisic acid, reduces gibberellins and zeatin and modulates their transporter genes in Marubakaido (*Malus prunifolia* Borkh. var. ringo Asami) apple rootstocks.

4.1 Introduction

In commercial apple (*Malus domestica* Borkh.) cultivation, tree height and planting density are critical. Consequently, the use of dwarfing rootstocks is widespread in the industry. Dwarfing rootstocks influence tree architecture, growth vigor, management practices and economic yield. Dwarfing rootstocks have been implicated in rootstock-induced growth restriction through the imbalance of carbohydrates, reduced cell growth and metabolism (Foster *et al.*, 2017), and contain higher proportion of bark and a reduced number of xylem cells with reduced diameters compared to invigorating rootstocks (Soumelido *et al.*, 1994). Relatedly, paclobutrazol (PBZ) has been found to have a major effect on plant height reduction, as well as the regulation of phytohormones levels (Edgerton, 1986; Zhu *et al.*, 2004; Fan *et al.*, 2018). The growth inhibitory response of PBZ is attributed to the interference of gibberellin (GA) biosynthesis by repressing the oxidation of ent-kaurene to ent-kauronoic acid through inactivating cytochrome P450-dependent oxygenase (Miki *et al.*, 1990). However, a detailed molecular understanding of PBZ-mediated dwarfing as a result of the regulation of phytohormone biosynthesis, transport, inactivation, and signaling has been largely unexplored in apple rootstocks.

Plant hormones are considered an essential component of regulating growth and development, and coordination of their biosynthesis, transport, degradation and regulation of signaling components is crucial in maintaining homeostasis in plant tissues (Sauer *et al*., 2013; Rademacher, 1997; Lange and Lange, 2006; Foster *et al*. 2017) (Fig 4.1). For example, suppression of gibberellin (GA) biosynthesis (feedback regulation) is mediated by decreased expression of *GA20OX* and *GA3OX*, which are GA biosynthetic genes, while increased catabolism (Feedforward regulation) occurs as a result of the upregulation of *GA2OX*, which is a GA catabolic gene (Yamaguchi *et al*., 2000).

According to the previously stated literature, the binding of GA to GID1-type receptors promotes interaction between GID1 and a DELLA protein leading to the destruction of DELLA and lifting of its inhibition on GA responses. NPF transporter family members have reportedly been involved in GA transport across the plasma membrane (Park *et al*., 2017). Recently, Zhang *et al*. (2019) also reported the expression of two GA transporter-like genes related to NPF3.1 in apples. An overview of the above processes is illustrated in Fig. 4.1A.

Auxin transporter proteins located on the plasma membrane provide active movement of IAA in a directional cell-to-cell movement called polar auxin transport (Grones and Friml, 2015) mediated by three classes of proteins: the AUX1/LAX family, the PIN-FORMED (PIN) family, and the P-glycoprotein ABC subfamily proteins (Petrasek and Friml, 2009; Fig. 4.1B). Overexpression of *ZmPIN1a* efflux transporter genes in maize transgenic lines markedly reduced height via the transport of more auxins from the shoot apex to the root meristem (Li *et al*., 2018). Increased polar auxin transport with elevated endogenous auxin levels in the basal end of citrus epicotyl cuttings appeared to be inhibitory for in vitro shoot organogenesis (Hu *et al*., 2017). Also, auxin response factors (ARFs) are considered components that confer specificity to the auxin response through the selection of target genes as transcription factors. ARFs regulate auxin-mediated transcriptional activation/repression (Li *et al*., 2016). Relatedly, it is documented that *ARF2* is a general repressor of cell division, elongation, and organ growth (Schruff *et al*., 2005).

The biosynthesis of abscisic acid (ABA) is mainly regulated by the rate-limiting step enzyme *9-cis*-epoxycarotenoid dioxygenase *NCED* (Lefebvre *et al*., 2006), while degradation is catalyzed by cytochrome P450 monooxygenase *CYP707A* (Kushiro *et al*., 2004). Growth cessation in poplar apical buds was observed after ABA levels peaked, and this was concomitant with the induction of genes related to ABA biosynthesis (*ABA1*, *NCED3*, and *ABA2*) (Ruttink *et al*., 2007). Meanwhile, to transfer ABA across the plasma membrane from the sites of synthesis to sites of action, ABA transporters are required (Kang *et al*., 2015). Several members of the nitrate transport1/peptide transport family (NFP) have been implicated to show ABA transport activity; for example, loss-of-

function mutants of nitrate transport1/peptide transport family (NPF) 4.6/ NRT1.2/ABA-importing transporter (AIT) 1 (*NFP4.6/AIT1*) became less sensitive to exogenous ABA application, thus revealing decreased inhibition of seed germination (Kanno *et al*., 2012). A summary of the steps involved in ABA biosynthesis, catabolism, and transport are shown in Fig. 4.1C.

Miyawaki *et al*. (2006) indicated that ATP/ADP isopentenyl transferases (IPTs) control the biosynthesis of isopentenyladenine (iP)- and *trans*-zeatin (*t*Z)-type cytokinins while *cis*-zeatin (*c*Z) is synthesized from tRNA IPTs. A systematic study of the IPT and cytokinin oxidase/dehydrogenase (CKX) gene families in apples by Tan *et al*. (2018) identified 12 *MdIPT*s and 12 *MdCKX*s in different tissues. They further showed that exogenous application of 6-benzylaminopurine upregulated six *MdIPT* genes (*MdIPT1*, *MdIPT2*, *MdIPT5*, *MdIPT6*, *MdIPT7*, and *MdIPT10*) with increased vigorous growth whereas a contrasting effect was observed after application of lovastatin, an inhibitor of cytokinin biosynthesis. The irreversible inactivation of cytokinin is catalyzed by CKX enzymes. Lower levels of cytokinin metabolites were observed in *Arabidopsis* plants overexpressing *35S:CKX7* (Köllmer *et al*., 2014). In apple dwarfing rootstocks, *CKX7* was upregulated (Foster *et al*., 2017). Among cytokinin transporters so far reported, several members of the purine permease (*PUP*) family have been suggested to be involved in cytokinin transport (Liu *et al*., 2019; Fig. 4.1D).

Previous studies aiming to discern PBZ-mediated dwarfing in fruit trees have mostly focused on endogenous phytohormone levels but focused less on the detailed molecular mechanisms governing phytohormone-related dwarfing. However, the integration of transcriptional analysis based on plant hormone biosynthesis, metabolism, signaling and, more importantly, transport, the molecular understanding of which is just emerging, will be of relevance to clearly unravel the PBZ involvement in dwarfing. Moreover, the loss of phytohormone transporter action is ascribed to impair developmental and physiological processes in plants. Therefore, the overall objective of this study was to investigate how PBZ induced Marubakaido apple rootstock dwarfing through phytohormone modulation and transport.

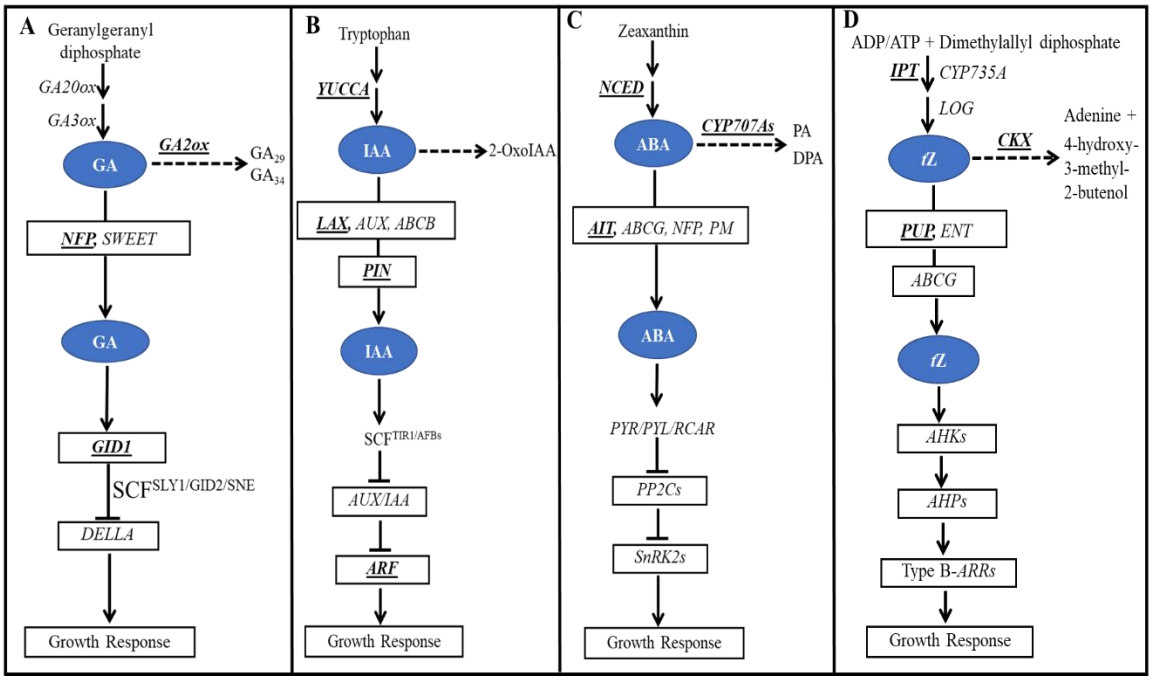


Fig. 4.1 A summary of a schematic representation of the biosynthesis, degradation, signaling, and transporter-related genes of GA, IAA, ABA and *trans*-zeatin (*t*Z). The genes analyzed in our study are underlined and bold. The dashed arrows represent the degradation of bioactive phytohormone. (A) GA-related genes: GA2ox; GA2 oxidase; NFP; nitrate transporter proteins (NRT1/PTR-family), SWEET; Sugars Will Eventually Be Exported Transporters, GID; gibberellin insensitive dwarf, DELLA; GA INSENSITIVE, REPRESSOR OF GA1-3, RGA-LIKE1 (RGL1), RGL2, and RGL3. (B) IAA-related genes: YUCCA; flavin monooxygenase protein, LAX; auxin transporter-like protein, PIN; PIN-FORMED, ARF; auxin response factor. (C) ABA-related genes: NCED; *9-cis*-epoxycarotenoid deoxygenase; CYP707A; ABA 8′-hydroxylase, AIT; ABA-IMPORTING TRANSPORTER. (D) Cytokinin (*t*Z)-related genes: IPT; isopentenyl transferase, CKX; cytokinin dehydrogenase/oxidase, PUP; purine permease.

4.2 Materials and Methods

4.2.1 Plant materials and treatment

One-year-old invigorating apple rootstock of Marubakaido (*Malus prunifolia* Borkh. var. ringo Asami) and dwarfing rootstock of M.9 (*Malus domestica* Borkh.) were used in this study. They were grown and managed at the Horticulture Farm of the Center for Environment, Health and Field Sciences, Chiba University, Kashiwa, Chiba, Japan (35°N, 140°E). The apple rootstocks were randomly divided into three groups with 27 rootstocks in each.

For each group, 9 rootstocks were considered to represent a single replication. *Marubakaido* apple rootstock stools were foliar-sprayed with or without 860 mg L^{-1} of PBZ dissolved in distilled water with 0.1% (v/v) of a surfactant (Approach BI, 50% polyoxyethylene hexitan fatty acid ester; Kao, Osaka, Japan) in a single application.

To clarify the dwarfing mechanism induced by PBZ, the M.9 apple rootstock cultivar (dwarf) was used as a positive control. The untreated controls were sprayed with distilled water containing 0.1% (v/v) Approach BI. The treatments and first sampling were both carried out on 15 May 2018 (0 days after treatment (DAT)). The second and third samplings were subsequently carried out fortnightly, corresponding to 14 and 28 DAT respectively. Thereafter, both the treated and untreated control Marubakaido rootstocks were excessively pruned and left to recover for at least two months, then sampled on 24 August, which was 99 DAT.

The terminal/apical shoots were sampled and transported to the lab under ice (0 ^{o}C). On arrival, the shoot apices (apical buds) were dissected from the main shoots, frozen immediately in liquid nitrogen and stored at -80 ^{o}C, then later used for gene expression analysis. The shoot bark (containing the cortex, epidermis, and layers of cambium cells) including 2–3 cm of the sub-apical part of the shoot (containing the xylem) were then quickly extracted from the shoot with a sharp blade, immediately frozen in liquid nitrogen, and stored at -30 ^{o}C for further analysis of endogenous plant hormones. The shoot bark was selected for analysis of basipetally-transported hormones (GAs, IAA, and ABA) while the sub-apical shoot was selected for acropetally-transported *t*Z.

4.2.2 Determination of rootstock growth

For the shoot length determination, 12–15 terminal shoots per replicate were measured, of which three replicates were used for each treatment. The numbers of internodes present on each shoot were recorded. The internode lengths were estimated by dividing the overall shoot length by the number of internodes.

4.2.3 Analysis of endogenous IAA and ABA concentrations

IAA and ABA were extracted and quantified by gas chromatography-mass spectrophotometry (GC-MS) according to Setha and Kondo (2009) with a few modifications. Approximately 0.5 g samples of frozen apple shoot bark were macerated in 20 mL 80% (v/v) methanol containing 0.5 g polyvinylpolypyrrolidone with butylhydroxytoluene (0.1 g L^{-1}) and ascorbic acid (0.1 g L^{-1}) as antioxidants. Prior to homogenization, the extraction buffer was fortified with 200 ng of each stable isotope of phenyl-$^{13}C_6$ IAA (Cambridge Isotope Laboratories, Andover, MA, USA) and 3′,5′,5′,7′,7′,7′-hexadeuterated ABA (ABA-d_6) as an internal standard (Ken Nelson, National Research Council of Canada, Saskatoon, Saskatchewan, Canada).

The samples were shaken for 1 h, centrifuged (15000 × g, 4 ºC, 15 min), filtered through Whatman No. 2 filter paper (pore size 2 nm, diameter 70 mm, Toyo Roshin Kaisha, Ltd., Japan), and re-extracted, and the supernatants were pooled. The supernatants were evaporated to dryness. The residue was suspended four times in 5 mL distilled water. The aqueous residue was adjusted to pH 2.5 with 0.1 M hydrochloric acid and partitioned 3 times with an equal volume of ethyl acetate. The ethyl acetate portion was evaporated to dryness, dissolved 3 times in 0.5 mL ethyl acetate, and reduced to dryness (40 ºC) in a stream of N_2. The dried residue was re-dissolved 3 times in 0.5 mL of 25% (v/v) acetonitrile containing 20 mM acetic acid. The solution was filtered through a nitrocellulose filter (pore size 0.22 μm, EMD MILLPORE Co., Billerica, MA, USA) and fractionated by high-performance liquid chromatography using an octadecylsilyl Mightysil RP-18 column (4.6 mm i.d. × 250 mm) with a gradient of 4.8–9.6 M acetonitrile containing 20 mM acetic acid over a 30-min period at a flow rate of 1.3 mL min^{-1}, and detected by ultraviolet absorption at 254 nm. The fractions from the HPLC corresponding to IAA elution (22–27 min) and ABA elution (27.5–31 min) were

collected separately and pooled together, evaporated to dryness, re-dissolved three times in 0.5 mL methanol and dried in a stream of N_2. The dry samples were re-dissolved with 1 mL of 10% (v/v) methanol in diethyl ether, methylated with diazomethane solution for 10 min, and thereafter dried in the stream of N_2. The methyl esters of IAA and ABA were quantified and identified by GC/MS selected ion monitoring (GC–MS-SIM; model QP5000; Shimadzu, Kyoto, Japan) using an InertCap 1MS column (GL Sciences, Tokyo, Japan; 0.25 mm i.d. × 30 m, 0.25 μm film thickness) and linear helium flow at 50.2 cm s^{-1}. The column temperature was a step gradient of 60 °C for 2 min, then 60 to 270 °C at 10 °C min^{-1}, and 270 °C for 35 min. Methyl-IAA and methyl-ABA were analyzed separately by electron impact GC-MS. Quantification was performed through selected ion monitoring by Labsolution data system software which involved monitoring responses to ions with mass-to-charge ratios *m/z* of 130 and 189 for methyl-IAA and 135 and 195 for methyl-[$^{13}C_6$]IAA, and 162 and 190 for methyl-ABA-d_0 and 166 and 194 for methyl-(ABA-d_6), respectively. The endogenous IAA and ABA concentrations were calculated from the peak ratios of *m/z* 130/135 and 190/194, respectively.

4.2.4 Analysis of gibberellins (GA_1 and GA_4) and cytokinin (zeatin)

The extraction and quantification of endogenous gibberellins and cytokinin was done according to Manzi *et al*. (2015) with some modifications. Frozen composite samples of the shoot bark and sub-apical shoot weighing about 0.5 g were macerated in 20 mL 80% (v/v) methanol containing butylhydroxytoluene (0.1 g L^{-1}) and ascorbic acid (0.1 g L^{-1}) as antioxidants. Each sample was fortified with 200 ng of deuterated isotopes (internal standards) of [$^2H_5$]-*t*Z, [$^2H_2$]-GA_1 and [$^2H_2$]-GA_4 at the beginning of extraction. After homogenization, the samples were left overnight at 4 ^oC. Thereafter, the homogenates were centrifuged for 15 min at 10000 × g and 4 ^oC. The extracts were vacuum-filtered through Whatman No. 2 filter paper (pore size 2 nm, diameter 70 mm, Toyo Roshin Kaisha, Ltd., Japan) and the filtrates were rotary-evaporated at 40 ^oC to dryness and suspended four times in 2 mL 0.1 M phosphate buffer at pH 8.5. Polyvinylpolypyrrolidone (0.5 g) was added to the extract and shaken for 20 min. After, the extract was again vacuum-filtered and evaporated to complete dryness. The dry residue was resuspended three times in 2 mL of distilled water and partitioned three times

using 3 mL *n*-hexane. The *n*-hexane layer was discarded while the recovered aqueous layer was evaporated to dryness and dissolved three times in 2 mL 1% (v/v) acetic acid. The resultant solution was purified using Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA, USA). Briefly, the cartridges were first conditioned with 5 mL of methanol in 1% acetic acid, then 5 mL of 1% acetic acid was eluted through them. The extracted sample was passed through the cartridges, and 5 mL of 1% acetic acid was again added with all the eluates discarded. The solution containing the phytohormones was eluted with 5 mL of 80% methanol containing 1% acetic acid with eluates collected and evaporated to dryness. The residue was dissolved five times with 1 mL of 1 M formic acid. The sample was loaded into an Oasis MCX column (60 mg sorbent; Waters Co.) which had been pre-conditioned with 5 mL methanol and equilibrated with 5 mL formic acid prior to sample addition. Thereafter, the column was washed with 5 mL formic acid with the eluent discarded. GA_1 and GA_4 were then eluted through the column with 5 mL methanol and collected for further purification. For cytokinin (*t*Z), 5 mL of 0.35 M ammonium hydroxide (NH_4OH) was subsequently added into the column and the filtrate was discarded. Thereafter, 5 mL of 0.35 M NH_4OH containing 60% methanol was used for eluting *t*Z. The two different fractions containing GAs and cytokinin were further purified as follows; the concentrated extract containing GAs after evaporation to dryness was dissolved in methanol. Before the extract was loaded into the Bond-Elute DEA (diethylaminopropyl) column (500 mg; Varian, Palo Alto, CA, USA), the column was pre-conditioned with methanol. After extract loading, methanol was again added to wash the column, and the GAs were eluted with 1% acetic acid and evaporated. For the *t*Z extract, the eluate was directly evaporated to dryness. The respective residues of the GAs and *t*Z were dissolved twice in 0.5 mL methanol, dried completely under a stream of nitrogen gas, and later reconstituted with methanol prior to LC-MS analysis. Analysis and quantification of the GA_1, GA_4, and *t*Z were performed using a LC/MS-2010EV (Shimadzu, Kyoto, Japan) consisting of a cooled autosampler and LC-10ADvp pump (Shimadzu, Kyoto) at a voltage of 1.5 kV, with the column temperature maintained at 40 ^oC, and connected to a mass spectrometer equipped with an Electron spray Ionization (ESI) source and operated in a positive analytical mode. The data acquisition software

was Labsolutions Ver. 3. Separation was achieved by an ODS Mightysil RP-18 column (150 mm × 2.0 mm i.d., 5 µm). The compounds were eluted isocratically with methanol/water containing 20 mM formic acid (80:20 v/v) at a flow rate of 0.3 mL min^{-1}. The injection volume was 1 µl. The LC-MS conditions were as follows; the ESI spray voltage was 4.5 kV, the curved desolvation line (CDL) temperature was 250 ^{o}C, the block heater temperature was 250 ^{o}C, and the nebulizer gas (N_2) flow was 1.5 mL min^{-1}. Quantification of the phytohormones was carried out using selected ion monitoring. The parent and fragment ions used for quantification were m/z = 347 and 349 for GA_1, 331 and 333 for GA_4, and 220 and 225 for *t*Z.

4.2.5 RNA extraction and quantitative reverse transcription polymerase chain reaction analysis

Total RNA was isolated from a composite of apices (0.1 g) of three biological replicates for each treatment using the cetyltrimethylammonium bromide and silica-column based extraction method by Henderson and Hammond (2013). The cDNA synthesis from the extracted RNA was carried out using the iScript™ Reverse Transcription Supermix for quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Bio-Red Laboratories Co., Ltd., California, United States) according to the manufacturer's instructions. qRT-PCR was performed on a StepOnePlus™ system (Applied Biosystems, CA, USA). The reaction mixture was prepared using a KAPA SYBR green fast ABI Prism qPCR kit (Kapa Biosystems, Wilmington, MA, USA) and the specific primers listed in Table S2. The reference genes used in our study were *SAND*, *ubiquitin* (*UBI*), and *Histone H3* (*HISH3*). The transcript levels of the target genes were estimated using the $2^{-\Delta\Delta Ct}$ algorithm and normalized against the transcript level of geometric averages of the three reference genes in each sample (Vandesompele *et al.*, 2002).

4.2.6 Statistical analysis

Statistical analysis was performed using SPSS 26 software (IBM, New York, NY, USA). The statistical significance of the experiment was determined either by single-factor analysis of variance and means separated by least significant difference (LSD) or by an independent two-tailed student's t-test with $P \leq 0.05$.

4.3 Results

4.3.1 Effect of PBZ foliar application on shoot and internode length

PBZ markedly suppressed shoot elongation of the *Marubakaido* rootstocks throughout the growth period (Fig. 4.2A). The PBZ effect was evident at 14 DAT, and this was comparable to the shoot elongation trend of the M.9 rootstock. Upon recovery after pruning at 99 DAT, the dwarfing effect of PBZ on *Marubakaido* rootstocks was still maintained compared to the untreated control. Relatedly, the internode length of PBZ-treated rootstocks was significantly suppressed compared to the untreated control throughout the experiment, and it was the same in M.9 at 28 DAT (Fig. 4.2B).

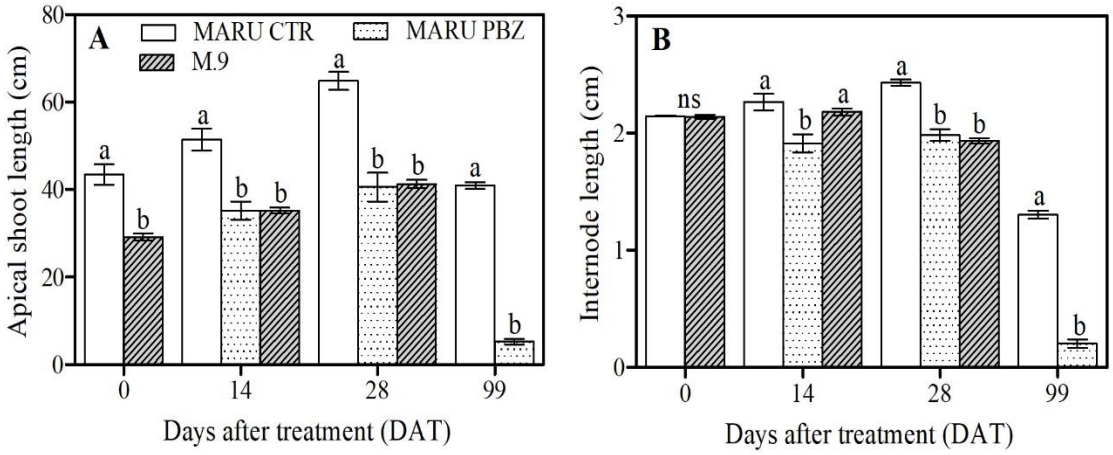


Fig. 4.2 Effect of paclobutrazol on growth of Marubakaido apple rootstocks. *Marubakaido* rootstock (MARU CTR) as an untreated control, Marubakaido with PBZ treatment (MARU PBZ (860 mg L^{-1})), and Malling 9 (M.9) as a positive control. Effect of PBZ on (A) shoot length and (B) internode length. Each bar represents the mean (± SE) of three replicates each with 12–15 shoots; means with different letters are significantly different at $P \leq 0.05$ by LSD or t-test; ns; not significant.

3.2. Endogenous concentrations of IAA, ABA, GA_1, GA_4 and zeatin

At 0 DAT, the IAA concentration in the shoot barks of Marubakaido rootstock was significantly higher than that in M.9 rootstock (Fig. 4.3A). Two weeks after the foliar application of PBZ (14 DAT), the treated *Marubakaido* rootstocks showed elevated IAA concentrations compared to both the untreated control and M.9 rootstocks. However, at

28 DAT, no effect of PBZ was observed on the IAA concentration in the treated rootstocks. But upon recovery after pruning, at 99 DAT, the IAA concentration in the treated rootstocks showed a significant increase compared to the untreated control.

The endogenous ABA concentration was not affected by the PBZ treatment at 14 DAT (Fig. 4.3B). At 28 DAT, the treated rootstocks displayed significantly higher ABA concentrations than the untreated rootstocks. Surprisingly, at 99 DAT, a dramatic 8.5-fold increase of ABA concentration was observed in the PBZ-treated rootstocks compared to the untreated control. Taken together, the above results indicate that PBZ increased both the IAA and ABA concentrations in the treated rootstocks, with a profound effect registered following the recovery after pruning (Figs. 4.3A and 4.3B).

At 14 DAT, PBZ had no effect on the GA_1 concentration in Marubakaido rootstocks, but the M.9 rootstocks showed the lowest levels compared to other treatments (Fig. 4.3C). In contrast, PBZ suppressed the GA_4 concentration in the treated rootstock barks at 14 DAT (Fig. 4.3D). At 28 and 99 DAT, both GA_1 and GA_4 concentrations were markedly reduced in the treated rootstocks compared to untreated control.

The *t*Z concentrations in the rootstock sub-apical shoots differed significantly among treatments. At 0 DAT, the *t*Z concentration was higher in the untreated rootstocks than in M.9. The concentrations were significantly decreased in PBZ-treated rootstocks at 14 and 28 DAT (Fig. 4.3E). However, PBZ had no effect on the rootstocks at 99 DAT. This result may suggest that the PBZ effect on the repression of cytokinin concentrations might have been negated with time.

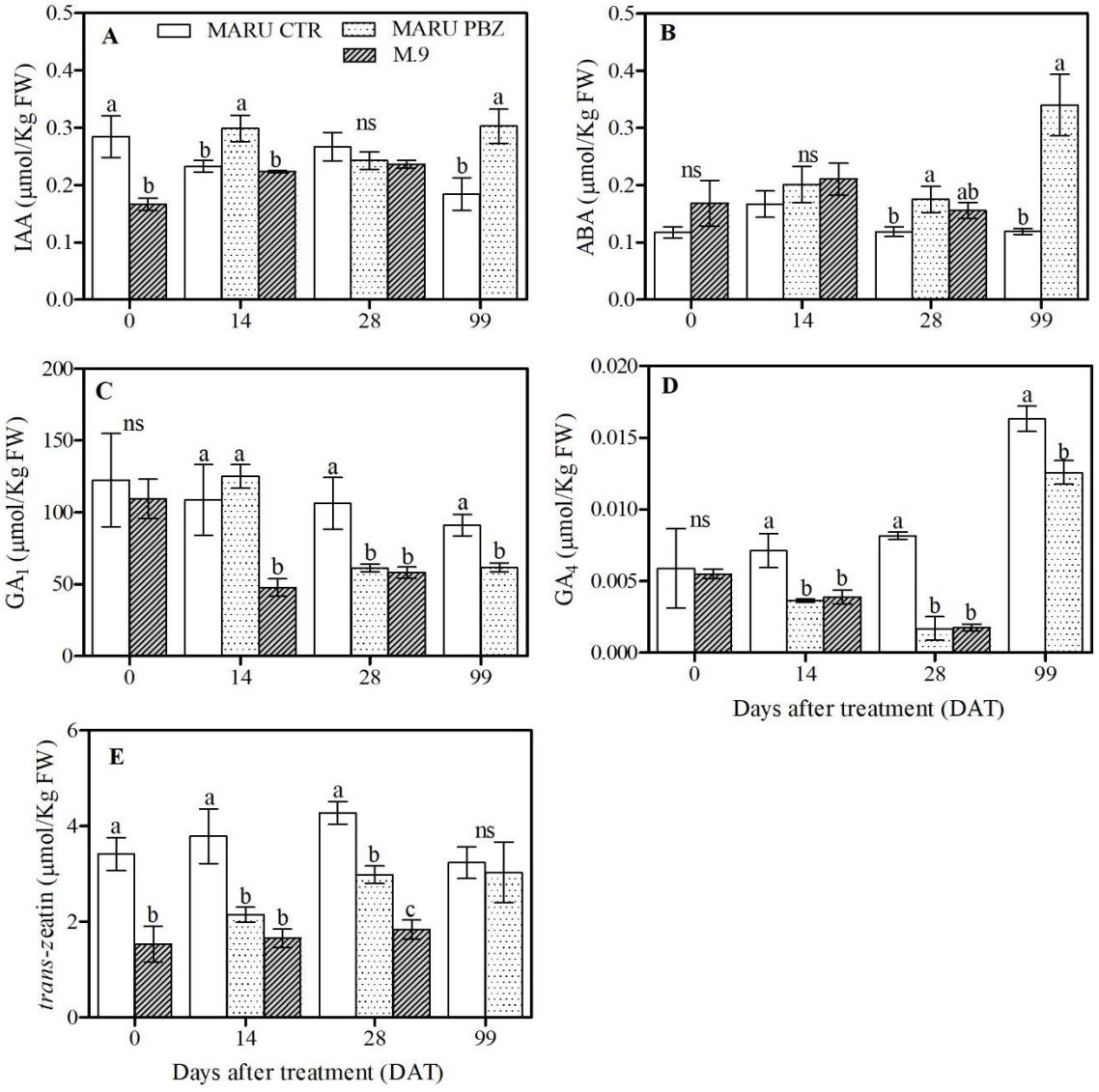


Fig. 4.3 Effect of paclobutrazol foliar application on endogenous auxin (IAA), abscisic acid (ABA), gibberellins (GA_1 and GA_4), and *trans*-zeatin (*t*Z) in Marubakaido apple rootstock shoot barks and sub-apical shoot. Marubakaido rootstock (MARU CTR) as an untreated control, Marubakaido with PBZ treatment (MARU PBZ (860 mg L^{-1})), and Malling 9 (M.9) as a positive control. Endogenous concentration of (A) IAA, (B) ABA, (C) GA_1, (D) GA_4, and (E) *t*Z. Each bar represents the mean (± SE) of three replicates each with 20–25 shoots; means with different letters are significantly different at $P \leq 0.05$ by LSD or t-test; ns; not significant.

4.3.3. Expression levels of efflux and influx transporter genes, ARF, and biosynthesis gene

The auxin efflux transporter gene *MdPIN1* was highly upregulated at 14 DAT by PBZ, but no effect was observed at 28 DAT (Fig. 4.4A). Upon recovery after pruning at 99 DAT, the *MdPIN1* transcripts generally declined, with PBZ-treated rootstocks revealing higher expression levels than the untreated control.

Furthermore, the auxin influx transporter gene *MdLAX1* was markedly expressed on 14 and 28 DAT in PBZ-treated rootstocks compared to both the untreated control and M.9 rootstocks (Fig. 4.4B). However, an opposite trend was observed at 99 DAT with the untreated control showing significantly higher expression levels than the PBZ-treated rootstocks.

Higher expression levels of the IAA signaling gene *MdARF2* in the PBZ-treated rootstocks was observed throughout the study period (Fig. 4.4C). However, statistical significance was registered at 28 and 99 DAT compared to the untreated control.

At 0 DAT, the untreated control showed a significant upregulation of the IAA biosynthesis gene *MdYUCCA10a* compared to the M.9 rootstocks (Fig. 4.4D). Additionally, at 14 DAT, a marked increase of *MdYUCCA10a* gene expression was observed in PBZ-treated rootstocks compared to other treatments. Thereafter, no PBZ effect was realized at 28 and 99 DAT.

Generally, at 14 DAT the expression levels of IAA-related genes in our study clearly corresponded to the elevated endogenous IAA concentration in the shoot barks of PBZ-treated Marubakaido rootstocks.

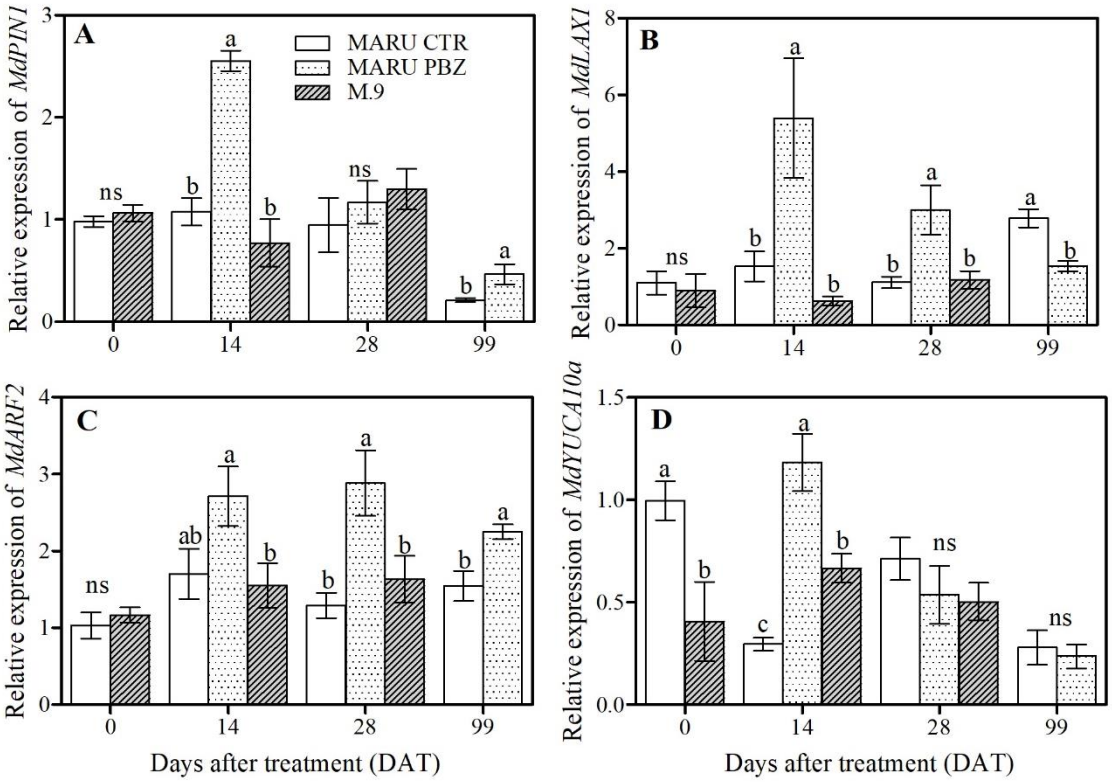


Fig. 4.4 Effect of paclobutrazol foliar application on expression of auxin (IAA) transporter-related genes, auxin response factor (ARF) gene, and biosynthesis gene in Marubakaido apple rootstock apical shoot apices. Marubakaido rootstock (MARU CTR) as an untreated control, Marubakaido with PBZ treatment (MARU PBZ (860 mg L^{-1})), and Malling 9 (M.9) as a positive control. Gene expression of (A) *MdPIN1*, (B) *MdLAX1*, (C) *MdARF2*, and (D) *MdYUCCA10a*. Each bar represents the mean (± SE) of three biological replicates each with 20–25 apices; means with different letters are significantly different at $P \leq 0.05$ by LSD or t-test; ns; not significant.

4.3.4. Expression of ABA transporter, biosynthesis, and degradation-related genes

The *MdAITb* transporter genes were highly expressed at 14 DAT in PBZ-treated and M.9 rootstocks, but with no statistical significance among the treatments (Fig. 4.5A). Thereafter, at 28 DAT *MdAITb* genes in the PBZ-treated rootstocks were significantly upregulated. The treated rootstocks maintained significantly higher expression levels compared to the untreated rootstocks at 99 DAT.

The expression of *MdNCED1* genes, which encode carotenoid deoxygenases, and the rate-limiting in ABA biosynthesis in plants were also examined. In the PBZ-treated rootstocks, *MdNCED1* gene expression was greatly upregulated at 14 DAT (Fig. 4.5B). Interestingly, at 28 DAT, the PBZ-treated and M.9 rootstocks showed lower expression levels than the untreated control but without any statistical difference. Upon recovery after pruning, at 99 DAT, the treated rootstocks showed a tremendous 4.8-fold increase in expression levels compared to the untreated control.

The ABA degradation gene *MdCYP707A1* in PBZ-treated rootstocks was significantly repressed at 28 and 99 DAT (Fig. 4.5C). However, at 14 DAT, the *MdCYP707A1* transcripts were unaffected in all treatments.

Collectively, the ABA-related genes assessed seemingly showed expression levels that corresponded to the endogenous ABA concentrations in Marubakaido rootstocks.

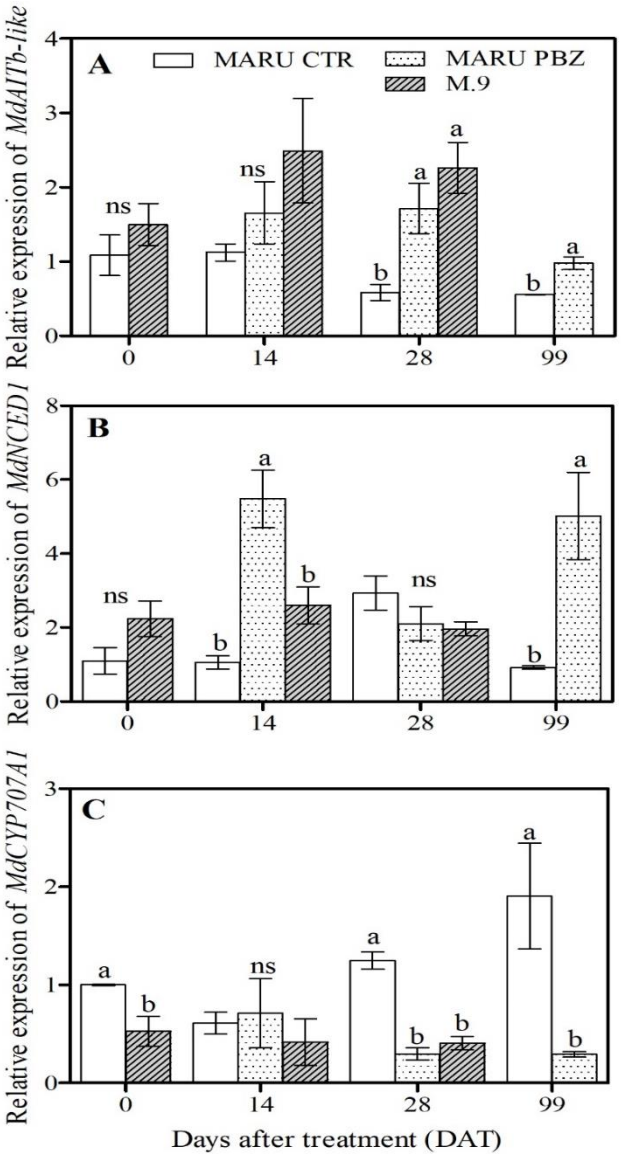


Fig. 4.5 Effect of paclobutrazol foliar application on expression of ABA transporter, biosynthesis, and degradation-related genes in Marubakaido apple rootstock in apical shoot apices. Marubakaido rootstock (MARU CTR) as an untreated control, Marubakaido with PBZ treatment (MARU PBZ (860 mg L^{-1})), and Malling 9 (M.9) as a positive control. Gene expression of (A) *MdAITb*-like (B) *MdNCED1*, and (C) *MdCYP707A1*. Each bar represents the mean (± SE) of three biological replicates each with 20–25 apices; means with different letters are significantly different at $P \leq 0.05$ by LSD or t-test; ns; not significant.

4.3.5. Expression levels of GA transporter, signaling, and metabolic-related genes

The transcript levels of *MdNFP3.1-like* genes in PBZ-treated rootstocks were significantly repressed throughout the growth period (Fig. 4.6A). Additionally, a stronger suppression of *MdNFP3.1-like* genes was observed at 99 DAT. *MdGID1b-like* signaling genes in the treated rootstocks followed a pattern similar to that of the *MdNFP3.1-like* genes except at 99 DAT, whereby PBZ had no effect on their expression (Fig. 4.6B).

The GA deactivation gene *MdGA2OX2* was significantly upregulated in both PBZ-treated and M.9 rootstocks compared to the untreated control at 14 and 28 DAT (Fig. 4.6C). However, after pruning, *MdGA2OX2* transcripts in the treated rootstocks showed higher levels, but without any statistical significance.

Generally, the GA-related genes followed a pattern like that of the endogenous GA_4 levels in Marubakaido rootstocks, thus clearly contributing to dwarfing in PBZ-treated rootstocks.

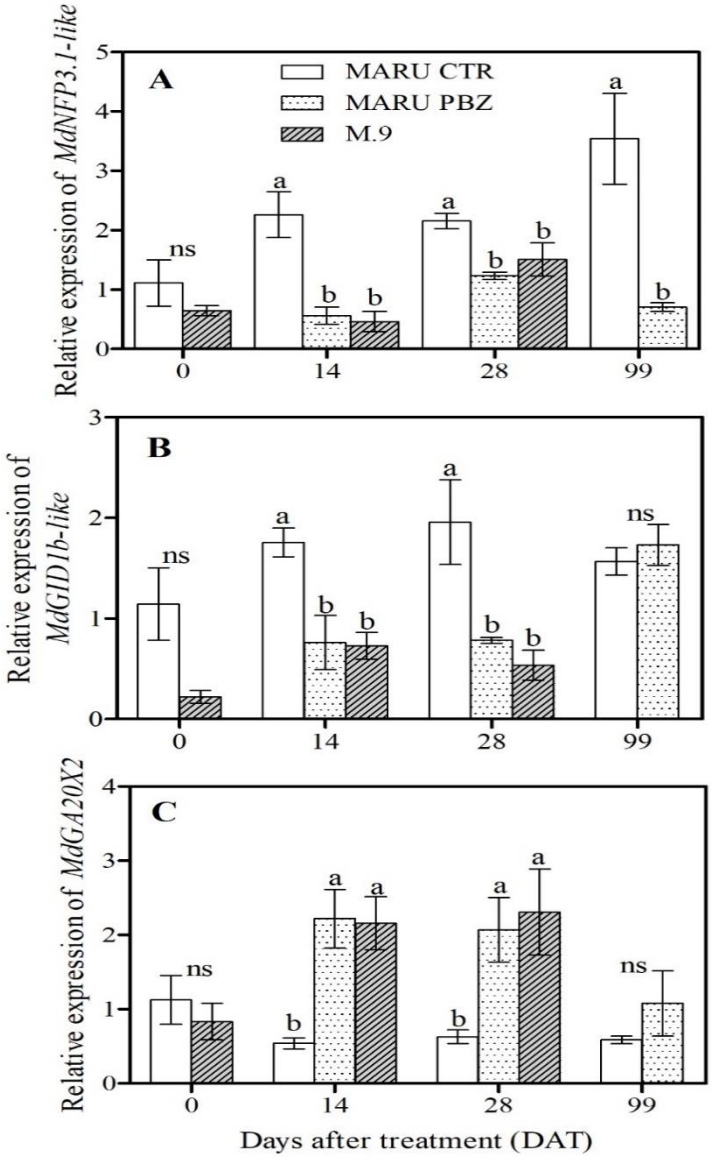


Fig. 4.6 Effect of paclobutrazol foliar application on expression of gibberellic acid transporter, signaling, and metabolism-related genes in Marubakaido apple rootstock apical shoot apices. Marubakaido rootstock (MARU CTR) as an untreated control, Marubakaido with PBZ treatment (MARU PBZ (860 mg L^{-1})), and Malling 9 (M.9) as a positive control. Gene expression of (A) *MdNFP3.1*-like (B) *MdGID1b*-like, and (C) *MdGA2OX2*. Each bar represents the mean (± SE) of three biological replicates each with 20–25 apices; means with different letters are significantly different at $P \leq 0.05$ by LSD or t-test; ns; not significant.

4.3.6. Expression levels of cytokinin biosynthesis, transporters, and degradation-related genes

In the PBZ-treated rootstocks, the *MdIPT6* gene was greatly downregulated at 14 DAT (Fig. 4.7A). At 28 and 99 DAT, there was no significant difference between the untreated control and the PBZ treatment. Additionally, *MdIPT3* and *MdIPT5* genes were not affected by the treatment (Data not shown).

Both *MdPUP7-like* and *MdPUP9-like* transporter genes were more highly expressed at 14 DAT than in the untreated control (Figs. 4.7B and 4.7C). The transcripts of the two transporter genes were significantly expressed under the PBZ treatment compared to the untreated control. At 99 DAT, PBZ had no effect on the *MdPUP7-like* gene expression levels (Fig. 4.7B). In contrast, *MdPUP9-like* gene expression in the untreated control revealed significantly higher levels than the treated rootstocks (Fig. 4.7C).

In PBZ-treated rootstocks, the cytokinin degradation gene *MdCKX7* was upregulated at 14 and 28 DAT (Fig. 4.7D). The *MdCKX7* transcripts were significantly expressed at 28 DAT compared to the untreated rootstocks. However, at 99 DAT, PBZ exerted no effect on the transcript levels of the *MdCKX7* gene. According to the results given above, PBZ had no effect on the expression of *MdIPT6*, *MdPUP7-like*, and *MdCKX7* genes at 99 DAT.

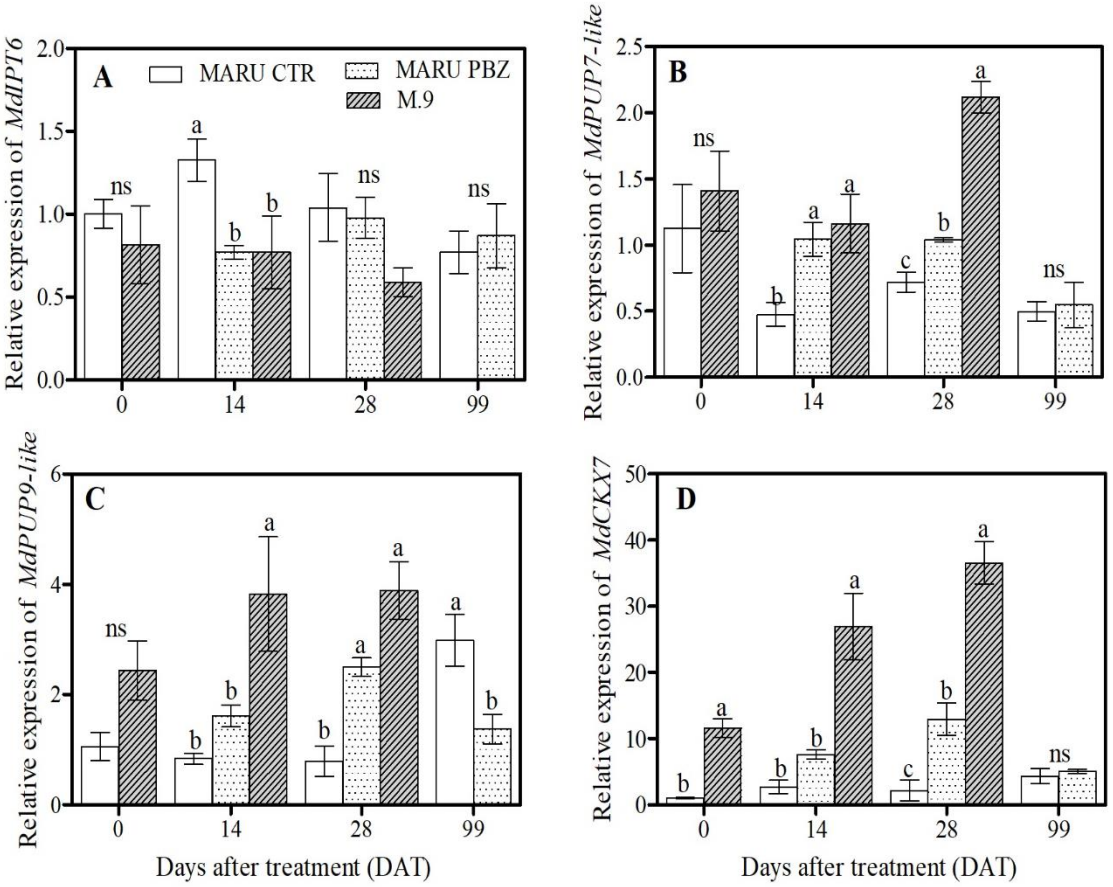


Fig. 4.7 Effect of paclobutrazol foliar application on expression of cytokinin biosynthesis, transporter, and degradation-related genes in Marubakaido apple rootstock apical shoot apices. Marubakaido root stock (MARU CTR) as untreated control, Marubakaido with PBZ treatment (MARU PBZ (860 mg L^{-1})), and Malling 9 (M.9) as a positive control. Gene expression of (A) *MdIPT6*, (B) *MdPUP7-like*, (C) *MdPUP9-like*, and (D) *MdCKX7*. Each bar represents the mean (± SE) of three biological replicates each with 20–25 apices; means with different letters are significantly different at $P \leq 0.05$ by LSD or t-test; ns; not significant.

4.4 Discussion

In our present study, the growth suppression of PBZ was observed a fortnight after foliar application on Marubakaido apple rootstocks. The morphological effects of the GA inhibitor PBZ were evidenced by a profound inhibition of both shoot and internode length elongation. In line with our findings, the growth-retarding effect of PBZ has been reported in apple (*Malus domestica* Borhk.), peach (*Prunus persica*), and sweet cherry (*Punus avium*) (Edgerton, 1986). A previous study by Fletcher and Hofstra (1990) reported that the growth-regulating properties of PBZ are mediated through the alteration of endogenous plant hormones such as GA, ABA, and cytokinin. These previous findings agree with our observations, which revealed elevated IAA and ABA concentrations with suppressed GAs and *t*Z levels in the PBZ-treated rootstocks. Taken together, these findings suggest that the dwarfing effect of PBZ on Marubakaido apple rootstocks was because of phytohormone regulation.

Upregulation or repression of genes encoding GA biosynthesis, metabolism, transport, and signaling could lead to the alteration of GA levels and may result in dwarf or tall phenotypes (Lange and Lange, 2006). The effects of PBZ on the rootstocks was obvious as it profoundly suppressed endogenous GA concentrations (GA_1 and GA_4) in the shoot bark. Also, the expression levels of downstream genes encoding the GA signaling gene *MdGID1b-like* in the apices were repressed by exogenous PBZ application. In contrast, the gene encoding GA catabolism, *MdGA2OX2,* was highly upregulated in PBZ-treated rootstocks. Previous studies have reported that PBZ application leads to the repression of GA biosynthesis mediated by decreased expression of the biosynthetic genes and of the signaling gene *GID1* and to the promotion of catabolism through the increased expression of *GA2OX,* which eventually leads to decreased endogenous GA levels and ultimately to the dwarfing of plants (Fan *et al*., 2018; Zhang *et al*. 2019). Several members of the NPF have been reported to transport GAs across the plasma membrane in addition to transporting numerous other substrates (Park *et al*., 2017). *NPF3.1* has been implicated to transport bioactive GAs (GA_1, GA_3, and GA_4), and it transports more GA_4 and to a lesser extent GA_1 and GA_3 in a pH-dependent manner (Tal *et al*., 2016). Recently, Zhang *et al*. (2019) reported the expression of two GA transporter-like genes related to NPF3.1, namely the *MdNPF3.1-like* genes, in the apple.

In our study, the expression of *MdNPF3.1-like* transporter genes was strongly suppressed in PBZ-treated rootstocks. This finding suggests that PBZ disrupted GA transport to appropriate sites, thus resulting in inhibited growth of Marubakaido rootstocks. Overall, PBZ-induced endogenous GA synthesis suppression reduced rootstock growth, and this strongly reduced internode elongation.

The localization of the influx and efflux carriers at the plasma membrane mediate IAA movement in and out (polar auxin transport), thus providing a unique machinery for transporting this hormone between different cells and tissues (Grones and Friml, 2015). In this study, the PBZ-treated rootstocks registered significantly higher expression levels of both *MdPIN1* and *MdLAX1* genes as efflux and influx IAA carrier-related genes, respectively. Furthermore, the *MdARF2* transcripts were upregulated in the PBZ-treated Marubakaido rootstocks as well as the IAA biosynthesis-related gene *MdYUCCA10a*. The IAA concentrations in the shoot barks happened to be concomitant with the expression levels of genes, except for the *MdLAX1* gene at 99 DAT (Fig. 4.2A). Elevated IAA concentrations in response to PBZ application might have resulted in the dwarfing of the rootstocks, which was evident at 14 DAT. Overexpression of *ZmPIN1a* in maize (*Zea mays* L.) plants corresponded to the increased IAA concentration in the shoots and root tips, and this eventually reduced the plant height, internode length, and ear height (Li *et al*., 2018). This tends to suggest that the overexpression of auxin transporter genes concomitant with elevated endogenous IAA levels may result in dwarfing, and this is consistent with our study results. Still, based on our study findings, we further suggest that increased IAA concentrations in the shoot bark in response to PBZ treatment may have occurred as a result of the accumulation of non-structural carbohydrates like starch (Zheng *et al*. 2012) and simultaneously decreased GA levels, thus dwarfing the rootstocks. Higher starch concentrations have been correlated with reduced growth in dwarfing apple rootstocks (Foster *et al*., 2017). Additionally, Schruff *et al*. (2005) observed the overexpression of *ARF2* identified as a mutant for the mega integument (*mnt*) gene in *Arabidopsis* as a repressor of cell division in many aerial plant organs. *AUX/IAA* proteins may dimerize with *ARF*s and repress the potential of *ARF*s to activate gene expression

(Li *et al*., 2016). However, we suggest further studies to unravel a clear mechanism by which PBZ leads to the above cascade of events and thus to growth inhibition.

The present study detected higher endogenous levels of biologically active ABA in the PBZ-treated Marubakaido rootstocks. The observed trend of ABA accumulation in this study is consistent with degradation and translocation of ABA from the roots to the shoots. This was clearly shown by upregulation of the rate-limiting biosynthesis gene *MdNCED1* and the transport-related gene *MdAITb-like* and downregulation of the degradation gene *MdCYP707A1*. The growth inhibitory response of PBZ has been associated with an increase in the biosynthesis of growth-limiting ABA. This occurs when PBZ blocks GA biosynthesis leading to the shunting and accumulation of more precursors in the terpenoid pathway, and eventually enhancing ABA biosynthesis (Rademacher, 1997). Besides biosynthesis, two key essential processes that control ABA-mediated plant growth regulation are ABA catabolism and transport. In the jack pine (*Pinus banksiana*), PBZ has been implicated in inhibiting normal ABA catabolism (Marshall *et al*., 1991). In plants, the ABA regulatory function is not limited to only vascular tissues or guard cells, although ABA biosynthesis and metabolism predominantly occur in these cells and tissues; thus, this suggests that ABA is transported throughout the plant (Dodd, 2013). There is overwhelming evidence that ABA transporters are necessary to transfer ABA from the sites of synthesis to sites of action (Kang *et al*., 2015). Consequently, Kanno *et al*. (2012) reported that AIT1 was preferentially localized to the plasma membrane of plant cells and concluded that this was an indicator of AIT1 function as an ABA transporter mediating cellular ABA uptake. They further observed that in the vascular tissues, the expression trends of AIT1 were comparable to the ABA biosynthetic enzymes such as AA03, ABA2, and NCED3. In our study, the mRNA transcripts of *MdAITb-like* transporter genes in the apices of the PBZ-treated rootstocks were markedly upregulated, suggesting that more ABA was transported from the site of synthesis to the apical end. In contrast, the untreated control showed lower expression, thus revealing attenuated ABA transport and increased shoot and internode length. Collectively, these findings imply that the PBZ effect on

biosynthesis, transport, and degradation processes leads to endogenous ABA accumulation in Marubakaido rootstocks and ultimately dwarfing.

Our study results revealed that the endogenous *t*Z was suppressed by PBZ treatment in Marubakaido rootstocks. In a finding consistent with this, Zhu *et al*. (2004) reported decreased Z and ZR levels in the xylem sap of PBZ-treated apple plants. Moreover, PBZ lessened acropetal translocation of root-borne *t*Z to the shoots and this possibly suppressed the growth of the rootstocks. To further support this notion, the cytokinin biosynthesis gene *MdIPT6* revealed lower expression levels in the apices and was consistent with decreased *t*Z in the sub-apical shoots. The IPT gene family has been shown to be involved in cytokinin biosynthesis in apple (Tan *et al.*, 2018). The expression of the *MdCKX7* gene, which encodes a cytokinin-degrading enzyme, was highly upregulated in response to PBZ treatment. In *Arabidopsis* and tobacco, overexpression of the *CKX* gene resulted in inhibited growth and dwarfing (Werner *et al*., 2008). Incidentally, Foster *et al*. (2017) reported upregulation of the *MdCKX7* gene in the dwarfing rootstocks. This result, therefore, suggests that PBZ influenced cytokinin concentration due to its effect on *MdCKX7* gene thereby influencing Marubakaido rootstock growth. Several members of the *PUP* family have been suggested to transport cytokinin; notably, *AtPUP14* homologue has been proposed as a cytokinin uptake transporter localized at the plasma membrane (PM) whose expression leads to the depletion of apoplastic cytokinin pools (Zurcher *et al*., 2016) or possibly located in the endoplasmic reticulum (ER) and pumps the cytokinin out of the ER lumen to cytosol according to Romanov *et al.* (2018). Herein, PBZ treatment upregulated the expression of two *PUP* transporter-like genes, *MdPUP7-like* and *MdPUP9-like*. This finding prompts us speculate that the upregulation of the two genes may have resulted in the inhibition of cytokinin perception by the PM localized receptors (Zürcher *et al*., 2016) or perhaps sequestration of cytokinin out of the ER (Romanov *et al.,* 2018) eventually leading to low signaling output hence the dwarfing of rootstocks. While localization of *PUP* family members is suggested to be on the PM mediating uptake, other members may reside in the endo-membranes to allow transport between organelles. Relatedly, it is worth considering that the understanding of the molecular mechanism of cytokinin transport is

in its infancy and localization of the cytokinin receptors is currently under consideration. However, Romanov *et al.* (2018) suggested that active cytokinin receptors can have different subcellular localizations and that ER and PM may be used in different tissues, organs, developmental stages or in response to signaling molecule of different origins. In line with the above supposition, it is worth noting that the functional relevance of PM versus ER-localized receptors is yet to be determined experimentally (Zürcher and Muller, 2016). Therefore, we suggest further studies on detailed characterization of *PUP* gene family members and their exact site of signaling perception. Notwithstanding, our finding sets a precedent for the further in-depth examination of various roles played by different *PUP* members in plants.

5. Conclusion

In our present study, the growth inhibition response induced by PBZ was exhibited at 14 DAT, and this was concomitant with the regulation of the morphological, physiological, and biochemical responses displayed by Marubakaido rootstocks. Altogether, the results of this study provide a likely model of how PBZ elevated endogenous IAA and ABA while suppressing GAs and *t*Z concentrations in the rootstocks, including a description of the role played by transporter genes in the modulation of rootstock growth. We established a molecular framework for PBZ-mediated action on different genes and eventually the consequential actions of IAA, ABA, GA, and zeatin, which led to the dwarfing of the rootstocks (Fig. 5).

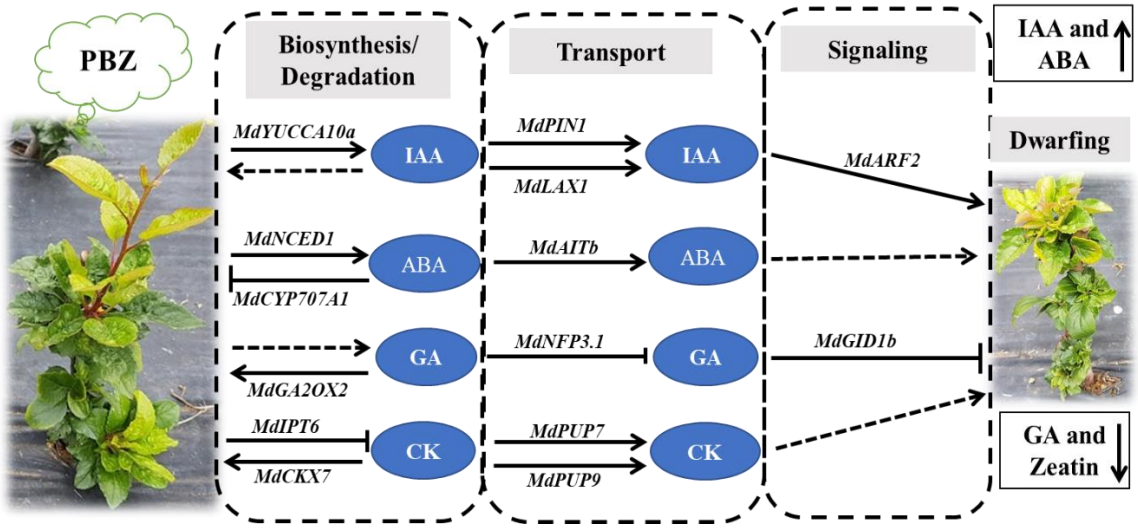


Fig. 5 A potential framework on regulation of genes because of PBZ-mediated dwarfing in Marubakaido apple rootstocks. Thick black lines represent genes analyzed in our study while dashed lines represent hypothetical genes not analyzed. Bold arrows facing up and down represent the decrease and increase of phytohormones, respectively.

CHAPTER 5

General discussion

Deciphering molecular mechanisms involved in AR formation in Marubakaido apple rootstock hard wood cuttings is of importance during vegetative propagation. A detailed understanding of AR formation has been greatly accelerated by RNA-seq analysis and expression of different genes in excised tissues obtained at different developmental stages (Wei *et al*., 2014; Liu *et al*., 2009). Through transcriptome analysis of the Marubakaido hard wood cuttings, an easy to root rootstock; *ABCG37*, *NOV*, and *LAX2* which are regarded as auxin-responsive and/ or related genes were identified during AR formation. Růžička *et al.* (2010) reported that ABCG37 auxin-efflux carrier mediates root auxin homeostasis and development in the root tip of *Arabidopsis*. A previous investigation by Tsugeki *et al*. (2009) revealed that *NOV* was required for cell type-specific expression of auxin-efflux carrier, *PINFORMED2, 3, 4,* and *7*, which modulate cell fate determination such as vascular differentiation and lateral root formation in *Arabidopisis* root. Additionally, *LAX2*-like gene has been implicated to be strongly expressed in the primary root cap and modulates the specification of quiescent center in *Arabidopsis* (Zhang *et al*., 2013). Furthermore, Kandasamy *et al*. (2001) and Casson *et al*. (2008) also suggested ACTIN*7* (*ACT7*) and *MERISTEM-DEFECTIVE* (*MDF*) are involved in auxin inducible callus formation and auxin responsive root stem meristem maintenance, respectively and these genes were as well identified in our study. Altogether, these results suggest that auxin homeostasis, such as cellular patterning of auxin, plays an important role in stem cell maintenance following root meristem morphogenesis.

McAdam *et al.* (2016), confirmed that ABA acted as a shoot derived signal which had a profound influence over root growth in well-watered plants. Literature states that *AP2C1*, which encodes clade B of PP2C-type phosphatase is known to promote ABA signaling (Brock *et al*., 2010). Induction of *AP2C1*-like gene was observed prior to AR formation in Marubakaido hard wood cuttings. Also, when the first visible AR showed, *MYB73*-like gene was upregulated. *MYB73*-like gene which is the R2R3-type *MYB* is

known to interact directly with the ABA receptor, PYL8, and regulate lateral root growth in *Arabisopsis* (Zhao *et al*., 2014), thus, suggesting the role ABA plays during AR formation in apple.

Recently, Hoang *et al*. (2020) reported the involvement of some stress-responsive genes like *ERF105* and *WRKY33* in cytokinin-dependent radial root growth in radish. In our study, MAPMAN functional analysis elucidated the expression of *ERF105*- and *WRKY33*-like before adventitious root formation. However, a clear relationship among the *ERF105* /*WRKY33*-like genes, cytokinin, and AR is still insufficient. *SCARECROW* (*SCR*)-like gene was identified as a DEG in our study and belongs to the transcription factor family. According to Sanchez *et al*. (2007), induction of increased mRNA transcript levels of *SCARECROW* (*SCR*) (SCR-like or SCL) genes of *Pinus radiata* and *Castanea sativa* species was observed within the first 24 h of root induction process when cell reorganization takes place, prior to establishment of AR primordium. Zhang *et al*. (2013) showed the cytokinin responsive expression pattern of *SCR* and the contribution of *SCR* to stem cell niche maintenance in quiescent center of *Arabidopsis*. Collectively, cytokinin signaling may affect the maintenance of root meristem following radial development of root.

The leucine-rich repeat receptor kinases (LRR-RKs) family perceive different peptide ligands, hormones, and can control multiple cellular processes such as cell division, proliferation, differentiation, stem cell balance (Torii, 2004). Among the LRR kinases identified in our study encoding peptide hormone receptor were *BAM3*- and *PXY*-like genes. Depuydt *et al*. (2013) demonstrated that CLV-like LRR-RLK BARELY ANY MERISTEM 3 (BAM3) and its putative ligand CLE45 are involved in guiding progression of protophloem development in the *Arabidopsis* primary root meristem, which determines the meristem's postembryonic growth capacity. Also, *Arabidopsis crinkly4* (*ACR4*) and *phytosulfokine receptor*-like genes were found in LRR kinases and were reported as CLE40 and plant peptide containing sulfated tyrosine 1 (PSY1) receptor, respectively. Stahl *et al*. (2009) reported that ACR4/CLE40 module control the stem niche in *Arabidopsis* root meristems. PSY1 was also known to be required for the activity of ROOT GROWTH FACTOR1, which was another peptide hormone controlling root

growth (Matsuzaki *et al*. 2010). The above result prompts us to speculate a relationship between peptide hormone and AR formation. However, it requires further studies to clarify this supposition. Further, Hohmann *et al*. (2017) demonstrated that the TRACHEARY ELEMENT DIFFERENTIATION FACTOR RECEPTOR/PHLOEM INTERCALATED WITH XYLEM (TDR/PXY) sense small, post translationally modified CLE peptide hormones to maintain plant cell populations in the shoot and the root. It has been reported that tracheary element differentiation inhibitory factor (TDIF; CLE41/44) signaling regulated xylem differentiation and lateral root development via crosstalk with auxin in *Arabidopsis* (Cho *et al*., 2014). As previously mentioned earlier, the relationship between vascular differentiation and auxin-*NOV* signaling was observed and the results strongly supported the relationship between TDIF and vascular development in apple.

In general, our study revealed that hormone related genes differentially fluctuated during adventitious root formation between two growing seasons and different analytical methods reflected the robustness of relationship between adventitious root formation and hormonal signaling. Therefore, these results might have suggested that adventitious root formation in apple is controlled by complex hormonal signaling, such as auxin, cytokinin, ABA, and plant peptide hormone. To clarify this possibility, a further study focusing on endogenous plant hormonal levels will be needed.

Modifying plant growth, architecture and yield has been a farming goal in professional agronomy and horticulture. In our study, the morphological effects of the GA inhibitor PBZ were evidenced by a profound inhibition of both shoot and internode length elongation and this is strongly linked to the significant height reduction in the Marubakaido apple rootstocks. The growth regulating properties of PBZ are mediated by altering the levels of plant hormones such as GAs, ABA and cytokinin (Fletcher and Hofstra, 1990). This is previous study concurs with our study finding and interestingly, PBZ elevated endogenous IAA concentrations in the shoot bark of Marubakaido rootstocks and ultimately resulted in the dwarfing of the apple rootstocks.

PBZ treatment has been well documented to be a GA biosynthesis inhibitor (Fan *et al*., 2018). Previous investigations related to biochemical, molecular and genetic

studies of GA have revealed that biosynthesis, metabolism, transport, and signaling are crucial for normal plant growth and once are interfered with, it leads to defects in growth (Zhu *et al*., 2004; Zhu *et al*., 2016). In our study, PBZ profoundly reduced endogenous concentrations of GA_1 and GA_4, reduced the expression levels of downstream GA signaling genes *MdGID1b-like*. In contrast, genes encoding GA catabolism *MdGA2OX2* were highly upregulated in PBZ-treated rootstocks. Additionally, PBZ repressed the expression of *MdNPF3.1-like* transporter genes. Previous studies have reported that PBZ application leads to repression of GA biosynthesis mediated by decreased expression of biosynthetic genes and signaling genes *GID1*; and promotion of catabolism by increased expression of *GA2OX* which eventually leads to decreased endogenous GA levels and ultimately dwarfing of plants (Fan *et al*., 2018; Zhu *et al*., 2016). Generally, PBZ-induced endogenous GA synthesis suppression, reduced rootstock growth and this was exemplified by a strongly reduced internode elongation.

Auxin plays a crucial role in the regulation of various aspects of plant growth and development (Benková *et al*., 2009). Alteration of endogenous auxin biosynthesis, transport, degradation and signaling may culminate into display of growth defects in plants. The findings in our study showed elevated endogenous IAA levels in the shoot bark of PBZ-treated rootstocks. Also, the efflux and influx auxin carrier related genes *MdPIN1* and *MdLAX1* genes respectively, as well as the IAA biosynthesis related genes *MdYUCCA10a* were upregulated by PBZ treatment in the apices of the Marubakaido rootstocks. Further, PBZ-treated rootstocks observed upregulation of IAA signaling gene *MdARF2*. Elevated IAA concentrations in response to PBZ application might have resulted in dwarfing of the rootstocks which was evident 14 DAT. Transgenic citrus plants expressing an auxin-inducible GUS reporter gene observed elevated auxin concentrations at the basal end of the epicotyl stem cuttings mediated by polar auxin transport and this was inhibitory for invitro shoot organogenesis (Hu *et al*., 2017). Also, *ZmPIN1a* overexpression in maize (*Zea mays*) plants corroborated with increased IAA concentration in the shoots and root tips and this eventually reduced plant height, internode length and ear height (Li *et al*., 2018). Additionally, Schruff *et al*. (2005) observed overexpression of *ARF2* identified as a mutant for mega integument (*mnt*) gene

in *Arabidopsis* as repressor of cell division in many aerial plant organs. Increased IAA levels because of PBZ treatment likely contributed to the dwarfing of the rootstocks, however, we recommend further studies to clarify the mechanism by which PBZ-mediated IAA elevation resulted in dwarfing of the Marubakaido rootstocks.

The sesquiterpene metabolite ABA plays a fundamental role in adaptation to abiotic stress as well as plant development (Nambara and Marion-Poll, 2005). The growth regulating properties of PBZ are mediated by altering the concentrations of ABA and other phytohormones (Fletcher and Hofstra, 1990). In our study, PBZ treatment increased endogenous ABA and its key biosynthesis gene *MdNCED1* but down regulated the catabolism gene *MdCYP707A1* in the apple rootstocks. In PBZ-treated rootstocks, the ABA transporter gene *MdAITb*-like gene was considerably upregulated. The shoot growth inhibition observed in PBZ-treated rootstocks is highly likely because of curtailed catabolism of ABA (Marshall *et al*., 2000). Conversely, PBZ blocks GA biosynthesis, thereby shunting and accumulating more precursors in the terpenoid pathway thus enhancing ABA biosynthesis (Rademacher, 1997). Al together, these findings infer that the PBZ effect on biosynthesis, transport, and degradation processes leads to endogenous ABA accumulation in Marubakaido rootstocks, hence dwarfing.

Apical dominance, shoot/root growth and leaf senescence are leaf and developmental processes affected by cytokinin and have also been implicated to potentially induce cell division in tissue culture (Sakakibara, 2006). Kamountsis and Sereli (1999) reported that PBZ has effect on cytokinin as a result influence plant growth. In our study, PBZ treatment markedly reduced *trans*-Zeatin (*t*Z) concentrations in the sub-apical part of the Marubakaido shoots. In contrast, Upreti *et al*. (2013), observed increased cytokinin in the flower buds of mango as it approached bud break but opposite trends in the leaves because of PBZ application. This discrepancy might have resulted on the specific roles exhibited by cytokinin at a given developmental stage and plant part, a case in point, flower initiation and vegetative growth. To agree with our study finding, Zhu *et al*. (2004) reported decreased Z and ZR levels in the xylem sap of PBZ-treated apple plants. Consistent with our result above, was the reduced expression of cytokinin biosynthesis gene *MdIPT6*. Additionally, PBZ-treatment upregulated the expression of

two *PUP* transporter-like genes, *MdPUP7-like* and *MdPUP9-like*. This finding prompted us speculate that the upregulation of the two transporter genes may have resulted in the inhibition of cytokinin perception by the PM localized receptors (Zürcher *et al*., 2016) or perhaps sequestration of cytokinin out of the ER (Romanov *et al*., 2018) eventually leading to low signaling output hence dwarfing the apple rootstocks. However, the supposition above is under consideration because according to Zürcher and Muller (2016), the functional relevance of PM vs ER- localized receptors is yet to be determined experimentally. Hence, further studies are suggested on characterization of *PUP* gene family members and their exact site of signaling perception.

CHAPTER 6

Conclusion

In our first study, before the visibility of first AR, the induction of auxin related genes *ABC transporter G family member-*, *NO VEIN-*, and *Auxin transporter-like protein 2*-like associated with cellular auxin distribution and maintenance of root meristem niche was observed. There was an association between AR formation and phytohormones such as cytokinin and ABA. Plant peptide hormone related genes were also involved in AR formation. Generally, in this study it was established that phytohormone signaling of auxin, cytokinin, ABA, and plant peptide hormone related genes were at play at different phases of AR formation.

The second study revealed that PBZ-mediated dwarfing of Marubakaido apple rootstocks was because of alteration of endogenous auxin, ABA, GAs, and zeatin. Notwithstanding, genes involved in biosynthesis, transport, inactivation and signaling of each respective phytohormone were modulated and ultimately restricted growth of the rootstocks.

SUPPLEMENTARY

Table S1 Primer sets used in the study; Association of auxin, cytokinin, abscisic acid, and plant peptide response genes during adventitious root formation in Marubakaido (*Malus prunifolia* Borkh. var. ringo Asami) apple rootstock

Target sequence	Sequences (top: forward primer, bottom: reverse primer)
ABCG37-like	5'-TGGCTCAGTTGGCAGGGG-3' 5'-CGCAAACTCGAGGCGTGG-3'
NOV-like	5'-GTCAGTCGGGCAAGCTGC-3' 5'-AGTCACATGGGGGCTGGC-3'
LAX2-like	5'-TGGATTCGGGGGTTGGGC-3' 5'-AAGACCATGGGCGGCAGC-3'
AP2C1-like	5'-GCAGGCTTCGGGTTTGGC-3' 5'-CCTCCACTCTCTCCGCCG-3'
MYB73-like	5'-TAACCAGCTGTCGCCGCA-3' 5'-TTGAGGAGGCGGGCGATG-3'
WRKY33-like	5'-CCGTGGGTTGTCCGGTGA-3' 5'-GCTGCGGGAACATCGTGG-3'
ERF105-like	5'-TGGGGCAAGTGGGTGTCC-3' 5'-TGATCGTCATGGCCGCCA-3'
BAM3-like	5'-CCCGAATCAGGGCAGTTT-3' 5'-GGTTTTTCGGGGTGTGCT-3'
PXY-like	5'-GGCGGCGTTTTGGGGAAG-3' 5'-CCGAAAGCAGCCGCCATG-3'
Histone H3a	5'-GTCAAGAAGCCCCACAGATAC-3' 5'-CTGGAAACGCAGATCAGTCTTG-3'
SAND	5'-CCCAGGACTTTGAGCTTTATGC-3' 5'-TATCACCATGAAAAGGGGCTTG-3'

EF1	5'-TCACATCAACATCGTGGTCA-3'	
	5'-TCGAACCTCTCAATCACACG-3'	

Table S2 Primers used for qRT-PCR analysis in the study; Paclobutrazol elevates auxin and abscisic acid, reduces gibberellins and zeatin and modulates their transporter genes in Marubakaido (*Malus prunifolia* Borkh. var. ringo Asami) apple rootstocks.

Gene name		Sequence (5'-3')	Accession no.
MdPIN1	(F)	GCGGGATCCATTGTCTCCATCC	XM_008395204
	(R)	CTGAGACCGCCGCGAGAAAATA	
MdLAX1	(F)	ATATGCTCACCTACAGAA	XM_029089637
	(R)	TATGAAGGAGTTGATTACG	
MdARF2	(F)	CGATAGACGAAGAGAAGAT	XM_008375953
	(R)	TCTGTGTAAGTTGGTTCA	
MdYUCCA10a	(F)	TCGACAAGGCTGTTGTGCTC	XM_008380434
	(R)	TGGCAAAGACCTGATGTCTCC	
MdAITb-like	(F)	CCGTGAGAGTACTCGGGTCA	XM_008341722
	(R)	CTGCTAACATTTTTGCCATCA	
MdNCED1	(F)	GTATCACGTCCAAATCACTGAAAC	XM_009356579
	(R)	ATTTGAGGTATGGCTTCTGAACG	
MdCYP707A1	(F)	GAAGAGGTATGCTTTTGATGTGG	XM_008358695
	(R)	TCAACAAGCCACCACTATCTTCT	
MdNFP3.1-like	(F)	TGCTAATGAGGTTTGTGAGAAGTTGG	XM_008341460
	(R)	TGCGTTGTCAAGTAGCTTATCATGT	
MdGID1b- like	(F)	ATGAAGTCAACGTCAATGAATCCAAG	NM_001294349
	(R)	AGGTTGTAAGCCAGCTTGAAATTGG	
MdGA2OX2	(F)	CACACAGACCCACAGATCATT	XM_008385257
	(R)	TTAGTCATCACCTGCAAGCAATC	
MdIPT6	(F)	GATGTGTAGGCAGCAAGGAGGAAG	XM_008388017
	(R)	GATTCAGGCACGACCAGAAGTTCA	
MdPUP7-like	(F)	CGGTCATGGACATGGTAGTG	XM_008385284
	(R)	CCTCCATCTCACCCTTCAAA	
MdPUP9-like	(F)	CTTCCTGTATCTACTTATTCC	XM_008373357
	(R)	CAATGTAAGCAGTGAACT	
MdCKX7	(F)	ATTGCCACACCAGCCACTCCA	XM_029092051
	(R)	TGCTGCGTGATTGTTGTTGTTGAA	
MdUBQ	(F)	CTCCGTGGTGGTTTTTAAGT	XM_008360582
	(R)	GGAGGCAGAAACAGTACCAT	
MdSAND	(F)	CCCAGGACTTTGAGCTTTATGC	XM_008356962
	(R)	TATCACCATGAAAAGGGGCTTG	
MdHISH3	(F)	GTCAAGAAGCCCCACAGATAC	XM_008345103
	(R)	CTGGAAACGCAGATCAGTCTTG	

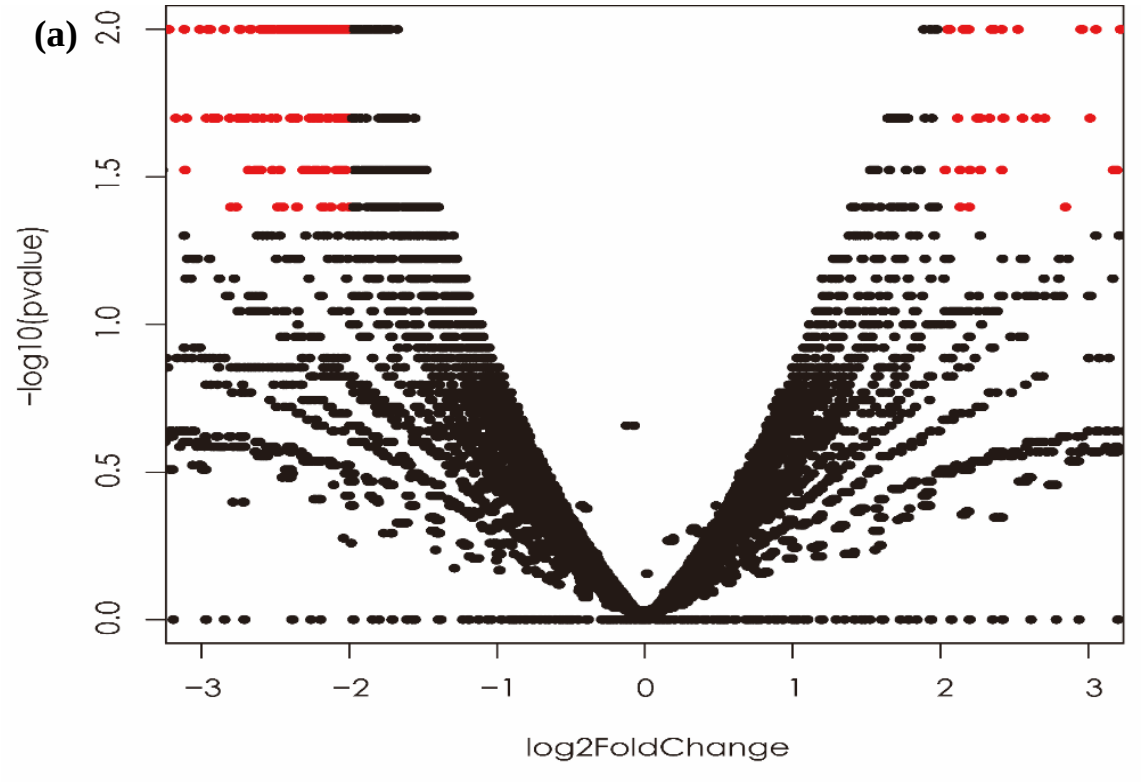


Fig. S1 (a) A summary of DEGs identification by volcano plot

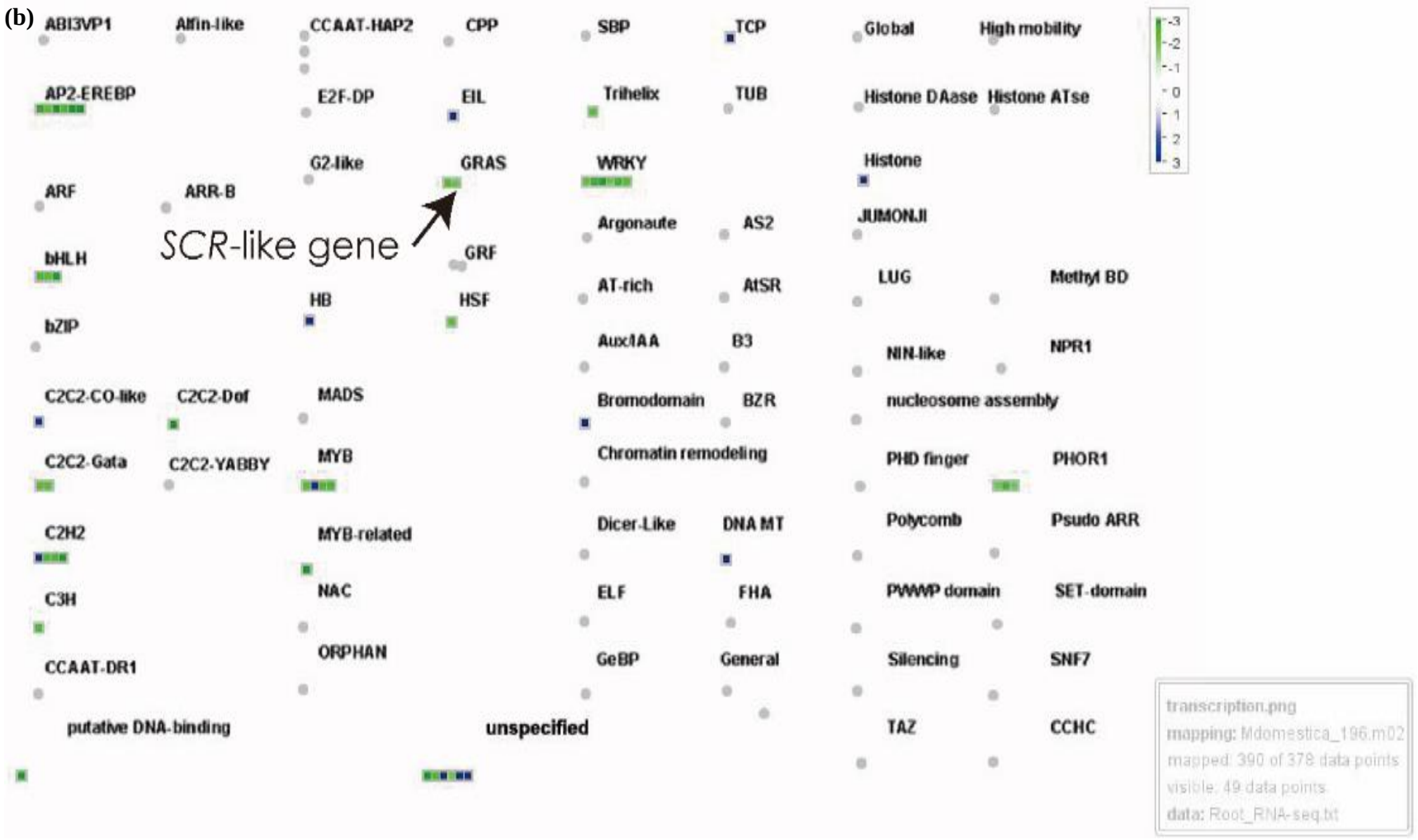


Fig. S1 (b) MapMan visualization showing changes of mRNA levels belonging to "transcription" based on log2-fold changes in C70 vs. R10 during 2016–2017 growing season. Blue and green represent highly expressed genes in C70 and R10, respectively.

(c)

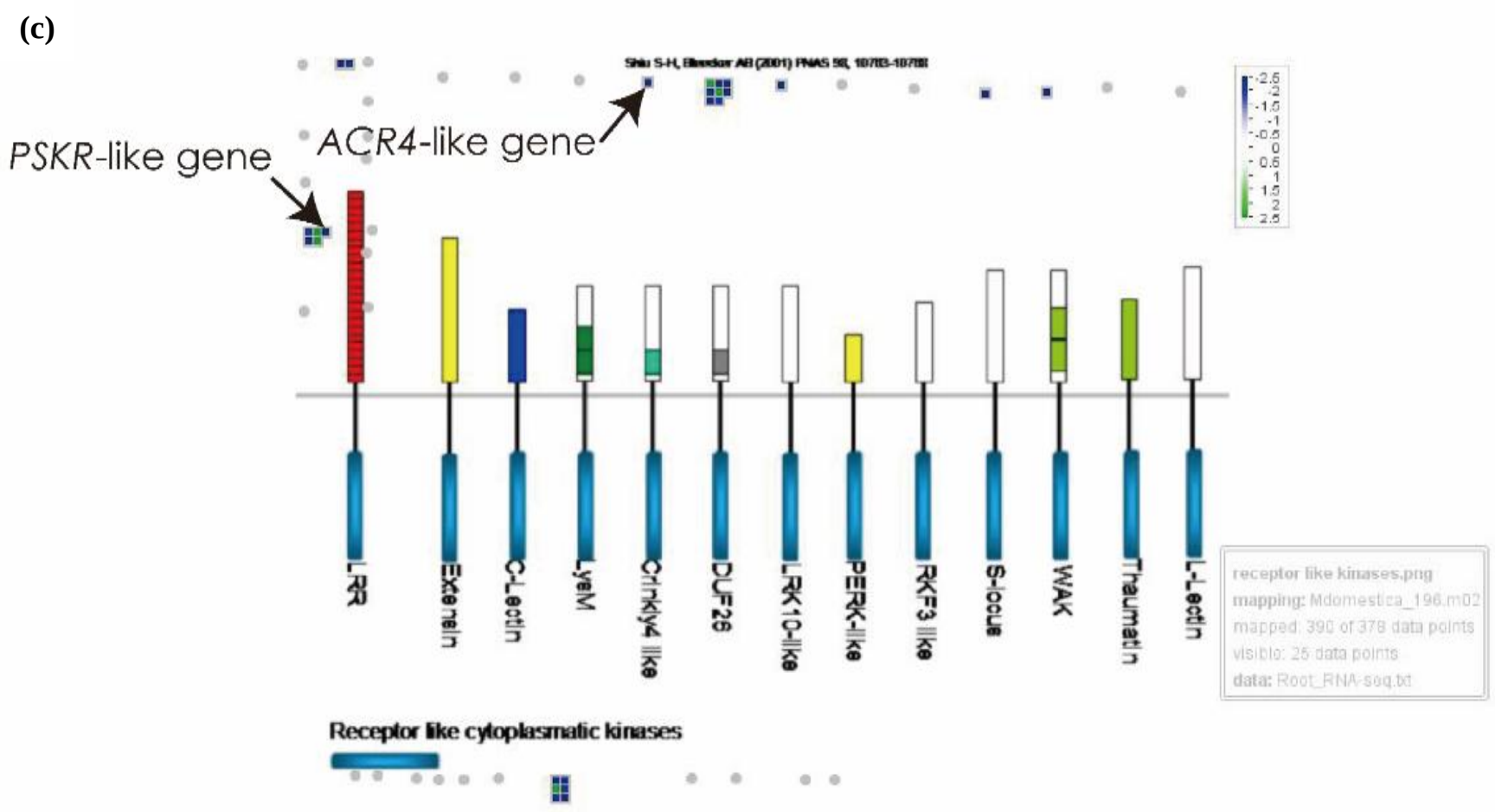


Fig. S1 (c) MapMan visualization showing changes of mRNA levels belonging to "receptor like kinases" based on log2-fold changes in C70 vs. R10 during 2016–2017 growing season. Blue and green represent highly expressed genes in C70 and R10, respectively

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