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# Using A "Superrooting"Cultivar of Taxus Chinensis Var. Mairei to Unravel Antioxidative Enzymes' and Micrornas' Role on Adventitious Rooting

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**Abstract**

Rooting of cuttings is very important for production of economically important plants. We produced thousands of plantlets in *Taxus chinensis* var. *mairei* using the technology of rooting of cuttings and identified two types of rooted cuttings, one with low rate of root formation and another with high rate of root formation. To determine the physiological role of antioxidative enzymes and microRNAs during the process of rooting, we measured the levels of these antioxidative enzymes and microRNAs in the stem portion, needles, roots, and basal portion of cuttings. Compared to the cuttings with low rate of root formation, cuttings with high rate of root formation had higher expression of polyphenoloxidase (*PPO*), catalase (CAT), peroxidase (*POD*), ascorbate peroxidase (APOX), glutathione reductase (GR), and superoxide dismutase (SOD) in the adventitious roots and basal portion of the rooted cuttings 77 days after planting. In the basal portion of cuttings, the content of thiobarbituric acid reactive substances (TBARS) and total phenols were decreased and the content of antioxidants was increased, but they did not changed in the needles of cuttings during planting. Analysis of microRNAs by quantitative real-time PCR demonstrated that expression of miR162, miR408, and miR857 increases in the basal portion of cuttings, but not in the stem portion of cuttings, 77 days after planting. Expression of miR408 and miR857 were also increased in the needles of cuttings 77 days after planting. Changes of these antioxidative enzymes and microRNAs associated with the rooting features of *T. chinensis* var. *mairei* cuttings and their functions have been discussed.

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## Introduction

Rooting of cuttings is very important for large-scale propagation of economically important plant species [1] [2] [3] [4]. Efficient propagation of these economically important plants is dependent on the efficiency of adventitious root formation in cuttings [5] [6] [7]. Cuttings of Taxus chinensis var. mairei are recalcitrant to root. The detailed cause of their low rooting ability is not clear. In general, environmental conditions and endogenous biochemical compounds are considered to affect adventitious root formation [8] [9] [10] [11]. Among the endogenous compounds, proteins, enzymes, and phytohormones have been used for the efficient propagation of cuttings in many horticultural and forestry plants [9] [12] [13].

Taxus is a world wide economically important and an endangered gymnosperm genus [6] [7] [14] [15] [16] [17] [18] [19]. The barks of these plants are very valuable anticancer medicinal resource such as the powerful anticancer agent paclitaxel [20]. Efficient propagation of these plants is critical to the scientific investigation and practical application in the field of conifer biotechnology [6] [14] [18] [19] [21]. It has been reported that anticancer agents have been obtained in samples of Taxus wallichiana var. mairei [5] and Taxus x media var. Hicksii [6]. In addition, the endophytic fungi in the bark of Taxus wallichiana var. mairei have been used to produce 10-deacetyl baccatin III [22]. Investigations of transcriptome and metabolome in plants of Taxus genus demonstrated that difference in gene expression is related to the plant tissue type [23]. However, biochemical and genetic studies in T. chinensis var. mairei are relatively rare because of lack of high efficient propagation system.

Antioxidative enzymes are known to affect morphogenesis in plants [24-34]. In the in vitro culture of many plant species, antioxidative enzymes can be biomarkers of adventitious root formation from microshoots [24] [25] [27] [28] [29] [30] [31]. On the other hand, antioxidative enzymes have regulatory effect on adventitious root formation in shoot culture of a large number of plant species [35-45] and in Vitis vinifera cuttings [46] [47] [48] [49]. The above research results indicate that antioxidative enzymes are involved in adventitious root formation. Although the effect of some antioxidative enzymes adventitious root formation has been confirmed in plants [29-33, 50, 51], physiological role of polyphenoloxidase (PPO), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APOX), glutathione reductase (GR), and superoxide dismutase (SOD) during the process of in vitro rooting in T. chinensis var. mairei cuttings has not reported.

MicroRNAs are known to affect morphogenesis in plants [52] [53] [54] [55]. However, the effect of microRNAs on adventitious root formation might be more complicated because the composition and concentration vary with the species and growth phase [13] [56] [57] [58] [59] [60] [61] [62]. The functions of microRNAs and their mechanisms in regulating plant growth and development are still unclear [52, 53, 63, 64]. Although the effect of some microRNAs on adventitious root formation has been confirmed and miR162, miR408, and miR857 have been reported to be involved in root initiation and growth in some plant species including model plants and crop plants [1] [2] [52] [53] [54] [55] [63] [64] [65] [66], physiological role of microRNAs miR162, miR408, and miR857 during the process of in vitro rooting in T. chinensis var. mairei cuttings remains to be determined. Therefore, we examined the expression of miR162, miR408, and miR857 during the process of in vitro rooting in T. chinensis var. mairei cuttings.

The aim of this study is to elucidate the effect of antioxidative enzymes PPO, CAT, POD, APOX, GR, and SOD and microRNAs miR162, miR408, and miR857 on the rooting ability of cuttings of T. chinensis var. mairei. Expression levels of PPO, CAT, POD, APOX, GR, SOD, miR162, miR408, and miR857 in the stem portion, needle, roots, and basal portion of cuttings with high rate of root formation were compared with those of cuttings with low rate of root formation.

## Materials and methods

### *Plant Material and Cuttings*

Taxus chinensis var. mairei [genotypes Baokang -normal rooting (NR) and Jinzhou-super rooting (SR)] was planted in a research field at Yangtze University and used in this experiment. Cuttings with 16 needles were obtained from these trees in early spring of the year and stored horizontally at 4$^{o}$C in a plastic bag until cutting.

Cuttings with one stem were prepared and cuttings with similar length and basal end diameter were planted in vermiculite in a planting box and placed in an unheated glasshouse. They were watered with 0.5-liter tap water per planting box every 3 days. Observations on rooting were made at 77 days after planting during each year. All cuttings with roots (>1 mm) were classified as rooted cuttings, and the roots (>1 mm) were used to prepare samples for analysis of antioxidative enzymes and micro RNA expression.

*Sample Preparation for Antioxidative Enzymes and microRNAs Analyses*

The number of cuttings used for antioxidative enzymes and microRNAs analyses were 756 (NR) and 649 (SR), respectively. The stem portion, needle, roots, and basal portions (2 cm) were collected from the cuttings on the planting day (day 0) and 77 days after planting. Each sample was chopped into small pieces with scissors, immediately frozen in liquid nitrogen, and then stored at −80°C until analysis. The levels of antioxidative enzymes and microRNAs were measured from samples of the stem portion, needles, roots, and basal portion of cuttings, respectively.

*Thiobarbituric Acid reactive Substances (TBARS) Determination*

Lipid peroxidation was determined as the amount of thiobarbituric acid reactive substances (TBARS) measured by the thiobarbituric acid (TBA) reaction as described previously [67] [68] [69]. Samples of stem portion, needle, roots, and basal portions (2 cm) of T. chinensis var. mairei cuttings were homogenized in 3 ml of 20 % (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 2,655 g for 20 min and mixed with 20 % TCA containing 0.5 % (w/v) TBA and100 ml 4 % BHT in ethanol at 1:1. After the extracts of plant tissues were heated at 95 °C for 30 min, they were cooled on ice for 5 minutes, centrifuged at 10,000 g for 15 min. The absorbance the extracts of different samples was measured at 532 nm. The control of non-specific absorption at 600 nm was subtracted from the samples. The value of TBARS was calculated using the method described previously [26] [67] [70].

*Measurement of total Phenols*

Analysis of phenols was conducted after harvested the samples. Samples (1 g) of stem portion, needle, roots, and basal portions (2 cm) of T. chinensis var. mairei cuttings were homogenized in 50mL cold 80% methanol using a biomixer (Nihonseiki, Tokyo). Samples were washed twice with 50mL of 80% methanol and filtered. 150mL of distilled water was added to the samples to obtain a crude extract of the phenols. The total phenols content was determined using the Folin-Ciocalteu method [46] [71-73]. After elimination of methanol in the crude extracts (180 mL) in vacuo, the aqueous phase was diluted five times with distilled water to the final volume of 900 mL. The diluted sample was mixed with 1N Folin-Ciocalteu reagent and then with 10% sodium carbonate 3 min later and placed at room temperature in darkness for 1 h. The absorbance of the reactants at 530 nm was measured. The total concentration of phenolic compounds was estimated by a standard curve obtained with chlorogenic acid (Wako Pure Chemical, Osaka).

*Determination of total antioxidant levels*

Total antioxidant levels were determined from samples (300 mg) of stem portion, needle, roots, and basal portions of T. chinensis var. mairei cuttings using an antioxidant assay kit (Sigma–Aldrich) following the product manual. Antioxidant reactions were performed in a 96-well plate by reading endpoint absorbance at 405 nm using a plate reader (BioTek, Synergy 2, Winooski, VT, USA).

*Measurement of Antioxidative Enzymes*

Analysis of PPO, CAT, and POD activity was conducted by following the previous protocol [29] [31] [32] [34] [50] [57] [74] [75]. Samples (6 g) of stem portion, needle, roots, and basal portions of T. chinensis var. mairei cuttings were immersed in 100mL of cold 80% methanol containing 1mM butylated hydroxytoluene (BHT) and 0.01% ascorbic acid and stirred for 24 h at 4°C. Activity of antioxidant enzymes PPO was measured by following the modified methods previously described [34]. The activity of CAT was determined spectrophotometrically as previously described [51]. The decomposition of 1 mmol $H_2O_2$ per gram FW in 1 min was defined as one unit. The activity of POD was determined according to the procedure [32] [51], with a Shimadzu UV-120IV spectrophotometer. The amount of POD catalyzing the oxidation of 1 mol guaiacol in 1 min was defined as one unit. The activities

of APOX, GR, and SOD were determined as described previously [39] [41] [42] [43]. Two grams of control and sampes of stem portion, needle, roots, and basal portions of T. chinensis var. mairei cuttings were homogenized under ice-cold conditions in 3 ml of extraction buffer, consisting of 50 mM phosphate buffer (pH 7.4), 1 mM EDTA, 1 g PVP, and0.5 % (v/v) Triton X-100 at 4 °C. The extracts were centrifuged at 10,000 ´ g for 20 min. The supernatant was used to determine the enzyme activity. APOX activity was measured immediately in fresh extracts and was assayed as described [44]. GR activity was determined by following the decrease in absorbance at 340 nm due to NADPH oxidation [42]. SOD activity was measured by the inhibition of the photochemical reduction of NBT, as described [39, 40].

*Total RNA isolation and Quantitative real-time PCR (qPCR)*

Total RNA was isolated from frozen samples of stem portion, needle, roots, and basal portions of T. chinensis var. mairei cuttings using TRIzol reagent according to the manufacturer's protocol (Invitrogen). The high quality cDNA were prepared using a TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). For miRNA expression analysis, TaqMan® MicroRNA Assays (Applied Biosystems) are carried out to amplify RNAs for quantitation of miRNAs. The controls used were as suggested by the manufacturer's manual. The U6 gene was used as an internal control. Samples were examined in triplicate on the Applied Biosystems 7900HT System, according to the manufacturer's manual.

*Analysis of Expression of microRNAs*

For the analysis of microRNAs (miR162, miR408, and miR857) in samples, the extraction procedure was used as previously described [26, 27, 53-55, 58, 60, 61, 68, 76]. Two specific primers were used to amplify each of miRNAs. Primers used for Real-Time PCR are R1: 5′-AGTGGTTTATCGATCTCTTCCTTG-3′ and F1: 5′-GTGGTTCAAGCGTTTTATTGTTG-3′ for miR162, R2: 5′GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGA-TACGACCATGCT-3′ and F2:5′-TCAGCACAGGGAACAAGCAG-3′ for miR408, and R3:5′GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTG GATACGACATACAC-3′ and

F3: 5′-GCGGCGTTTTGTATGTTGAAG-3′ for miR857

*Statistical Analyses*

Mean values were used to determine the significant differences among different groups with the Least Significant Difference test at 5% level of probability. Statistic analysis of data was performed by Student's t-test or one-way ANOVA using GraphPad Prism 6 (GraphPad Software Inc., CA).

## Results

*Adventitious Root Formation in cuttings of Taxus chinensis var. mairei*

During the in vitro rooting process in T. chinesis var. mairei, we used two different genotypes of T. chinensis, one with normal rooting and the other with super rooting phenotypes, which originated from sampling at two sites. Cuttings of T. chinensis with normal rooting (NR) are from BaoKang. Cuttings of T. chinensis with super rooting (SR) are from Jingzhou. 3436 cuttings of Baokang were planted at day zero and 756 cuttings of them actually rooted. 832 cuttings of Jingzhou were planted at day zero and 649 cuttings of them actually rooted at 77 days of in vitro rooting. Rooted cuttings (Baokang, 756 plantlets) has low rate of adventitious root formation [named normal rooting (NR) cuttings, Fig. 1a], and another type of rooted cuttings (Jinzhou, 649 plantlets) has high rate of adventitious root formation [named super rooting (SR) cuttings, Fig. 1b]. To determine the difference of these two types of rooted cuttings, percentage of rooting types (Fig. 1c), number of adventitious roots per cuttings (Fig. 1d), number of branch per cuttings (Fig. 1e), and growth rate (Fig. 1f) were measured. Our results demonstrated that percentage of rooting in super rooting cuttings is 3.9 fold higher than in normal rooting cuttings (Fig. 1c). There are 3.7 fold increase in number of adventitious roots per cuttings (Fig. 1d), 2.1 fold increase in branch number per cuttings (Fig. 1e), and 2.6 fold increase in growth rate (Fig. f) in rooted cuttings with high rate of adventitious root formation, compared to rooted cuttings with low rate of adventitious root formation. Our results indicated that number of adventitious roots (Fig. 1d) and number of branch (Fig. 1e), as well as growth rate (Fig.1f), are significantly increased in rooted cuttings

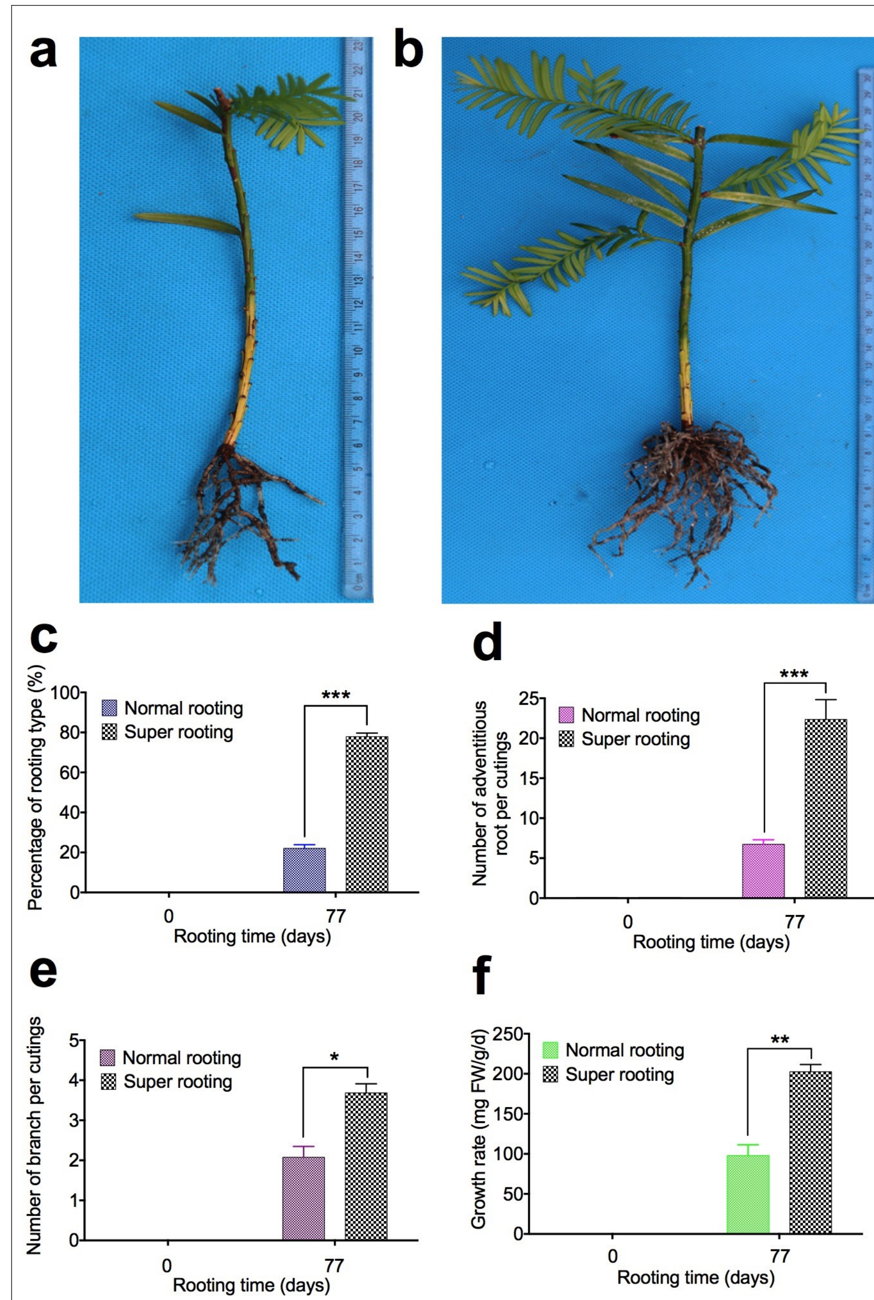


**Figure 1.** Adventitious root formation in cuttings of *Taxus chinensis* var. *mairei*. During the in vitro rooting process in *T. chinesis*, we identified two genotypes of rooted cuttings at 77 days of in vitro rooting. One type of rooted cuttings has low rate of root formation with low number of adventitious roots and lateral roots (Fig. 1a, normal rooting cuttings), another type of rooted cuttings has high rate of root formation with high number of adventitious roots and lateral roots (Fig. 1b, super rooting cuttings). Percentage of rooting types (Fig. 1c), Number of adventitious roots per cuttings (Fig. 1d), number of branch per cuttings (Fig. 1e), and growth rate (Fig. 1f) were measured for rooted cuttings. The statistically significant difference between groups was determined by one-way ANOVA. Data are presented as means of five independent experiments. Error bars represent standard error. The asterisk indicates significant differences compared to the rooted cuttings with low rate of root formation, as assessed by a *t*-test. ***P<0.001, significant relative to control. Vertical bars indicate standard error.

with high rate of adventitious root formation 77 days after planting.

*TBARS, total phenols, and Antioxidants content in shoot cuttings*

To evaluate the effect of TBARS, total phenols, and antioxidants content on root formation, TBARS, total phenols, and antioxidants content in different portions of rooted cuttings of T. chinensis var. mairei was determined at 0 and 77 days after planting. TBARS (Figs. 2a, 2b, and 2c), total phenols (Figs. 2d, 2e, and 2f), and antioxidants content (Figs. 2g, 2h, and 2i) was measured from samples of stem portion, basal portion, and needles of rooted cuttings, respectively. Our results demonstrated that TBARS was significantly lower from day 0 to day 77 after planting in stem portion (Fig. 2a) and basal portion (Fig. 2b) of both NR cuttings and SR cuttings, total phenol was significantly higher from day 0 to day 77 after planting in stem portion (Fig. 2d), but was significantly lower from day o to day 77 of planting in basal portion (Fig. 2e) of both NR cuttings and SR cuttings. Content of antioxidants significantly increased in base portion (Fig. 2h) of SR cuttings, compared to NR cuttings 77 days after planting. TBARS, total phenols, and antioxidants content were not changed in stem portion and needles of two types of cuttings 77 days after planting.

*PPO, CAT, and POD activity in cuttings*

To examine the effect of PPO, CAT, and POD activity on root formation, PPO, CAT, and POD activity in shoot cuttings of T. chinensis var. mairei were measured at 0 and 77 days after planting. PPO (Figs. 3a, 3d, 3g, and 3j), CAT (Figs. 3b, 3e, 3h, and 3k), and POD activity (Figs. 3c, 3f, 3i, and 3l) were examined in stem portion, basal portion, needles, and adventitious roots of rooted cuttings of T. chinensis var. mairei at 0 and 77 days after planting. Our results demonstrated that activity of PPO, CAT, and POD was not changed in stem portion of cuttings between normal rooting and super rooting cuttings (Figs. 3a-c). However, activity of PPO, CAT, and POD was significantly higher in basal portion (Figs. 3d-f) and adventitious roots (Figs. 3j-l) of SR cuttings, compared to NR cuttings 77 days after planting. Activity of PPO and CAT in needles of SR cuttings is similar to that of NR cuttings 77 days after planting (Figs. 3g and h). Activity of POD significantly increased in needles (Fig. 3i) of SR cuttings, compared to NR cuttings 77 days after planting.

*APOX, GR, and SOD activity in cuttings*

To examine the effect of APOX, GR, and SOD activity on root formation, APOX, GR, and SOD activity were measured in shoot cuttings of T. chinensis var. mairei at 0 and 77 days after planting. APOX (Figs. 4a, 4d, 4g, and 4j), GR (Figs. 4b, 4e, 4h, and 4k), and SOD activity (Figs. 4c, 4f, 4i, and 4l) in stem portion, basal portion, needles, and adventitious roots of rooted cuttings of T. chinensis var. mairei at 0 and 77 days after planting. Our results demonstrated that activity of APOX, GR, and SOD in stem portion (Figs. 4a-c) and needles (Figs. 4g-i) of SR cuttings were similar to that of NR cuttings. However, activity of APOX, GR, and SOD in adventitious roots was significantly higher in SR cuttings (Figs.4j-l), compared to NR cuttings 77 days after planting. Activity of APOX, GR, and SOD in basal portion (Figs. 4d-f) of NR cuttings was higher than SR cuttings before planting (0 day). Activity of APOX, GR, and SOD in basal portion (Figs. 4d-f) of NR cuttings with was similar to that of SR cuttings 77 days after planting.

*Expression of microRNAs in cuttings*

To examine the effect of expression of microRNAs on root formation, expression of microRNAs were measured in shoot cuttings of T. chinensis var. mairei at 0 and 77 days after planting. MicroRNA162 (Figs. 5a, 5d, 5g, and 5j), miR408 (Figs. 5b, 5e, 5h, and 5k), and miR857 (Figs. 5c, 5f, 5i, and 5l) in stem portion, basal portion, needles, and adventitious roots of rooted cuttings of T. chinensis var. mairei at 0 and 77 days after planting. Our results demonstrated that expression of miR162, miR408, and miR857 was not changed in stem portion (Figs.5a-c) of SR cuttings, compared to NR cuttings. However, expression of miR162, miR408, and miR857 significantly increased in basal portion (Figs.5d-f) of SR cuttings, compared to NR cuttings. Expression of miR162 was not changed in needles (Fig.5g) and roots (Fig. 5j), but expression of miR857 significantly increased in needles (Fig.5i) and roots (Fig. 5l) of SR cuttings, compared to NR cuttings

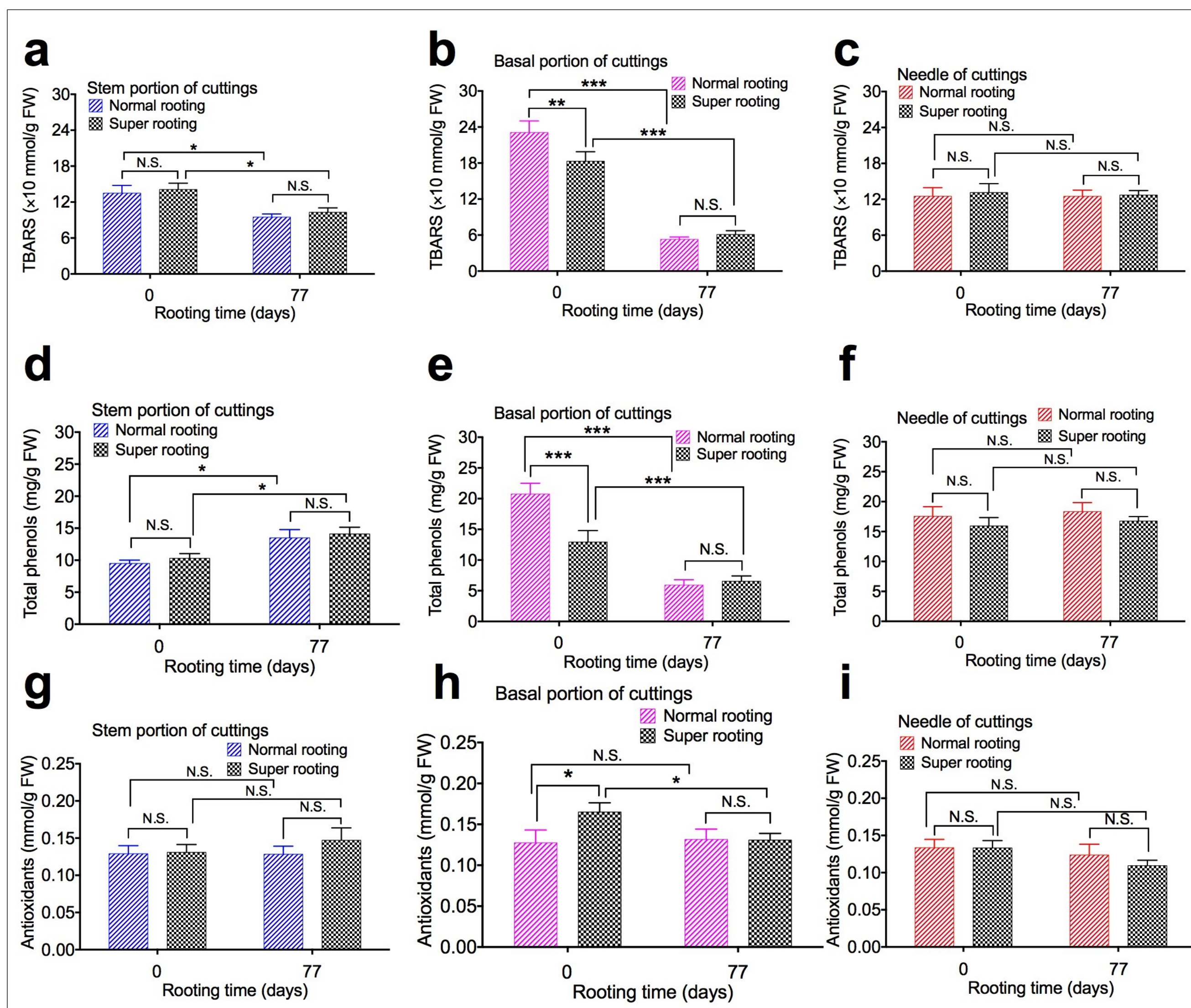


**Figure 2.** TBARS, total phenols, and antioxidants content of shoot cuttings of *T. chinensis* var. *mairei* at 0 and 77 days after planting. TBARS (Figs. 2a, 2b, and 2c), total phenols (Figs. 2d, 2e, and 2f), and antioxidants content (Figs. 2g, 2h, and 2i) was measured from samples of stem portion, basal portion, and needles of rooted cuttings. Experiment was repeated five times. The statistically significant difference between groups was determined by one-way ANOVA. Data are presented as means of five independent experiments. Error bars represent standard error. The asterisk indicates significant differences compared to the Phase I, as assessed by a *t*-test. *P<0.05, **P<0.01, ***P<0.001, significant relative to rooted cuttings with low rate of root formation. N.S., no statistics significance. Vertical bars indicate standard error.

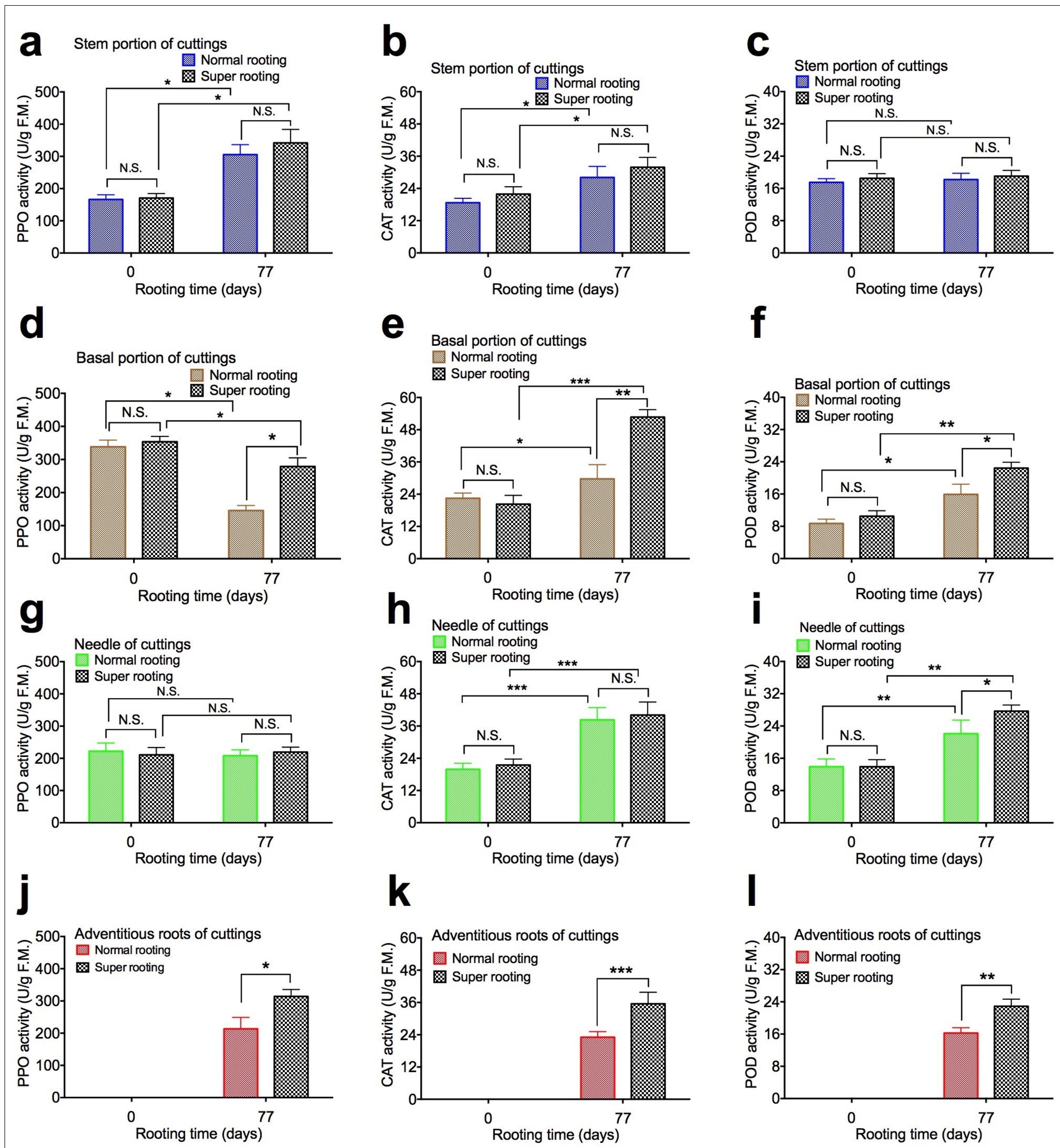


**Figure 3.** PPO, CAT, and POD activity in shoot cuttings of *T. chinensis* var. *mairei* at 0 and 77 days after planting. PPO (Figs. 3a, 3d, 3g, and 3j), CAT (Figs. 3b, 3e, 3h, and 3k), and POD activity (Figs. 3c, 3f, 3i, and 3l) in stem portion, basal portion, needles, and adventitious roots of rooted cuttings of *T. chinensis* var. *mairei* at 0 and 77 days after planting. Experiment was repeated five times. The statistically significant difference between groups was determined by one-way ANOVA. Data are presented as means of five independent experiments. Error bars represent standard error. The asterisk indicates significant differences compared to the rooted cuttings with low rate of root formation, as assessed by a *t*-test. *P<0.05, **P<0.01, ***P<0.001, significant relative to Phase I. N.S., no statistics significance. Vertical bars indicate standard error.

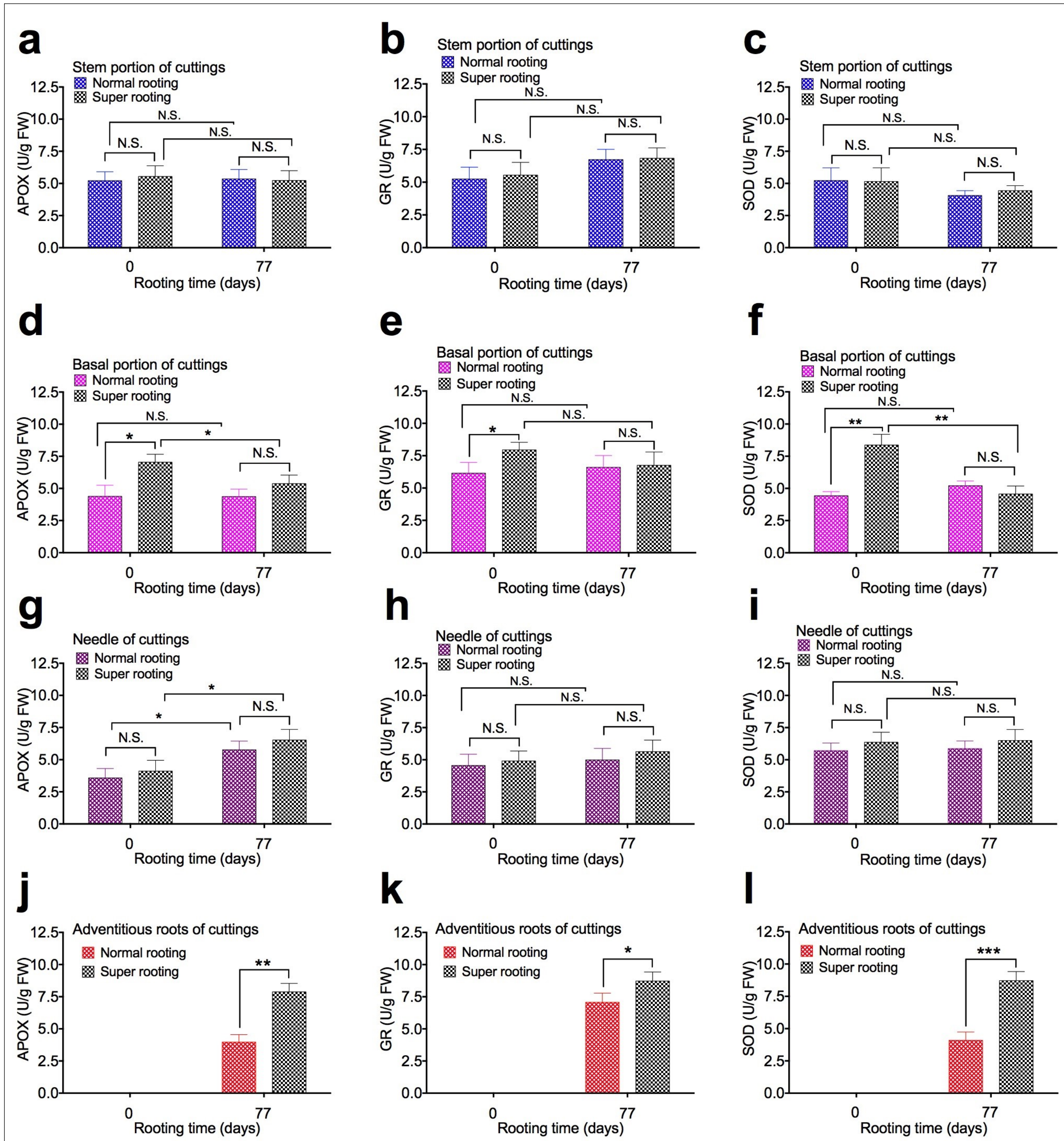


**Figure 4.** APOX, GR, and SOD activity in shoot cuttings of *T. chinensis* var. *mairei* at 0 and 77 days after planting. APOX (Figs. 4a, 4d, 4g, and 4j), GR (Figs. 4b, 4e, 4h, and 4k), and SOD activity (Figs. 4c, 4f, 4i, and 4l) in stem portion, basal portion, needles, and adventitious roots of rooted cuttings of *T. chinensis* var. *mairei* at 0 and 77 days after planting. Experiment was repeated five times. The statistically significant difference between groups was determined by one-way ANOVA. Data are presented as means of five independent experiments. Error bars represent standard error. The asterisk indicates significant differences compared to the rooted cuttings with low rate of root formation, as assessed by a *t*-test. *P<0.05, **P<0.01, ***P<0.001, significant relative to Phase I. N.S., no statistics significance.

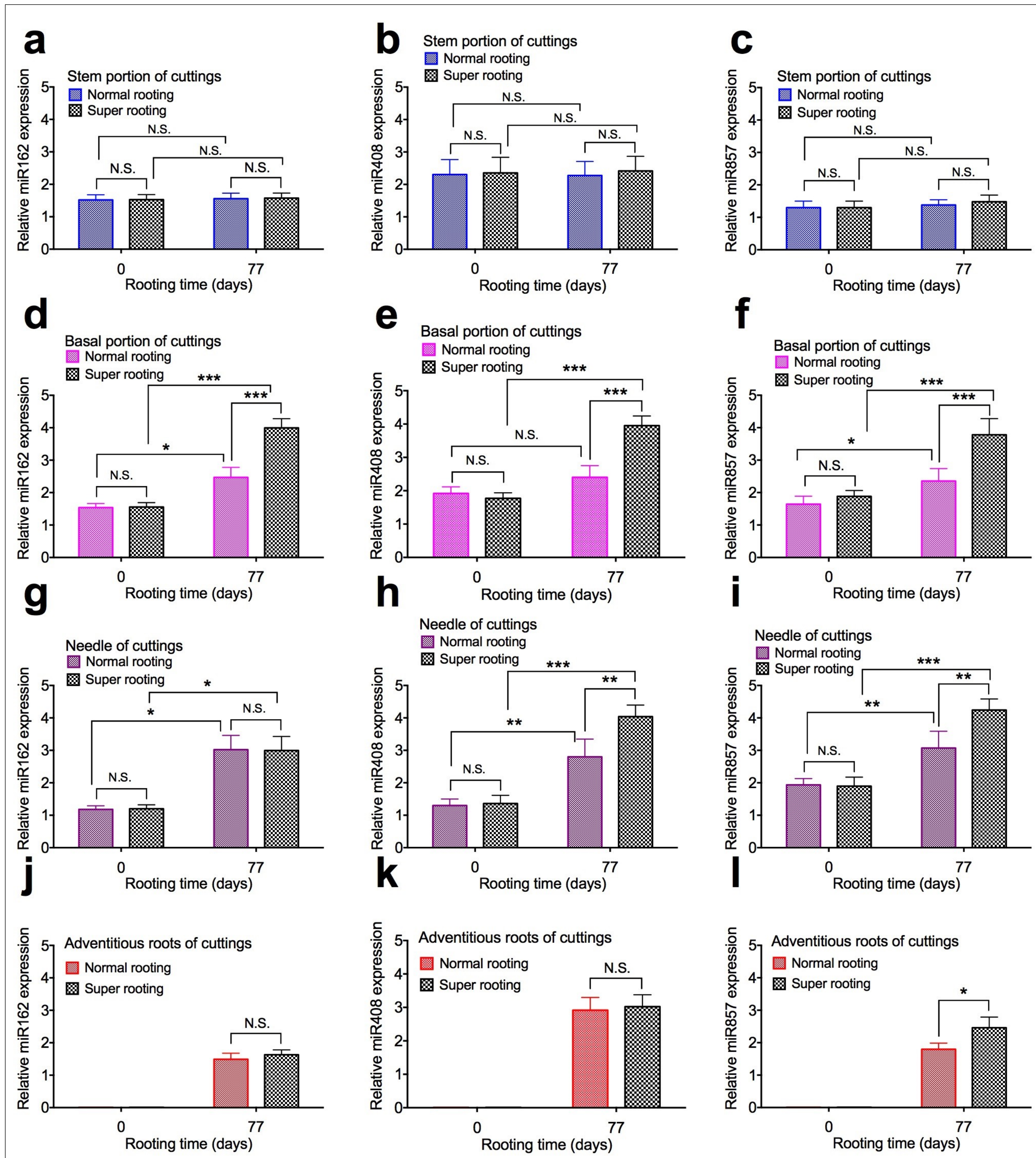


**Figure 5.** Expression of microRNAs in shoot cuttings of *T. chinensis* var. *mairei* at 0 and 77 days after planting. MicroRNA162 (Figs. 5a, 5d, 5g, and 5j), miR408 (Figs. 5b, 5e, 5h, and 5k), and miR857 (Figs. 5c, 5f, 5i, and 5l) in stem portion, basal portion, needles, and adventitious roots of rooted cuttings of *T. chinensis* var. *mairei* at 0 and 77 days after planting. Experiment was repeated five times. The statistically significant difference between groups was determined by one-way ANOVA. Data are presented as means of five independent experiments. Error bars represent standard error. The asterisk indicates significant differences compared to the rooted cuttings with low rate of root formation, as assessed by a *t*-test. *P<0.05, **P<0.01, ***P<0.001, significant relative to Phase I. N.S., no statistics significance.

77 days after planting. Expression of miR408 was not changed in roots (Fig.5k), but significantly increased in needle (Fig. 5h) of SR cuttings, compared to NR cuttings 77 days after planting.

**Discussion**

Environmental conditions and endogenous biochemical compounds are important in regulating adventitious root formation. For example, the endogenous factor IAA regulates mitotic activity for root initiation in the cuttings of grape [49] [72] [73] [77]. The IAA level in grape cuttings at planting is higher in easy-to-root varieties than in difficult-to-root varieties [78] [79] [80]. Increased IAA level of cuttings during planting is a more important factor for rooting than the higher IAA level at planting [8]. High level of IAA was observed in the cuttings of chestnut with high rooting ability in the period of four days after planting [81], suggesting that accumulated IAA in basal portion of cuttings may regulate root architecture during rooting. Reduction of auxin signaling by the auxin antagonist α-(phenyl ethyl-2-one)-IAA rapidly decreases the expression of several core cell cycle genes [82] [83]. Overexpression of the mitotic cyclin CYCA2;3, which inhibits endocycle onset, promotes the termination of endoreplication [84]. IAA signaling is critical for determining the timing of the transition to the endocycle [8] [12] [13] [82]. IAA plays a crucial role in stem cell specification in roots [83] [84] [85]. In addition, reduction in RETINOBLASTOMA-RELATED protein (RBR) expression in Arabidopsis roots increases the number of stem cells without changing the duration of cell cycles in the meristem [86] [87]. Overexpression of RBR results in a loss of stem cells, indicating that an appropriate level of RBR is essential for maintaining the appropriate number of stem cells [86] [87], indicating that the amount of IAA in basal portion of cuttings may affect rooting ability. Polyamines are another important endogenous factor in the maintenance of rooting ability in plants [26] [69] [70] [76] [88] [89]. It has been reported that an increase in free Put could be correlated with the formation of root primordial in cuttings of grape [73] [78], and that Put may be a good marker for root differentiation [26] [27]. An increase in free Put levels was observed at an early stage of rooting of cuttings [26] [27]. Concentrations of Spd may affect root formation after planting in woody plants. It has been reported that the high level of Spd can be an indicator of the rooting ability of these species [25] [26] [27]. Conjugated Put and Spd may have an effect on rooting. Conjugated Put and Spd were found to accumulate in Chrysanthemum leaf explants on a medium promoting root formation, followed by a decrease during the period of root emergence [25] [26] [27]. In addition, changes in the levels were more rapid and substantial in the conjugated Put and Spd than in the free Put and Spd [25] [26] [27]. Considering effects of auxin and polyamines on root formation have been extensively investigated in plants, we focus on influence of antioxidative enzymes and microRNAs on root formation in T. chinensis cuttings.

The rooting ability of T. chinensis cuttings depends on the varieties and cultivars. So far, little information on the rooting ability of T. chinensis cuttings is available. To analyze the effects of the endogenous factors on root formation in T. chinensis cuttings, we measured the levels of antioxidative enzymes and microRNAs in the stem portion, needles, roots, and basal portion of cuttings during the process of adventitious rooting in two cultivars of this species, known to have different adventitious rooting patterns. Our results demonstrated that antioxidative enzymes and microRNAs in the basal portion of the cuttings with high rate of root formation is an important factor affecting root formation in cuttings of T. chinensis. It has been reported in cuttings of some woody plants that formation of adventitious roots requires a certain level of antioxidative enzyme sand microRNAs [29] [31-34] [50,51]. The activity of different anti-oxidative enzymes like glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APO), peroxidase (POD) and polyphenol oxidase (PPO) were examined in cultivars of tea [32] and wheat [29]. These enzymes were reported to affect seed germination and root growth of several plant species including soybean roots [33]. In the present study, we identified that activity of PPO, CAT, and POD was significantly increased in basal portion (Figs. 3d-f) and adventitious roots (Figs. 3j-l) of rooted SR cuttings, compared to NR cuttings 77 days after planting.

To counteract the effects of environmental

stresses, the plants use antioxidant detoxification to avoid the accumulation of damaging free oxygen radicals and changes in the antioxidative machinery in plants under stress conditions played important role in root formation [39-44]. POD, APX, GR, and lipid peroxidation are directly interrelated with different factors to modulate activities of various stress markers [39]. The activities of antioxidative enzymes (SOD, CAT, POD, GR, APOX, MDHAR and DHAR) and contents of proteins and glutathione were increased in leaves of B. juncea plants after stress treatment [41]. SOD, APOX, GR accumulated in plants at comparably higher levels than their counterparts under dry soil conditions in peanut [42]. Inhibition of CAT, SOD, GPOX, APOX, GR, as well as increasing MDA concentration results in decreasing of fine roots volume [43]. In the present study, we identified that activity of APOX, GR, and SOD in adventitious roots significantly increased in SR cuttings (Figs.4j-l), compared to NR cuttings 77 days after planting.

MicroRNAs are known to affect root formation in plants. In Chlamydomonas reinhardtii, expression of multiple microRNAs, miR397, miR408, and miR857, involved in copper homeostasis [53] [54] [55]. In I. campanulata, miR398, miR168, miR858, miR162 and miR408 were up-regulated, while miR394 and miR171 were down-regulated under stress. In J. pentantha, miR394, miR156, miR160, miR164, miR167, miR172, miR319, miR395, miR396, miR403 were up-regulated and miR157 was down-regulated under stress. Basal miRNA levels and their drought-mediated regulation were very different between the two species [58] [60] [61]. Plants utilize complex gene regulation mechanisms to tolerate abiotic stresses. MicroRNAs are important regulators of gene expression acting at post-transcriptional level. Expression levels of miR408 regulated drought responsive genes and rooting -related genes [90-94]. Expression of microRNAs is related to the nutrition. Upon N starvation, the expression of miR169, miR171, miR395, miR397, miR398, miR399, miR408, miR827, and miR857 was repressed, whereas those of miR160, miR780, miR826, miR842, and miR846 were induced. Among these N-starvation-responsive miRNAs, several were involved in cross-talk among responses to different nutrient (N, P, S, Cu) deficiencies. miR160, miR167, and miR171 could be responsible for the development of Arabidopsis root systems under N-starvation conditions [53] [54] [55] [63] [64]. In the present study, we identified that expression of miR162, miR408, and miR857 significantly increased in basal portion (Figs.5d-f) of SR cuttings, compared to NR cuttings.

## Conclusion

In the present study, changes in the levels of antioxidative enzymes and microRNAs during the process of rooting were observed in the cuttings of T. chinensis. Significant increase in antioxidative enzymes and microRNAs expression levels was found in basal portion, stem portion, tips of adventitious roots, and tips of lateral roots in SR cuttings 77 days after planting in T. chinensis. Compared to NR cuttings, SR cuttings had higher expression of polyphenoloxidase (PPO), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APOX), glutathione reductase (GR), and superoxide dismutase (SOD) in the adventitious roots and basal portion of the rooted cuttings 77 days after planting. In the basal portion of cuttings, the content of thiobarbituric acid reactive substances (TBARS) and total phenols were decreased and the content of antioxidants was increased, but they did not changed in the needles of cuttings during planting. Analysis of microRNAs by quantitative real-time PCR demonstrated that expression of miR162, miR408, and miR857 increases in the basal portion of cuttings, but not in the stem portion of cuttings, 77 days after planting. Expression of miR408 and miR857 were also increased in the needles of cuttings 77 days after planting. Changes of these antioxidative enzymes and microRNAs are associated with the rooting features of T. chinensis var. mairei cuttings. Our research revealed that the levels and changes in endogenous factors affecting the rooting of cuttings differ between easy-to-root and recalcitrant-to-root T. chinensis. The individual features of antioxidative enzymes and microRNAs would benefit the development of efficient propagation methods for cuttings of T. chinensis.

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