ORIGINAL ARTICLE

Root predominant overexpression of *iaaM* and *CKX* genes promotes root initiation and biomass production in citrus

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Abstract

Promoting initiation and biomass production of roots is significant for plant-based industries including using roots as bioreactors. Two citrus genotypes, Carrizo and US-897, were used as model plants to test the effects of root-predominantly overexpressed the *iaaM* (indoleacetic acid-tryptophan monooxygenase) gene and a *CKX* (a cytokinin oxidase/dehydrogenase) gene. The *iaaM* transgenic lines exhibited markedly faster root initiation, more root numbers, and higher root biomass compared to their wild-type counterparts. The transgenic *iaaM* + *CKX* plants also exhibited similar phenotypes, albeit to a lesser extent than the *iaaM* plants. Molecular analysis revealed an auxin-responsive *CsGH3.1* gene was up-regulated in the *iaaM* roots and *iaaM* + *CKX* roots, and a cytokinin-responsive gene *CsARR5* gene was down-regulated in the *iaaM* + *CKX* roots. Our results demonstrate that root predominant overexpression of the *iaaM* or both the *iaaM* and *CKX* genes drastically enhances the initiation, growth and biomass production of roots. These results provide additional support that manipulation of auxin and cytokinin levels in roots via transgenic or gene-editing technologies may benefit production of high-value secondary metabolites using roots as bioreactors and also improve rooting of recalcitrant plant species.

Key message

Root-predominant overexpression of an auxin synthetic gene and a cytokinin degradation gene in citrus enhance initiation and biomass production of roots.

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Abbreviations

SbUGT	*Scutellaria barbata* flavonoid glycosyltransferase gene
iaaM	Indoleacetic acid-tryptophan monooxygenase gene
CKX	Cytokinin oxidase/dehydrogenase gene
GUS	β-Glucuronidase
X-gluc	5-Bromo-4-chloro-3-indolyl-b-D-glucuronic acid
nptII	Neomycin phosphotransferase gene
6-BA	6-Benzylaminopurine
NAA	Naphthaleneacetic acid
IBA	Indole-3-butyric acid
OD	Optical density
AS	Acetosyringone

Introduction

Roots, 'the hidden half' of the plant, directly mediate nutrient and water uptake and thus influence overall plant growth and development (Verma et al. 2021). A robust root system provides stronger support of the plant to strong winds and floods, and enhanced tolerance to abiotic stress and biotic stress (Koevoets et al. 2016). Plant roots have also been widely used as a source of bioactive molecules that are used as pharmaceuticals, agrochemicals, flavors, fragrances, pigments, bio-pesticides, and food additives (Ardalani et al. 2021; Murthy et al. 2008).

The development of complex root architectures has been a notable outcome in the evolution and adaptation of higher plants in their surroundings, enabling their colonization of land and allowing them to meet nutrients and water requirements (Verma et al. 2021). Vegetative or clonal propagation is an important practice in horticulture and forest industries to produce large numbers of homogeneous plants efficiently and cost-effectively (Guan et al. 2015; Lakehal and Bellini 2019). Adventitious roots, which mainly arise from stems and shoots, are essential for newly propagated plants to survive and are a major component of the mature root system of many plant species. Also, adventitious root development is a crucial step in clonal multiplications, micropropagations and genetic transformation (Legué et al. 2014; Rigal et al. 2012). Often, the adventitious root initiation is a limiting factor for may plant species particularly woody species including some citrus varieties that are difficult to root (De Almeida et al. 2017; Park et al. 2017).

Current advances in plant biotechnology provide an opportunity to culture plant cells, tissues, and organs to produce many important secondary metabolites in a shorter time than whole plant cultivation in the field (Baque et al. 2012; Jeong et al. 2009a, b). Roots, particularly adventitious roots, are an excellent source of highly valuable metabolites (Hussain et al. 2022; Murthy et al. 2008). Adventitious root culture has successfully been used to scale up production of secondary metabolites for many medicinal plants, such as *Panax ginseng* (Jeong et al. 2006; Wang et al. 2013), *Oploanax elatus* (Jiang et al. 2015), *Eurycoma longifolia* (Fan et al. 2021), *Echinacea angustifolia* (Cui et al. 2013), *Plantago ovata* (Budzianowska et al. 2022), and *Rehmannia glutinosa* (Rahmat et al. 2021). Adventitious root culture has been show to produce high yield and high quality secondary metabolites (Wang et al. 2013; Wu et al. 2011). For example, when adventitious roots of *Echinacea purpurea* were cultured in air-lift bioreactors, both biomass and secondary compounds increased by tenfold after 4 weeks of culture (Jeong et al. 2009a, b). Adventitious roots of *Astragalus membranaceus* produced significantly higher total polysaccharide and total saponin content compared to three-year-old roots grown in the field (Wu et al. 2011). In addition, Wang et al. (2013) reported that the total saponin content in adventitious roots was much higher than in native ginseng cell and hairy root tissue culture. Furthermore, a cluster analysis revealed that the quality of saponins produced from adventitious roots was mostly similar to that of native ginseng, and the extracts of ginseng adventitious roots exhibited a similar cellular immunoregulatory effects on mice as those of native ginseng roots.

The development and growth of adventitious roots are complex process regulated by both environmental and endogenous factors (Agulló-Antón et al. 2014), among which the plant hormone auxin and cytokinins play a central role (Da Costa et al. 2013; Della Rovere et al. 2013; Pan et al. 2021; Pacurar et al. 2014). Auxin is an essential plant hormone involved in growth and developmental process, including but not limited to cell elongation, cell division, vascular differentiation, and root growth and development (Barbez et al. 2017; Di et al. 2021; Kasahara 2016; Li et al. 2016; Wang et al. 2008; Li and Jia 2022; Camalle et al. 2022; Niu et al. 2022). Overexpressing of *YUC* and *TAA1* genes, which are involved in auxin biosynthesis in *Arabidopsis*, significantly promotes formation of adventitious and lateral roots (Mashiguchi et al. 2011). When adventitious roots are used as bioreactors, auxin has always been used to enhance initiation and biomass production of roots (Jeong et al. 2006; Wang et al. 2013; Jeong et al. 2009a, b; Cui et al. 2010). The *Agrobacterium tumefaciens* indoleacetic acid-tryptophan monooxygenase (*iaaM*) gene encodes an enzyme that catalyzes the conversion of amino acid tryptophan (Trp) into indole-3-acetamide, which is hydrolyzed to indole-3-acetic acid (IAA) in plant cells (Weijers et al. 2005; Zhai et al. 2021). Overexpression of the *iaaM* gene in petunia (Klee et al. 1987), tobacco cv *Samsun* (Romano et al.

1991), and tobacco cv *Xanthi* (Li et al. 2017) led to drastic increases in adventitious and lateral roots.

Cytokinins are another important regulator of adventitious root development (Agulló-Antón et al. 2014). As a negative regulator in root growth and branching, lowering cytokinin levels often lead to larger root systems (Jameson and Song 2020). With root-specific expression of a cytokinin oxidase/dehydrogenase (*CKX*) gene in barley, the transgenic plants developed larger root systems (Ramireddy et al. 2018). In chickpea, root-specific expression of chickpea cytokinin oxidase/dehydrogenase 6 (*CaCKX6*) gene enhanced root growth (Khandal et al. 2020). Root-predominant expression of *AtCKX2*, a cytokinin oxidase/dehydrogenase gene from *Arabidopsis*, in poplar enhanced the root growth of poplar trees (Li et al. 2019).

However, relatively little has been done to genetically manipulate auxin and cytokinin levels in woody plants, particularly in a root specific manner. In this study, we report production and characterization of transgenic plants that overexpress an *iaaM* gene and in combination with a *CKX* gene predominately in roots of two commercial important citrus genotypes, Carrizo and US-897.

Materials and methods

Ti-plasmids and *Agrobacterium*

The − 102 to + 86 relative to the transcription start site of a flavonoid glycosyltransferase gene (*SbUGT*) promoter sequence from *Scutellaria barbata* (Chiou et al. 2010), was used to drive the expression of *Arabidopsis* cytokinin oxidase/dehydrogenase 2 gene (*AtCKX2*). The *SbUGT::AtCKX2* and terminator were synthesized as one fragment and subcloned into the *SbUGT*::*iaaM* (abbreviated as *iaaM)* plasmid (Li et al. 2017) to create the *SbUGT::iaaM + SbUGT::AtCKX2* construct (abbreviated as *iaaM + CKX*). In *SbUGT::iaaM, SbUGT::iaaM + SbUGT ::AtCKX2*, and *SbUGT::GusPlus* constructs (Li et al. 2019), the *nptII* gene and the catalase intron inserted plant-specific expressed *GusPlus* (Li et al. 2017; Vickers et al. 2007) gene were used as the select reporter. All those three constructs were introduced into *Agrobacterium tumefaciens* strain EHA105 and used to transform Carrizo and US-897 seedlings, respectively.

Citrus explant preparation and *Agrobacterium*-mediated transformation

The Carrizo (*Citrus sinensis* x *Poncirus trifoliata*) and US-897 (*C. reticulata* x *P. trifoliata*) seeds were purchased from Lyn Citrus Seed, Inc. in Arvin, CA, USA and stored at 4 °C prior to use. Healthy seeds were selected, and their outer seed coats were carefully removed. The seeds were then sterilized by immersing them in 75% ethanol for one minute, followed by freshly prepared sodium hypochlorite solutions (1% sodium hypochlorite active gradients, pH adjusted to 7.0–7.2 with 1 M HCl, and several drops of Tween-20 added before use) for 15 min and rinsing with sterilized distilled water for five to six times.

Inner seed coats were removed aseptically, and then seeds were cultured on a M519 medium (purchased on https://phytotechlab.com) containing 30 g/L sucrose and 7 g/L agar, with a pH 5.8 (abbreviated as MS medium later on) in a growth chamber at 28 °C in complete darkness for 3–4 weeks. Epicotyls of etiolated seedlings were cut 1 cm in length. The EHA105 *Agrobacterium* harboring *SbUGT::iaaM*, *SbUGT::iaaM + SbUGT::AtCKX2,* and *SbUGT::GusPlus* constructs were cultivated until the OD_{600} around 0.6–0.7 and then resuspend in the MS liquid medium containing 50 mg/L Acetosyringone (AS). The suspended solutions were incubated at 28 °C with shaking at 200 rpm for 30 min and then used to infect the epicotyl segments for 20 min. After removal of residual liquid, the infected epicotyl segments were transferred onto the MS medium containing 50 mg/L AS.

After a 3-day co-cultivation in dark, explants were transferred to the MS medium that was supplemented with 6-BA (1 mg/L), NAA (0.1 mg/L), kanamycin (50 mg/L), and timentin (150 mg/L) (Zhang et al. 2017). The explants were then cultured in at 26 ± 1 °C and a 16/8-h light/dark cycle, with a photon flux intensity of 60 μmol m^{-2} s^{-1}. Every 3–4 weeks, the explants were sub-cultured.

Histochemical GUS activity assay

Sliced leaves of regenerated shoots (putative *SbUGT:: iaaM or SbUGT*::*iaaM + SbUGT::AtCKX2* transgenic shoots)*,* as well as intact rooted plants (putative *SbUGT::GusPlus* transgenic plants), were histochemically stained for GUS activity by incubation in X-Gluc solutions (Zhai et al. 2021) at 37 °C overnight. After the staining, the tissues or entire plants were treated with ethanol to remove chlorophylls and other pigments, until the mesophyll tissues turned white. Visual inspection was conducted for GUS positive tissues or plants.

Molecular confirmation of transgenic plants

Genomic DNA was extracted from leaves of wild-type and representative GUS-positive *SbUGT::iaaM* and *SbUGT ::iaaM + SbUGT::AtCKX2* independent transgenic lines using Macherey–Nagel NucleoSpin Plant II Genomic DNA extraction kit (Macherey–Nagel, Allentown, PA). The purified genomic DNAs were used as template for PCR, with the *iaaM* and *AtCKX2* genes being amplified using specific primer pairs. The *iaaM* gene primer pair (iaaM-F: 5′-GTC

TACCAAGGCGTCCAATAC-3′ and iaaM-R: 5′-CAGATG TGACCACCACCTTATC -3′) produced a 595 bp amplicon, while the *CKX* gene primer pair (CKX-F: 5′-CCGGTT TCTTGGACGGATTA-3′ and CKX-R: 5′-TCCGGTTTG TTGGATAGAGAAG-3′) produced an amplicon of 787 bp. The PCR reaction mix (20 μL) contained 10 μL PCR buffer (EmeraldAmp® GT PCR Master Mix, Takara), 0.25 μM of each primer, and 200 ng of DNA. The amplification protocol consisted of an initial denaturation step at 95 °C for 3 min, followed by 32 cycles of 95 °C for 30 s, 60 °C for 30 s, and 68 °C extension for 40–50 s, and a final extension at 68 °C for 5 min.

Characterization of root initiation and growth

Shoots approximately 1.3 cm in length from the wild-type and GUS-positive *SbUGT*::*iaaM* and *SbUGT::iaaM + SbUGT::AtCKX2* plants were cultured in MS medium supplemented with 150 mg/L timentin. After 2 months of culture, the number of roots per plant was recorded.

Based on the preliminary characterization of rooting, about 1.3 cm long shoot of wild-type, representative *SbUGT::iaaM* and *SbUGT::iaaM + SbUGT::AtCKX2* transgenic Carrizo and US-897 lines were cultured in MS medium supplemented with 150 mg/L timentin. The number of days that the first root in each shoot could be observed were recorded, and the number of roots per plant was counted after 2 months of culture. After 6 months for Carrizo plant lines or 4 months for US-897 plant lines, dry root biomass was measured.

Roots of Carrizo wild-type, *SbUGT::iaaM* expresser *iaaM*#7, and *SbUGT::iaaM + SbUGT::AtCKX2* expresser *iaaM + CKX*#13, were cut into approximately 1 cm lengths and cultured on MS medium at 25°C under complete darkness. Three biological replicates were performed. Each replicate has four to eight root segments from wild-type, *iaaM*#7, and *iaaM + CKX*#13 plants, respectively. The number and length of new roots that produced were recorded after 8 weeks of culture.

Real-time quantitative reverse transcription PCR (RT-qPCR) analysis

Total RNAs were extracted from roots of Carrizo wild-type, representative *SbUGT::iaaM*, and *SbUGT::iaaM + SbUGT::AtCKX*2 lines using the NucleoSpin plant RNA Mini kit (Macherey–Nagel, Allentown, PA) according to the manufacturer's instructions. cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, Richmond, CA). 10× diluted cDNA was used as a template for qPCR using the iQ™ SYBR® Green Supermix kit (Bio-Rad Laboratories,

Hercules, CA) on a CFX96TM RealTime PCR detection system (Bio-Rad Laboratories, Hercules, CA). The specific gene expression levels were measured using the following primers: iaaM-qF: 5′-TGGATTTCTCCGAAGCAC A-3′, iaaM-qR: 5′-CCCGGTAACGCATTTCAT-3′ (Li et al. 2017), CKX-qF: 5′-TCTACCGATCCTTCCATCATCT-3′, CKX-qR: 5′-CGTATTGGAGGAGACGAGAGATA-3′, GH3.1-qF: 5′ TGAGTTCTTGCCTCACGACC-3′, and GH3.1-qR: 5′-TGGGGAGCCGAGTTGTAGTA-3′ (Zou et al. 2019), ARR5-qF: 5′-CAACGGCTCGTCAAGGAA -3′, and ARR5-qR: 5′-ACTATCGTCGACAGCAAGAAC-3′, Actin-qF: 5' -TCTCTTGAACCTGTCCTTGGA-3′, and Actin-qR: 5′-AGTGCCGATACGCTGTCTA-3′ (Hu et al. 2016).

The Actin-qF and Actin-qR primers were used to amplify the internal reference gene *ActB*, a highly conserved β-actin gene in citrus, to normalize the samples. Three replicates were performed on each sample.

Results

Activity of the SbUGT promoter in citrus and production of transgenic *iaaM*, *iaaM + CKX* Carrizo and US-897 plants

To monitor the activity of the *SbUGT* gene promoter, we produced transgenic Carrizo plants using the *SbUGT*::*GUSPlus* gene. Histochemical staining of the GUS activity in the *SbUGT::GUSPlus* plants revealed that the *SbUGT* promoter was highly active in roots (Fig. 1A). Based on the results of GUS activity staining and PCR confirmation using genomic DNA as templates (data not shown), 48 and 64 independent lines of Carrizo expressing the *SbUGT*::*iaaM* gene and the *SbUGT*::*iaaM + SbUGT*::*AtCKX2* genes were identified, respectively. We also produced 69 and 73 independent lines of US-897 expressing the *SbUGT*::*iaaM* gene, the *SbUGT* ::*iaaM + SbUGT*::*AtCKX2* genes, respectively.

Overexpression of the *iaaM* or *iaaM + CKX* genes promotes root initiation

To investigate the effects of root-predominant expression of *SbUGT::iaaM* and *SbUGT::iaaM + SbUGT::AtCKX2* on root initiation, 64 wild-type Carrizo shoots and 61 wild-type US-897 shoots, along with shoots of all *iaaM* and *iaaM + CKX* transgenic lines, were cultured on a hormone-free medium for 2 months. Overexpression of the *iaaM* or *iaaM + CKX* gene led to 69% and 62% of all independent transgenic Carrizo lines produced roots, respectively when compared to 44% of the wild-type plants (Fig. 1B). For US-897 plants, 38%, 56%, and 86% of the wild-type, *iaaM*,
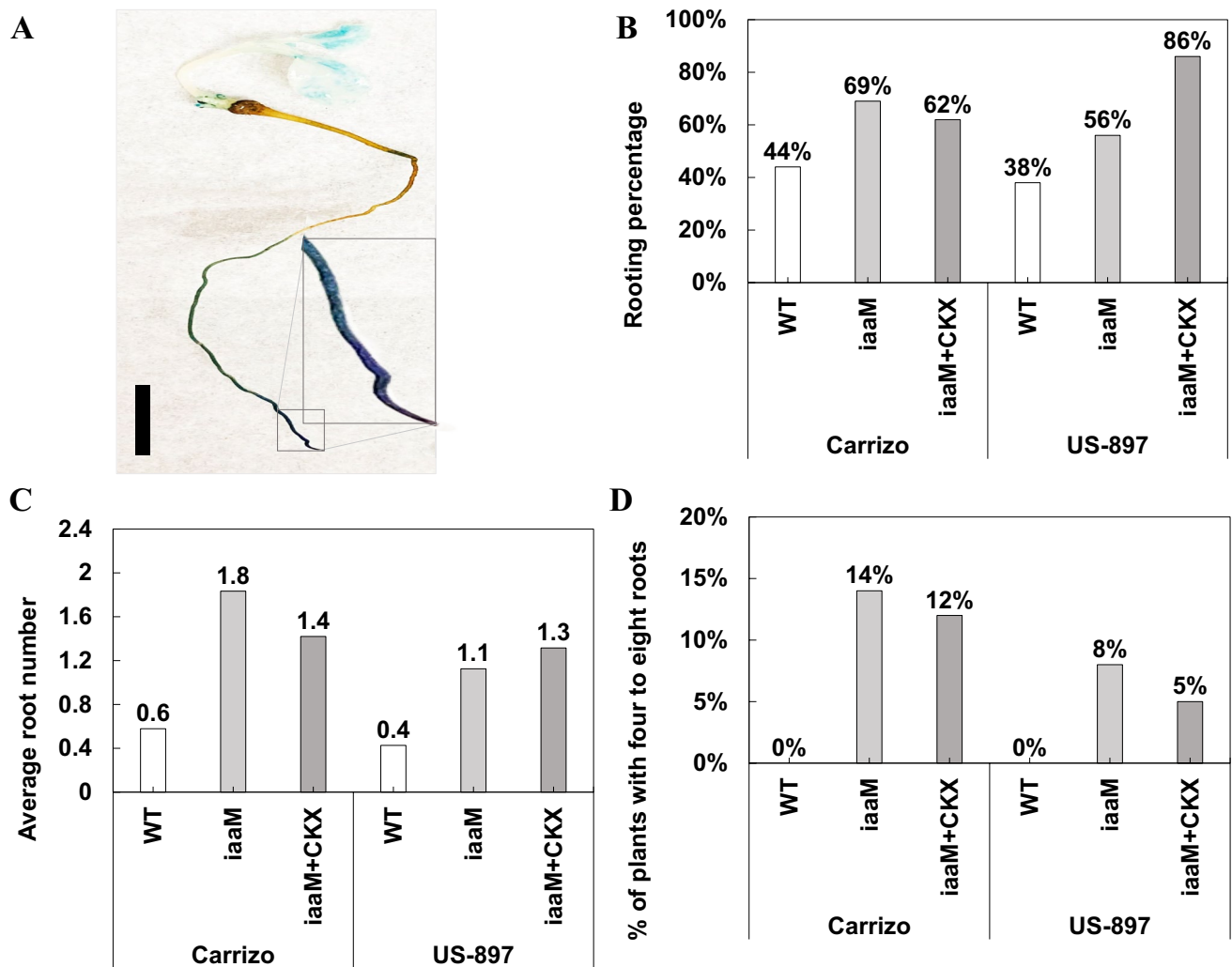
Fig. 1 *SbUGT* promoter is predominantly expressed in root and root-predominant overexpression of the *iaaM* or *iaaM + CKX* genes promotes rooting. Histochemical staining of GUS activity in a rooted *SbUGT::GusPlus* Carrizo plant, indicating that the *SbUGT* promoter is predominantly active in the root (**A**). In (**A**), bar = 1 cm. Expression of *SbUGT::iaaM* or *SbUGT::iaaM + SbUGT::AtCKX2* in both Carrizo and US-897 plants increased percentage of rooting (**B**), average root number of all transgenic lines produced (**C**), and percentage of all transgenic lines with four to eight roots (**D**). For (**B**–**D**), 64 Carrizo wild-type plants, 48 Carrizo independent *iaaM* lines, 69 Carrizo independent *iaaM + CKX* lines, 61 US-897 wild-type plants, 64 US-897 independent *iaaM* lines, and 73 US-897 independent *iaaM + CKX* lines were used for testing. For (**C**), the bars represent means ± standard deviation (SD). Asterisks indicate significant differences from the wild-type as determined using two-tailed Student's *t* test of Microsoft Excel software (*$P < 0.05$; **$P < 0.01$)

and *iaaM + CKX* independent lines produced roots, respectively (Fig. 1B).

On average, the shoots of the Carrizo *SbUGT::iaaM* and the *SbUGT::iaaM + SbUGT::AtCKX2* transgenic lines produced 3.0- and 2.3-fold more roots than these of the wild-type plants (Fig. 1C), while the US-897 *iaaM* and *iaaM + CKX* lines had 2.8- and 3.3-fold more roots than these of the US-897 wild-type stem cuttings, respectively (Fig. 1C). Furthermore, 14% and 12% of the Carrizo *iaaM* or *iaaM + CKX* overexpressing shoots had four to eight roots, whereas none of the wild-type shoots produced more than three roots per plant (Fig. 1D). For US-897, 8% and 5% of *iaaM* and *iaaM + CKX* independent transgenic lines have

more than four roots, and none of the wild-type plants produced more than three roots (Fig. 1D).

Root-predominant overexpression of the *iaaM* or *iaaM + CKX* genes increases growth from root segments

Root segments of wild-type Carrizo, transgenic plants of lines *SbUGT::iaaM#7,* and *SbUGT::iaaM + SbUGT::AtCKX2*#13 were cultured in the MS medium to exam their growth rate. After an 8-week culture, 50% of the *iaaM#7* and 41.7% of the *iaaM + CKX*#13 root segments produced new roots, while only 8.3% of the wild-type root segments developed

new roots (Fig. 2A). The *iaaM* and *iaaM + CKX* root segments had 7.9- and 5.3-fold more lateral roots compared to the wild-type root segments (Data not shown), respectively. The *iaaM* and *iaaM + CKX* root segments produced nearly 18-and 24-fold more root biomass compared to the wild-type root segments (Fig. 2B).

Compared to the *iaaM* overexpressed root segments, the new roots produced from the *iaaM + CKX* root explants were 34% longer (Data not shown). Based on our observations, the root tip of new roots produced from wild-type root segments turned brown gradually and ceased growth when the root length reached to 5–6 cm after 10 weeks of culture (Data not shown). In contrast, the new root tissues produced from the *iaaM*#7 or *iaaM + CKX*#13 root segments remained healthy and continued to grow for over 6 months. These results suggest that the *iaaM* and *iaaM + CKX* root tissues possess enhanced growth rate and vigor compared to the wild-type root tissues.

Root predominant overexpression of the *iaaM* or *iaaM + CKX* genes promotes rooting from shoot explants

As shown in Fig. 3A–F and Table 1, transgenic *SbUGT::iaaM* #7, and *SbUGT::iaaM + SbUGT::AtCKX2*#13 shoots exhibited significantly faster rooting and produced more and longer roots compared to the wild-type plants. In the case of Carrizo (Fig. 3A–C), the average rooting time, the day of the first root appeared from a shoot explant, of the *iaaM* lines was 17 days earlier than the wild-type plants. The average rooting time of the *iaaM + CKX* shoots was 7 days earlier than the corresponding wild-type plants (Table 1).

After 2 months of culture, the *iaaM* and *iaaM + CKX* transgenic plants had 4.1- and 2.8-fold increases in number of roots compared to wild-type plants (Table 1). The Carrizo *iaaM* and *iaaM + CKX* transgenic lines produced roots that had 13.6- and 4.5-fold increases in total root length, respectively, than those of the wild-type plants (Data not shown).

The US-897 *SbUGT::iaaM* and *SbUGT::iaaM + SbUGT::AtCKX*2 shoots also exhibited enhanced rooting and root growth (Fig. 3D–F). The rooting of the US-897 *iaaM* and *iaaM + CKX* lines observed was 16 and 9 days earlier than the wild-type controls, respectively (Table 1). After 2 months of culture, the US-897 *iaaM* and *iaaM + CKX* lines produced 4.4- and 2.8-fold more roots compared to the wild-type plants, respectively (Table 1). The total root length of the US-897 *iaaM* were 7.1-fold longer than those of the wild-type plants, while the total root length of *iaaM + CKX* lines were 6.6-fold longer than that of the wild-type plants (Data not shown). For some strong expressers of the *iaaM* gene, we also observed that both the Carrizo and US-897 *iaaM* transgenic lines displayed leaf epinasty and stunted growth. However, the overexpression of the *CKX* along with the *iaaM* gene effectively neutralized the negative effects of the *iaaM* expression.

Root predominant overexpression of the *iaaM* gene and *iaaM + CKX* genes enhance root biomass production

The *SbUGT::iaaM* and *SbUGT::iaaM + SbUGT::AtCKX2* transgenic plants exhibited drastic increase in root growth (Fig. 4). The representative *iaaM* transgenic lines showed 9.8- and 12.1-fold more root biomass in Carrizo and
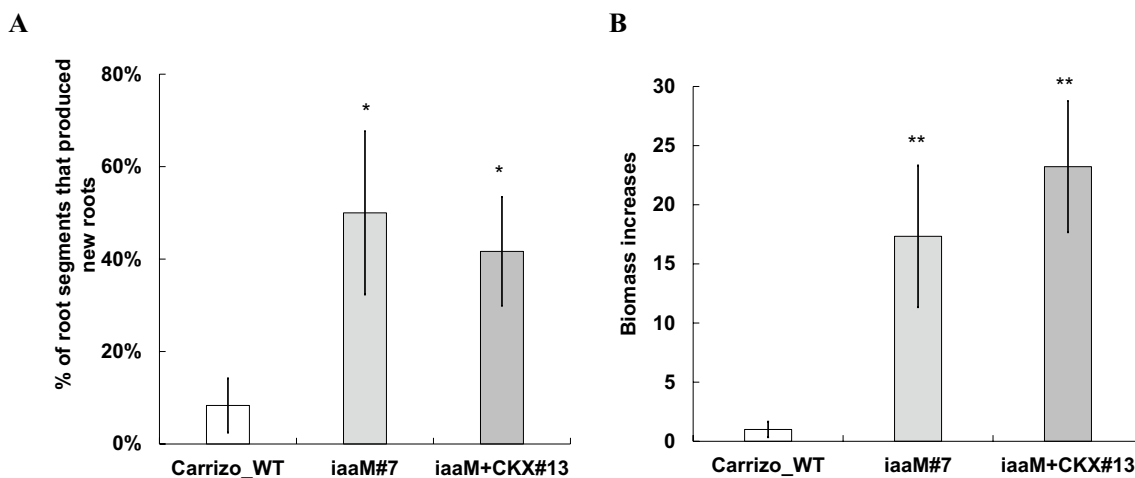
Fig. 2 Root-predominant overexpression of the *iaaM* or *iaaM + CKX* genes increased growth from root segments. **A** The *iaaM*#7 and *iaaM + CKX*#13 root segments showed significantly higher rate of growth than wild-type root tissues after 8 weeks of culture. **B** The *iaaM*#7 and *iaaM + CKX*#13 root segments had significantly more

root biomass increases. The values represent means ± S.D. Asterisks indicate significant differences from the wild-type as determined using two-tailed Student's *t*-test of Microsoft Excel software (*$P < 0.05$; **$P < 0.01$)
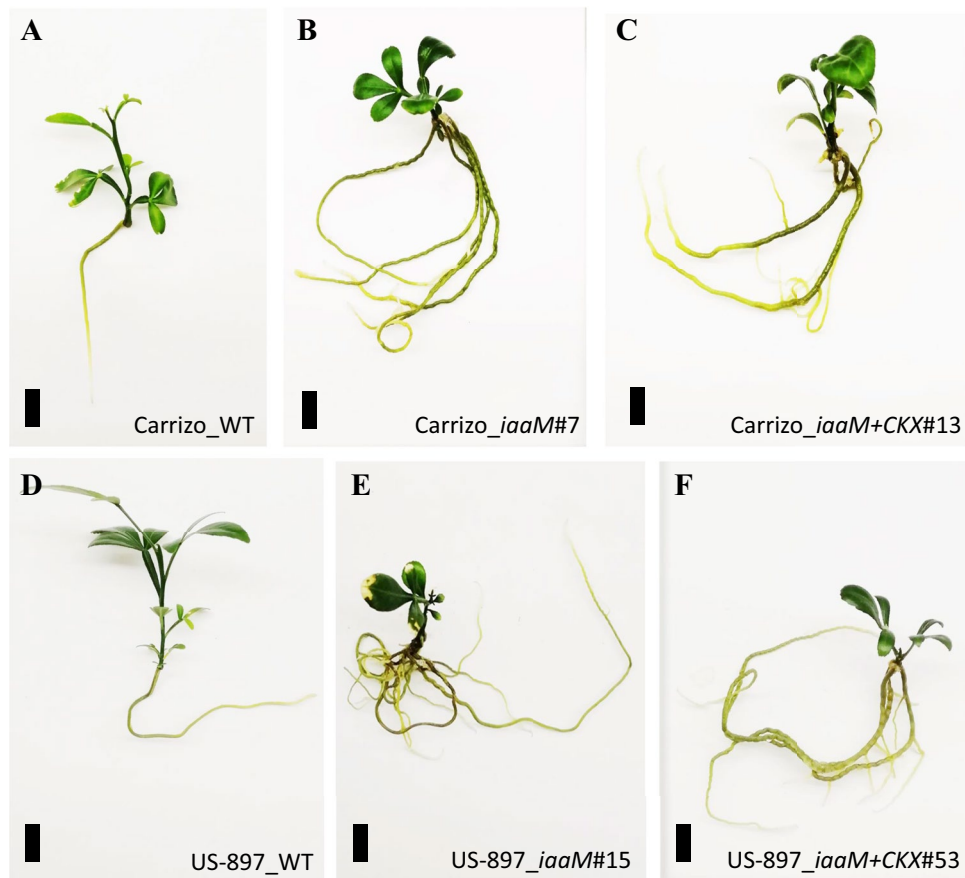
Fig. 3 Root predominant overexpression of the *iaaM* gene or *iaaM + CKX* genes promoted root initiation and root growth. **A–C** Two-month-cultured Carrizo wild-type plant (**A**), the *SbUGT::iaaM* expresser, line *iaaM*#7 (**B**), and the *SbUGT::iaaM + SbUGT::AtCKX2* expresser, line *iaaM + CKX*#13 (**C**). **D–F** Two-month-cultured US-897 wild-type plant (**D**), *iaaM*#15 transgenic line (**E**), and *iaaM + CKX*#53 transgenic line (**F**). In **A–F**, bars = 1 cm

Table 1 Rooting characteristics of transgenic Carrizo and US-897 plants

	Plant	Number of plants/lines used*	Time of the first root appears (day)	Root numbers
Carrizo	WT	8 plants	32.00 ± 5.90	1.40 ± 0.49
	iaaM	7 plants (i.e., 7 representative independent lines)	14.29 ± 4.98**	5.71 ± 1.75**
	iaaM + CKX	8 plants (i.e., 8 representative independent lines)	24.63 ± 4.24*	3.88 ± 0.93**
US-897	WT	5 plants	33.80 ± 5.74	1.60 ± 0.49
	iaaM	4 plants (i.e., 4 representative independent lines)	17.80 ± 2.64**	7.00 ± 2.61**
	iaaM + CKX	4 plants (i.e., 4 representative independent lines)	24.25 ± 4.02*	4.50 ± 0.50**

The root numbers of each plant were counted after 2 months of culture. Values represent the means ± SD. Asterisks indicate significant differences from the wild-type plants determined using two-tailed Student's *t* test of Microsoft Excel software (*$P < 0.05$; **$P < 0.01$)

*Lines with four to eight roots (Fig. 1D) under the preliminary selection stage were selected for rooting test. None of the WT plants produced more than three roots per plant

US-897, respectively, compared to the corresponding wild-type plants (Fig. 4A, B, D, E, F, H). The negative impact of the *iaaM* gene overexpression on growth of aerial parts becomes more pronounced with time in culture. For some strong *iaaM* expressers, their aerial parts stopped to grow after 4 months, even though their roots grew vigorously and healthily (Fig. 4B, F). However, the *CKX* gene overexpression neutralizes the negative effects of the *iaaM* gene overexpression (Fig. 4G), which suggests that a combined use of both *SbUGT::iaaM + SbUGT::AtCKX2* genes can enhance
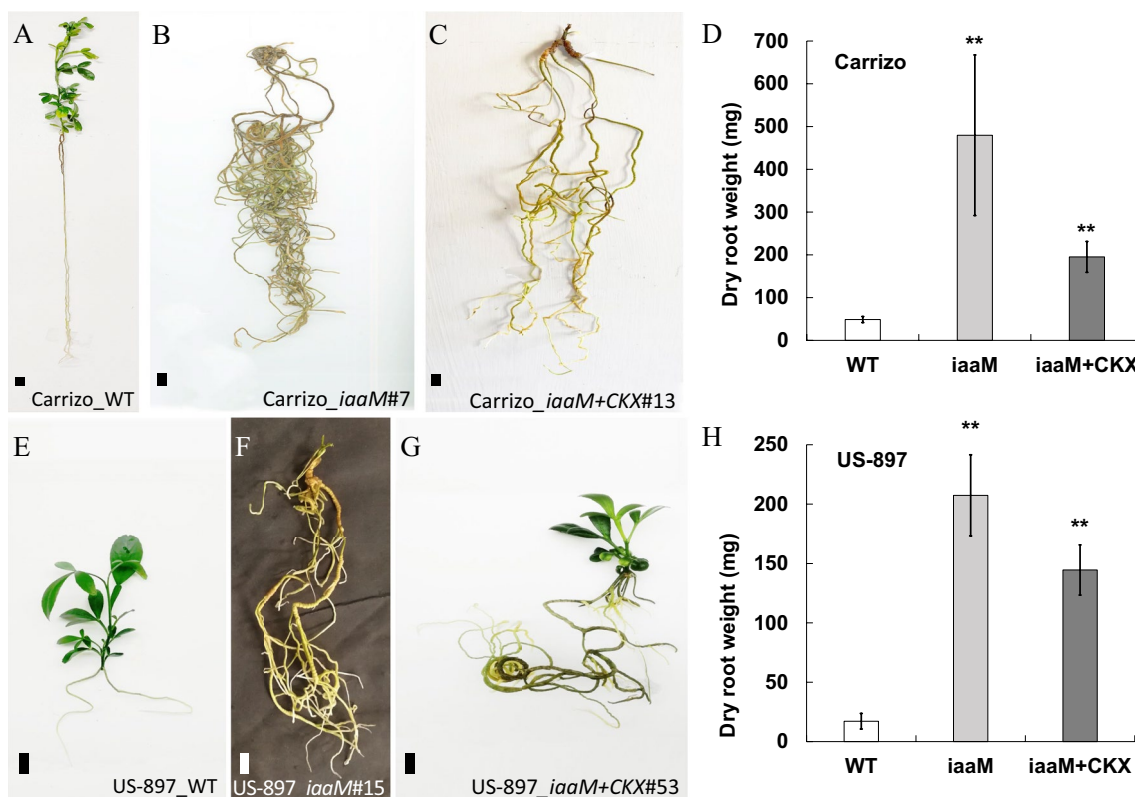
Fig. 4 Root predominant overexpression of the *iaaM* gene or the *iaaM* + *CKX* genes increased root biomass production of Carrizo and US-897 plants. **A–C** Six months cultured Carrizo plants, **A** a wild-type; **B** a *SbUGT::iaaM* expresser, *iaaM#7*; **C** a *SbUGT::iaaM* + *SbUGT::AtCKX2* expresser, *iaaM* + *CKX*#13. **D** Average dry root weight of 6 months grown Carrizo wild-type plants and the *iaaM* and *iaaM* + *CKX* independent lines. As shown in Table 1, for Carrizo wild-type plants, N=5; the *iaaM* transgenic lines, N=7; and the *iaaM* + *CKX* transgenic lines, N=8. **E–G** Four month old cultured US-897 plants, a wild-type plant (**E**), a *SbUGT::iaaM* expresser, *iaaM* #15 (**F**), a *SbUGT::iaaM* + *SbUGT::AtCKX2* expresser, *iaaM* + *CKX* #53 (**G**). Average dry root weights of four-month cultured US-897 wild-type, *iaaM*, and *iaaM* + *CKX* independent lines (**H**). As shown in Table 1, for US-897 wild-type plants, N=5; the *iaaM* transgenic lines, N=4; and the *iaaM* + *CKX* transgenic lines, N=4. In **A–C** and **E–F**, Bars=1 cm. In **D** and **H**, bars represent means±SD. Asterisks indicate significant differences from the wild-type as determined using two-tailed Student's *t*-test of Microsoft Excel software (*$P<0.05$; **$P<0.01$)

root initiation and root biomass production but the negative effects on of the aerial parts are minimal.

Overexpression of the *iaaM* and *CKX* genes alter expression of auxin- and cytokinin-responsive genes

Carrizo representative *SbUGT::iaaM* and *SbUGT::iaaM* + *SbUGT::AtCKX2* expresser: *iaaM*#31, *iaaM*#7, *iaaM* + *CKX*#13, and *iaaM* + *CKX*#27 lines were used to determine altered expression levels of auxin-responsive gene *CsGH3.1* and cytokinin-responsive *CsARR5* genes. The *iaaM*#7 plant that showed a more pronounced root growth phenotype compared to the *iaaM*#31 plant, had a much higher *iaaM* gene expression level (Fig. 5A). Consistently, expression of the *CsGH3.1* gene was up-regulated in both *iaaM*#7 and *iaaM*#31 roots, with 6.1-fold and 1.6-fold increases, respectively, suggesting an increases in

endogenous auxin levels in these transgenic lines (Xiao et al. 2020). In the *iaaM* + *CKX* transgenic roots, both *iaaM* and *CKX* genes were expressed simultaneously (Fig. 5C, D). Up-regulation of the *CsGH3.1* gene expression was observed in both *iaaM* + *CKX*#13 and *iaaM* + *CKX*#27 transgenic roots (Fig. 5E). The *CsARR5* gene, a cytokinin responsive-gene (Ye et al. 2021), was significantly down-regulated in these two transgenic lines (Fig. 5F). These results demonstrate that the overexpression of the *iaaM* and *CKX* genes leads to alterations in genes that are responsive to changes in auxin and cytokinin concentrations.

Discussion

In present study, our results showed that the root-predominant overexpression of either the *iaaM* gene or both the *iaaM* and the *AtCKX2* genes under the control of the *SbUGT*
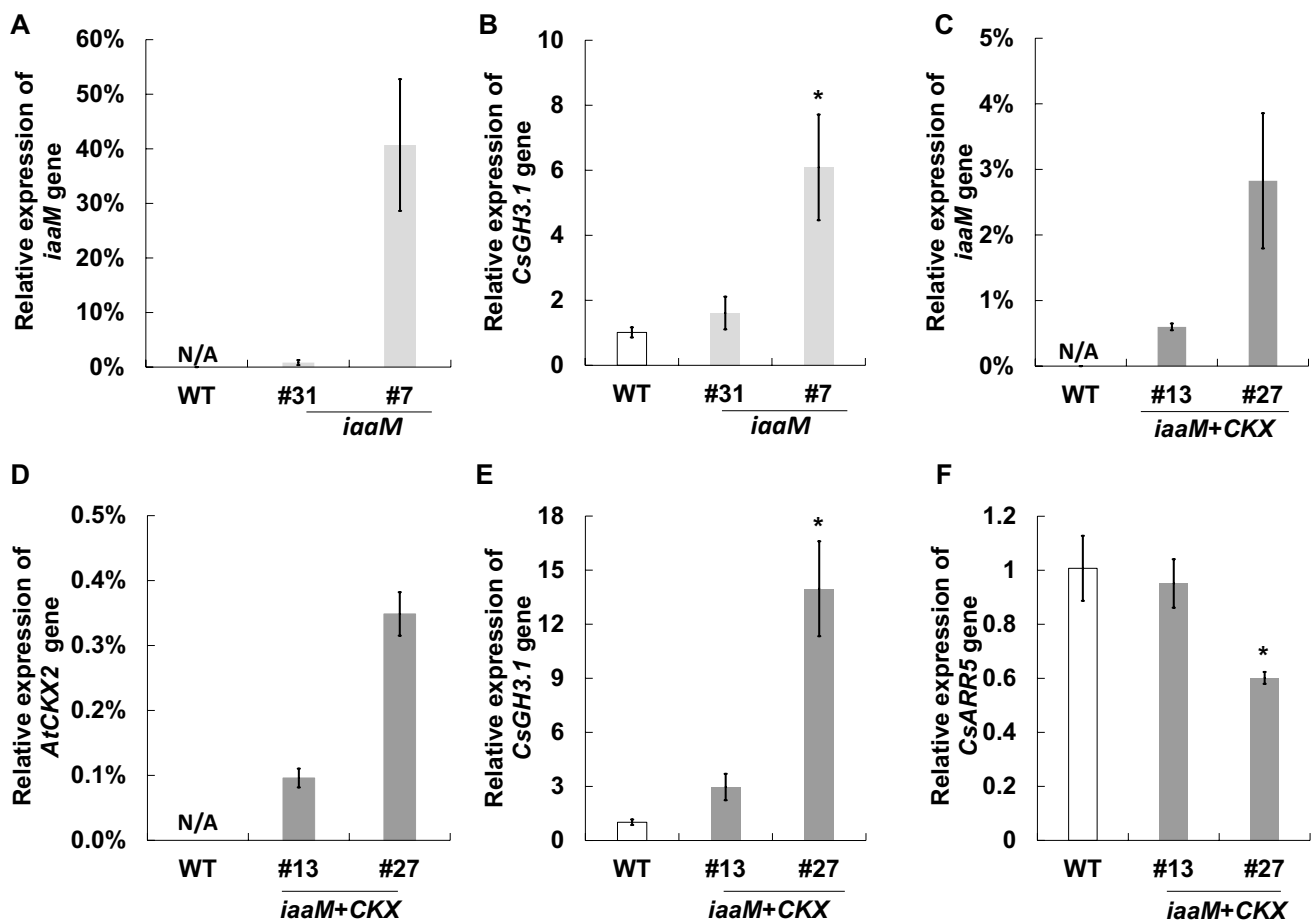
Fig. 5 Root predominant overexpression of the *iaaM* and *iaaM + CKX* genes altered expression levels of auxin-and cytokinin-responsive genes. **A** Relative *iaaM* gene expression levels and **B** elevated expression levels of auxin-responsive *CsGH3.1* gene in the 6-month-cultured *iaaM* Carrizo roots. Relative expression level of *iaaM* gene (**C**), *CKX* gene (**D**), auxin-responsive *CsGH3.1* gene (**E**), and **F** cytokinin-responsive *CsARR5* gene in 6-month-cultured Carrizo *iaaM + CKX* roots. The overexpression of the *iaaM* and *CKX* gene increased the expression level of auxin-responsive *CsGH3.1* gene and decreased the cytokinin-responsive *CsARR5* gene's expression level. The relative gene expression of the *iaaM* and *CKX* genes were compared with the corresponding citrus β-actin gene expression level in the same sample. The relative gene expression fold of the *CsGH3.1* and the *CsARR5* were normalized by citrus β-actin gene *ActB* and performed by the $2^{-\Delta\Delta Ct}$ method. N/A represents no data applicable. Bars represent standard deviation (SD). Asterisks indicate significant differences from the wild-type as determined by two-tailed Student's *t* test in Microsoft Excel software (*$P < 0.05$, **$P < 0.01$)

promoter significantly enhanced initiation and biomass production of roots. The overexpression of the *iaaM* gene promoted root initiation as evidenced by shortened rooting time in both transgenic Carrizo and US-897 plants. Also, the root-predominant overexpression of the *iaaM* gene led to increases in root length and number, and therefore biomass. We have also previously reported similar results in tobacco (Li et al. 2017). Thus, we conclude that the root-predominant overexpression of the *iaaM* and *CKX* genes should lead to enhanced initiation and more vigorous growth of roots in both herbaceous and woody species.

Roots have often been used as bioreactors for biologically active substances of high value including many pharmaceuticals (Murthy et al. 2008; Baque et al. 2012). However, efficient production of root biomass is a challenge in many plant species, particularly in some woody species (Zobayed & Saxena 2003). In addition to the increases in root biomass production from stem cuttings, under this study we have also demonstrated that isolated *iaaM* or *iaaM + CKX* overexpressing root segments can initiate new roots faster, and have drastic increases in root number and biomass. The *iaaM* or *iaaM + CKX* genes we used in this study can therefore be beneficial in enhancing initiation and biomass production of roots of plant species that are of value as bioreactors, which can be particularly beneficial for the plant species that are difficult to root and have poor root growth (Agulló-Antón et al. 2014; Cui et al. 2013).

Stem cuttings are wildly used as efficient, and cost-effective starting materials for vegetative plant propagation, especially for ornamental plants and orchard trees (Agulló-Antón

et al. 2014) or for those that are difficult to propagate from seeds. The formation of adventitious roots is a critical step of vegetative propagation. For large scale vegetative propagation of plants, growth regulators and other manipulations are often used to promote root initiation (De Almeida et al. 2017; Kentelky et al. 2021; Wendling et al. 2010). Even so, it is also difficult to root some woody plants, such as *Eucalyptus gunnii* Hook. f. (*Myrtaceae*) (Di Battista et al. 2019). We have shown that root predominant overexpression of the *iaaM* gene can drastically enhance root initiation from stem cuttings. On the other hand, the overexpression of the *iaaM* gene reduced shoot growth in some of the transgenic lines with strong phenotype, likely due to high levels of auxin inhibit shoot growth. However, the inhibition of shoot growth due to overexpression of the *iaaM* gene can be reduced or eliminated in the transgenic plants that also expressed a *CKX* gene. Therefore, our results show that the *CKX* gene expression can neutralize the negative effects caused by the *iaaM* gene expression in perennial woody citrus plants, similar to the results previously reported in tobacco (Li et al. 2017).

In citrus, the most devastating disease called the greening disease (also called Huanglongbing, HLB) has caused more than 40% yield reduction in citrus planting area in Florida (Graham et al. 2020). One major symptom is that HLB reduces root growth of the infected citrus trees a couple of years before the trees are dead, suggesting weaken root system is involved in the death of infected trees (Munir et al. 2018; Wang and Trivedi 2013). Further studies have shown that more vigorous root growth can drastically delay the development of many HLB symptoms and therefore provide significant protection for the infected trees. Our results reported here strongly suggest that the *iaaM* + *CKX* transgenic citrus trees we have developed when used as rootstocks may provide an excellent component for an integrative approach for managing the HLB disease.

Due to the global warming, drought has become the major abiotic stress factor limiting plant growth and development (Ilyas et al. 2021). Significant yield losses of crop plants are caused by drought stress worldwide every year. It has also been shown that vigorous root growth can improve productivity of crop plants under drought and also non-drought conditions (Guo et al. 2023). Previously, it has been shown that overexpression of *CKX* gene can enhance root growth and plant's drought tolerance (Wang et al. 2020a, b). Here, our results further demonstrate that more vigorous root growth may be achieved if an *iaaM* gene is also overexpress in roots specifically along with overexpression of a *CKX* gene.

For strong *iaaM* expressers, we have observed reduced shoot growth and leaf epinasty similar to previously reported (Guilfoyle et al. 1992, 1993; Klee et al. 1987). Even though the *SbUGT* promoter is predominately active in roots, there are some activity in leaf tissues. It is possible that a more root specific expression of the *iaaM* gene may eliminate the effects on shoots and leaves. On the other hand, auxin could be transported upwards to shoots if elevated auxin is accumulated in roots (Zhai et al. 2021). However, the combined use of the *iaaM* and *CKX* genes as we have showed in this study can effectively reduce the negative effects observed in the transgenic plants that overexpress the *iaaM* gene alone.

In summary, our results have demonstrated that the root-predominant overexpression of the *iaaM* gene or and *CKX* gene can drastically enhance root initiation and growth of citrus tree, which is the first report in woody plants. We suggest that the root-specific expression of both an *iaaM* and a *CKX* gene may be useful in genetic improvement of crop plants for various applications, such as improving rooting, vegetative propagation of reluctant woody plants, and scaling up the industrial culture of adventitious roots.

Declarations

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