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Adventitious root formation of *in vitro* peach shoots is regulated by auxin and ethylene

Sang-Ho Park[a,1], Mohamed Elhiti[a,1,2], Huaiyu Wang[a], Anna Xu[a,3], Dan Brown[b,*], Aiming Wang[a,*]

[a] London Research and Development Centre, Agriculture and Agri-Food Canada,1391 Sandford Street, London, Ontario, N5 V 4T3, Canada
[b] Canadian Centre for Agri-Food Research in Health and Medicine,351 Tache Avenue, Winnipeg, Manitoba, R2H2A6, Canada

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ABSTRACT

Adventitious root formation is a critical step in micropropagation and genetic transformation. However, it is often a limiting factor for some crop species, particularly woody plants. In this report, we studied adventitious rooting using *in vitro* shoots of peach, a fruit tree species that is notoriously recalcitrant to genetic transformation and adventitious root induction. We found that culture age affected adventitious rooting efficiency. Hormone analysis revealed that the peach shoots maintained *in vitro* for 2 years (yr) with a higher rooting rate contained more endogenous indole-3-acetic acid (IAA) than those grown *in vitro* for 1yr under the same growth conditions.

Treatment: of peach shoots with Aminoethoxyvinylglycine (AVG), a potent auxin biosynthesis inhibitor, inhibited adventitious rooting. To explore the association of gene expression with adventitious rooting, we performed a comparative transcriptome analysis. We found that genes encoding key enzymes in auxin biosynthesis were up-regulated in 2yr shoots. In contrast, genes involved in ethylene biosynthesis and its signaling pathway were down-regulated in 2yr shoots. Addition of 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate metabolic precursor of ethylene, significantly inhibited adventitious rooting in a dose-dependent manner. Therefore, auxin and ethylene act antagonistically on adventitious rooting. Taken together, our results shed new insights into the mechanism regulating adventitious rooting of peach shoots, and may help develop novel rooting methods for peach and related woody plants.

1. Introduction

Biotechnologies such as *in vitro* micropropagation techniques have been widely used for the multiplication of diverse plant species including *Prunus* fruit species (Martínez-Gómez et al., 2005). However, rooting is often the bottleneck of *in vitro* propagation, particularly for woody plants (Díaz-Sala 2014; Guan et al., 2015; Legué et al., 2014). Previous studies have suggested that adventitious root formation of woody plants is linked to the action of endogenous auxin and can be triggered by the application of exogenous auxin such as indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), or α-Naphthaleneacetic acid (NAA) (Blakesley 1994; Krikorian 1995; Ludwig-Müller et al., 2005; Pacurar et al., 2014; Pamfil and Bellini 2011). However, the physiological ages of shoots are complex and the rooting response to exogenous auxin depends on the plant species and cultivars. As the most studied and abundant natural auxin, IAA was the first used to promote adventitious root formation. IBA has become the most widely used auxin for inducing adventitious root in many species. IBA is more potent than IAA and is suggested to a precursor to IAA (Cooper 1935; Kurepin et al., 2011; Woodward and Bartel, 2005).

Although auxin plays a central role in adventitious rooting, phytohormones interact with one another and the complex cross-regulatory interaction network between auxin and many different phytohormones controls root development (Bellini et al., 2014; Pacurar et al., 2014). Indeed, the complex interactions between auxin and ethylene affect primary root elongation, lateral root development, and root hair initiation and elongation (Muday et al., 2015; Muday et al., 2012). Auxin promotes and ethylene inhibits lateral root development, while both have a negative effect on primary root elongation (Alarcón et al., 2013; Mockaitis and Estelle 2008; Růžička et al., 2007; Swarup et al., 2007). Ethylene and auxin act synergistically on root hair initiation and elongation (Pitts et al., 1998; Rahman et al., 2002; Strader et al., 2010). These studies indicate that ethylene negatively regulate root elongation and lateral root formation and positively affect root hair initiation and

* Corresponding authors.
E-mail addresses: danielbrown500@gmail.com (D. Brown), Aiming.Wang@AGR.GC.CA (A. Wang).
[1] These authors contributed equally to this work.
[2] Current address: Department of Botany, Faculty of Science, Tanta University, Tanta, 31527, Egypt.
[3] Current address: Leslie Dan Faculty of Pharmacy, University of Toronto, 144 College St, Toronto, Ontario, M5S 3M2, Canada.

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Table 1
The effect of IBA, exposure time and sugar source on adventitious rooting of peach shoots (2yr old).

Treatment[a]	IBA (mg/L)	Exposure time (day)	sugar (g/L)	Rooting rate (%)[b]	Callus forming rate (%)[b]
T2-1	1	3	sucrose 10	59.0 ± 6.0^{bc}	00.00 ± 00.00^{e}
T2-2	1	5	fructose 10	54.0 ± 5.1^{bc}	11.66 ± 02.11^{cde}
T2-3	1	7	fructose10 + glutose5	45.0 ± 5.0^{c}	23.82 ± 07.46^{bcd}
T2-4	3	3	fructose10	82.2 ± 4.9^{a}	17.28 ± 05.16^{bcde}
T2-5	3	5	fructose10 + glutose5	68.2 ± 6.8^{ab}	38.72 ± 11.50^{b}
T2-6	3	7	sucrose 10	45.4 ± 5.6^{c}	33.62 ± 06.23^{bc}
T2-7	5	3	fructose10 + glutose5	64.7 ± 3.9^{abc}	36.90 ± 07.94^{b}
T2-8	5	5	sucrose 10	54.9 ± 3.9^{bc}	37.28 ± 07.33^{b}
T2-9	5	7	fructose 10	60.5 ± 7.3^{bc}	86.18 ± 05.14^{a}
T2-10	0.5	23	fructose10 + glutose5	53.5 ± 3.6^{bc}	07.00 ± 00.00^{de}

[a] 9 treatments (T2-1 to T2-9) were generated according to the orthogonal array $L_9(3^4)$ and T2-10 was set arbitrarily.
[b] All values are expressed by means ± standard error of mean with a minimum of 10 shoots per replicate (5 replications; the total shoot number was over 500). Different letters in the same column indicate statistically significant differences. Raw numerical data were analyzed by ANOVA and the significance of differences among means was carried out using Duncan's Multiple Range Test.

elongation. However, the effects of ethylene on adventitious root formation are more complex and differ among species. In tomato, ethylene enhances adventitious root formation, but treatment with the immediate precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC) and mutations that cause increased ethylene synthesis reduce adventitious rooting in *Arabidopsis* (Negi et al., 2010; Sukumar 2010). These results showed that ethylene has an opposite effect on adventitious root formation in these two species, while auxin positively regulates adventitious root formation in both (Clark et al., 1999; Li et al., 2009; Ludwig-Müller et al., 2005; Sorin et al., 2005; Sorin et al., 2006; Tyburski and Tretyn 2004).

Accumulated genetic and physiological evidence suggests that biotic and abiotic cues may regulate primary, adventitious, and lateral root system architecture via modulating phytohormone homeostasis and/or signaling (Jung and McCouch, 2013; Koltai 2013; Malamy 2005; Malekpoor Mansoorkhani et al., 2014; Osmont et al., 2007; Rogers and Benfey 2015). The physiological characteristics, and the age of propagation materials in particular are also closely linked to adventitious root formation (de Klerk et al., 1999; Osterc et al., 2009). Juvenile materials are easier to culture and are more suitable for successful rooting (Morgan et al., 1980; Osterc and Štampar, 2011; Osterc et al., 2009). Juvenile cuttings enhance root development apparently because they contain higher concentrations of free IAA than mature cuttings at the time of severing (Osterc et al., 2009). Although adventitious root formation of stockplant materials has been discussed in many plants, the evaluation of physiological age on root development on *in vitro* cultured shoots remains poorly understood.

Prunus species include several very important fruit trees whose genetic improvement is hindered by their recalcitrance to genetic transformation and adventitious root induction (Felek et al., 2017; Wang et al., 2011; Wang et al., 2013). Of them, peach (*Prunus persica* L. Batsch) is recognized as one of the most difficult-to-root fruit trees (Hammerschlag et al., 1987; Pérez-Clemente et al., 2004). In peach, it has been reported that the length of *in vitro* culture time greatly influenced rooting rate in all cultivars tested (Hammerschlag et al., 1987). However, the underlying mechanism remains obscure.

In this study, we found a significantly low rooting capacity of peach shoots cultured *in vitro* for 1 year (1yr) compared with the shoots maintained in culture for 2yr and identified the best exogenous hormone combinations for rooting of peach shoots. Furthermore, we conducted hormonal analysis, comparative transcriptome profiling and pharmacological studies to gain insights into the molecular mechanisms regulating adventitious rooting.

2. Materials and methods

2.1. Preparation of in vitro peach shoots

In vitro shoot explants (*Prunus persica* cv. Loring) were prepared as described (Elhiti et al., 2016). Peach branches (45–70 cm in length) were collected from Vineland Station, Ontario, Canada. Branches were wiped with 70% ethanol and buds (explants) were cut from the branches with woody base. The buds were sterilized in 70% ethanol for 30 s and washed with sterile ddH_2O. The buds were placed in 15% commercial bleach for 20 min, followed by washes with sterile ddH_2O with shaking at 120 RPM. The buds were incubated in the QL liquid medium (Quoirin & Lepoivre, basal salt supplemented by 0.2 mg/L BA, 10 g/L fructose, 5 g/L glucose at 25 °C under 1500 ~2000 lx light with 16 h photoperiod for 2 weeks (Quoirin and Lepoivre 1977). When the buds opened and green leaves emerged, the explants were transferred to the QL solid medium (Quoirin & Lepoivre basal salt, 0.5 mg/L BA, 10 g/L fructose, 5 g/L glucose). Shoots were propagated on the QL proliferation medium supplemented with 1 mg/L BA, 10 g/L fructose, and 5 g/L glucose (Pascual and Marin, 2005). Shoots were subcultured onto fresh media every three to four weeks by segmenting individual shoots or shoot clusters.

2.2. Optimization of in vitro rooting

To evaluate the relative importance of selected factors (IBA, exposure time, and sugar) on root formation, peach shoots from the QL proliferation medium were exposed to the Rose modified rooting basal medium (PhytoTechnology Laboratories, KS, USA) with treatments designed according to the L9(3^4) and L16(4^5) orthogonal arrays (Hedayat et al., 2012; Taguchi 1986; Taguchi 1987), also known as the Taguchi's method (four factors at three levels and five factors at four levels, respectively; Table 1, 2 and 3). The orthogonal array design consisted of the following parameters: IBA (1, 3 and 5 mg/L), exposure time (3, 5, and 7 days) and sugar (Sucrose 10 g/L, fructose 10 g/L, and fructose 10 g/L + glucose 5 g/L) from Table 1, IBA (0.5, 1, 3 and 5 mg/L), IAA (0, 0.5, 0.1 and 0.3 mg/L), NAA (0, 0.02, 0.05 and 0.1 mg/L), Phloroglucinol (0, 5, 10 and 15 mg/L) and exposure time (3, 5, 7, and 14 days) from Table 2 and IBA (3,4, and 5 mg/L), exposure time (3, 5, and 7 days) and AVG (0, 10 and 30 μM) from Table 3. To examine the role of ethylene on adventitious rooting, peach shoot explants were tested on A-7 media (5 mg/L IBA, 0.3 mg/L IAA, 0.03 mg/LNAA, and 5 days of exposure) in the presence of 50 and 100 μM of ACC.

2.3. IAA quantification by HPLC-ES-MS/MS

Peach shoots cultured for one year and two years on the QL proliferation medium were collected and washed by deionized water three