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July 2015. Horticultural Plant Journal, 1 (1): 48–54.

Horticultural Plant Journal

Available online at www.sciencedirect.com

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The Physiological Mechanism of Improved Formaldehyde Resistance in *Petunia hybrida* Harboring a Mammalian *cyp2e1* Gene

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Received 25 March; Received in revised form 11 May 2015; Accepted 2 July 2015

Abstract

Cytochrome P450 CYP2E1 is mainly present in hepatocytes in the livers of mammals, where it plays an important role in the metabolism of xenobiotic organic substances. Previous studies showed that transgenic petunia (*Petunia hybrid*) plants harboring a mammalian *cyp2e1* gene (designated *cyp2e1*-transgenic petunia) exhibited increased resistance to formaldehyde stress. In this study, we used *cyp2e1*-transgenic petunia plants to analyze physiological indexes related to formaldehyde stress responses. The results indicated that under formaldehyde stress, the malondialdehyde content in *cyp2e1*-transgenic petunia plants was lower than in *β*-glucuronidase gene (*gus*)-transgenic and wild-type petunia plants. The activities of both superoxide dismutase and peroxidase in the *cyp2e1*-transgenic plants were higher than in *gus*-transgenic and wild-type plants. The alcohol dehydrogenase activity was slightly increased and more glutathione was consumed. Additionally, under formaldehyde stress, the levels of plant hormones including indole-3-acetic acid, zeatin and abscisic acid in *cyp2e1*-transgenic petunia plants displayed decreasing trends, whereas the level of gibberellic acid displayed an increasing trend. In contrast, the indole-3-acetic acid, zeatin and abscisic acid levels in *gus*-transgenic and wild-type petunia plants displayed increasing trends, whereas the gibberellic acid level displayed a decreasing trend. At 72 h after incubation of 0.5 g of *cyp2e1*-transgenic petunia plants in 40 mL of treatment solution containing formaldehyde at 50 mg · L^{-1}, the formaldehyde content remaining in the treatment solution was close to zero while approximately half of original formaldehyde remained in the treatment solutions containing *gus*-transgenic and wild-type petunia plants.

Keywords: *Petunia hybrida*; cytochrome P450 CYP2E1; formaldehyde stress; transgenic plant

1. Introduction

Cytochrome P450s are a class of heme-containing enzymes that can interact with heavy metals and play important roles in the metabolism of xenobiotic organic substances. Cytochrome P450 2E1 (CYP2E1), which is encoded by the *cyp2e1* gene, is a member of the cytochrome P450 super family and is mainly distributed in the hepatocytes of mammalian livers. In mammalian livers, CYP2E1 catalyzes alcohol oxidation and p-hydroxylation of molecules such as phenylamine, and thus has a very important detoxifying capability (Lieber, 1997; Leng and Qiu, 2001; Gonzalez, 2007).

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http://dx.doi.org/10.16420/j.issn.2095-9885.2015-0005

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While some plants have the capability to detoxify formaldehyde, the efficiency is generally quite low. Transgenic technology can be used to generate and cultivate transgenic plants that have the capability to remove organic pollutants in the environment with high efficiency (Abhilash et al., 2009). Doty's research group at the University of Washington in the United States was the first to generate transgenic plants with a mammalian *cyp2e1* gene (Doty et al., 2000). With the *Agrobacterium rhizogenes*-mediated transgenic technology, Zhang et al. (2011) introduced the rabbit hepatic *cyp2e1* gene into petunia and found that *cyp2e1*-transgenic petunia plants exhibited significantly increased resistance to formaldehyde. A study by Li et al. (2012) indicated that in *cyp2e1*-transgenic tobacco plants, the endogenous NADPH P450 oxidoreductase and cytochrome b5 played roles similar to those of their mammalian counterparts and were involved in the electron transfer chain during the catalysis process by CYP2E1, which was related to CYP2E1-mediated detoxification in the transgenic plants. However, the cellular and physiological mechanisms by which the transgenic *cyp2e1* gene enhances the capability of transgenic petunia to resist formaldehyde stress are currently unclear. In the present study, we treated the previously generated *cyp2e1*-transgenic petunia plants with formaldehyde and conducted cellular and physiological analysis on *cyp2e1*-transgenic, *gus*-transgenic, and wild-type petunia plants to explore the physiological responses of *cyp2e1*-transgenic plants to formaldehyde stress.

2. Materials and methods

2.1. Experimental materials and pre-treatments

The *cyp2e1*-transgenic, *gus*-transgenic, and wild-type petunia plants (without transgenes) were generated in our previous study (Zhang et al., 2011). These plants were sub-cultivated on 1/2 MS culture medium for about 15 days. Uniform healthy seedlings of petunia were used as experimental materials. The experiments were conducted in the Key Laboratory of Plant Science of Hangzhou Normal University during 2013–2014 (Hangzhou, Zhejiang, China).

Approximately 0.5 g of the upper branches of the sub-cultivated petunia seedlings was taken and put into an ampoule containing 40 mL of formaldehyde treatment solution (MS culture medium plus 50 mg · L^{-1} formaldehyde) or 40 mL of MS liquid culture medium (Fig. 1) and incubated at (20 ± 2) ℃ with continuing illumination at an intensity of 100 mol · m^{-2} · s^{-1} and shaking at 100 r · min^{-1} for 24 h.

The samples were taken out and washed with water. The excess water residue was absorbed with a paper towel. The samples were put into a pre-cooled homogenizer and 2 mL of pre-cooled 0.05 mol · L^{-1} phosphate buffer (pH 7.8) was added. The samples were homogenized on an ice bath to generate 20% homogenates, which were then centrifuged at 4 000 r · min^{-1} and 4 ℃ for 20 min. The supernatants were saved and used as crude enzyme extracts for subsequent assays of physiological indexes.

2.2. Measurement of malondialdehyde (MDA) content

The MDA content in the crude enzyme extract was measured with a plant MDA ELISA kit (Shanghai Xinran Bio-Tech Co., Ltd, Shanghai, China). A micro-well plate was coated with purified anti-plant MDA antibody to generate a solid antibody matrix. MDA solution was loaded into the wells pre-coated with antibody and then bound to horseradish peroxidase (HRP)-labeled anti-MDA antibody to form a complex of antibody-antigen-enzyme labeled with antibody. After thorough washing, the substrate tetramethylbenzidine (TMB) was added to develop the color. Under HRP catalysis, TMB was converted to a blue substance, which was further converted to a yellow substance under the action of an acid. The depth of the yellow color was positively proportional to the MDA content present in the samples. The absorbance at 450 nm (OD_{450}) was read with a microplate reader and a standard curve was drawn. The MDA content in the samples was calculated according to the standard curve.

2.3. Assays of superoxide dismutase (SOD) activity

Total SOD activity in the crude enzyme extract was assayed with a total SOD assay kit (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, Jiangsu, China). By measuring the superoxide radical (O_2^-) generated in the xanthine-xanthine oxidase system, which is oxidized to form nitrite and a substance with an amaranth color, the absorbance can be measured with a visible spectrophotometer.

When SOD is present in the samples, it causes specific reduction of the superoxide radical and reduces the formation of nitrite; thus, the absorbance of SOD-containing samples is lower than that of controls without SOD and the SOD activity in the samples can be calculated. One unit (U) of SOD activity was defined as the amount of SOD required for an inhibitory rate greater than 50% for 1 g of tissue in 1 mL of reaction solution.

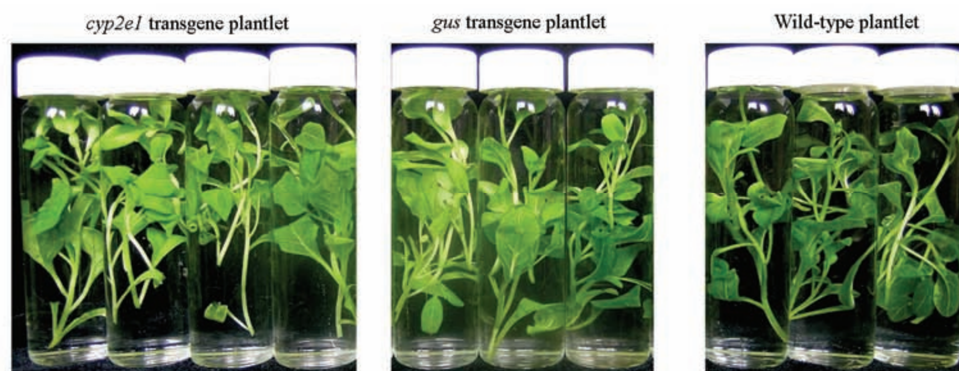

Fig. 1 Pre-treatment of samples

2.4. Assay of peroxidase (POD) activity

POD activity in the crude enzymatic extract was assayed with a plant POD ELISA assay kit (Shanghai Xinran Bio-Tech Co., Ltd). A micro-well plate was coated with purified anti-plant POD antibody to generate a solid antibody matrix. POD was added into the wells pre-coated with antibody and then bound to HRP-labeled anti-POD antibody to form a complex of antibody-antigen-enzyme labeled with antibody. After through washing, the substrate TMB was added to develop the color. Under HRP catalysis, TMB was converted to a blue substance, which was further converted to a yellow substance under the action of an acid. The depth of the color was positively proportional to the POD content in the samples. The OD_{450} was read with a microplate reader and a standard curve was drawn. The POD content in the samples was calculated according to the standard curve.

2.5. Assay of alcohol dehydrogenase (ADH) activity

ADH activity in the crude enzyme extract was assayed with a plant ADH ELISA assay kit (Shanghai Xinran Bio-Tech Co., Ltd). The protocol for ADH activity assay was the same as described for the POD activity assay in section 2.4 above.

2.6. Assay of the glutathione content

The glutathione content in the crude enzyme extract was measured with a glutathione assay kit (Nanjing Jiancheng Bioengineering Institute). 5,5′-dithiobis-(2-nitrobenzoic acid) reacts with glutathione to form 2-nitro-5-thiobenzoic acid and oxidizes glutathione to form glutathione disulfide. The 2-nitro-5-thiobenzoic acid product has a yellow color. The glutathione content can be determined by measuring the absorbance at 405 nm (OD_{405}). Under the action of glutathione disulfide reductase, glutathione disulfide is reduced back to glutathione, which then reacts with 5,5′-dithiobis-(2-nitrobenzoic acid) to form 2-nitro-5-thiobenzoic acid and causes cyclic reactions. Thus, the total glutathione content (glutathione plus glutathione disulfide) in the samples can be measured.

2.7. Assays of plant hormone contents

The contents of plant hormones including indole-3-acetic acid (IAA), zeatin (ZR), abscisic acid (ABA) and gibberellic acid (GA) were measured with a plant hormone ELISA kit (Shanghai Xinran Bio-Tech Co., Ltd). A micro-well plate was pre-coated with the corresponding purified anti-plant hormone antibodies (IAA, ZR, ABA or GA) to generate a solid antibody matrix. The corresponding hormone was loaded into the wells pre-coated with antibody and then bound to HRP-labeled anti-hormone antibody to form an antibody-antigen-hormone complex labeled with antibody. After thorough washing, the substrate TMB was added to develop the color. Under HRP catalysis, TMB was converted to a blue substance, which was further converted to a yellow substance under the action of an acid. The depth of the yellow color was positively proportional to the hormone content in the samples. The OD_{450} was read with a microplate reader and a standard curve was drawn. The content of each hormone in the samples was calculated according to the corresponding standard curve.

2.8. Assay of formaldehyde absorption

The amount of formaldehyde remaining in the formaldehyde-treatment solution was measured according to the "Water Quality-Determination of Formaldehyde-Acetyl Acetone Spectrophotometric Method" in The State Environmental Protection Standards (http://www.zhb.gov.cn/gkml/hbb/bgg/201102/W020110216389531775025.pdf), and was measured once every 8 h up to 72 h.

3. Results

3.1. Changes in the MDA content of cyp2e1-transgenic petunia plants

At 24 h after treatment with MS + 50 mg · L^{-1} formaldehyde,

the MDA contents in *cyp2e1*-transgenic, *gus*-transgenic and wild-type petunia plants were significantly increased (Table 1), but the increase in *cyp2e1*-transgenic plants was significantly lower than in the wild-type and *gus*-transgenic plants ($P < 0.05$).

3.2. Changes in SOD, POD and ADH activities in cyp2e1-transgenic petunia plants

Compared with the wild-type and *gus*-transgenic plants, SOD activity in the *cyp2e1*-transgenic petunia plants was increased (Fig. 2). Under formaldehyde stress, the SOD activities in the wild-type, *gus*-transgenic and *cyp2e1*-transgenic petunia plants all displayed significantly increasing trends ($P < 0.01$).

The POD activity in *cyp2e1*-transgenic petunia plants was higher than in wild-type and *gus*-transgenic plants (Fig. 2). However, under the formaldehyde stress at 50 mg · L^{-1}, the POD activities in all three groups of plants were decreased ($P <$ 0.01).

Compared with the wild-type and *gus*-transgenic plants, the ADH activity in *cyp2e1*-transgenic petunia plants was slightly increased. After formaldehyde stress, the ADH activities in wild-type and *cyp2e1*-transgenic petunia plants were slightly increased ($P < 0.05$).

Table 1 Changes of MDA content in *Petunia hybrida* after formaldehyde treatment

Formaldehyde treatment/(mg · L^{-1})	Sample	Line	MDA content/(nmol · L^{-1} FW)
0	Wild-type		0.199 ± 0.01 a
	gus-transgenic		0.198 ± 0.01 a
	cyp2e1-transgenic	Line 1	0.190 ± 0.01 b
		Line 2	0.189 ± 0.01 b
		Line 3	0.184 ± 0.01 b
50	Wild-type		0.217 ± 0.01 b
	gus-transgenic		0.227 ± 0.01 a
	cyp2e1-transgenic	Line 1	0.197 ± 0.01 c
		Line 2	0.194 ± 0.01 c
		Line 3	0.191 ± 0.01 c

Note: Different letters in the same column for the same treatment indicate significant difference among treatments at the 0.05 level.

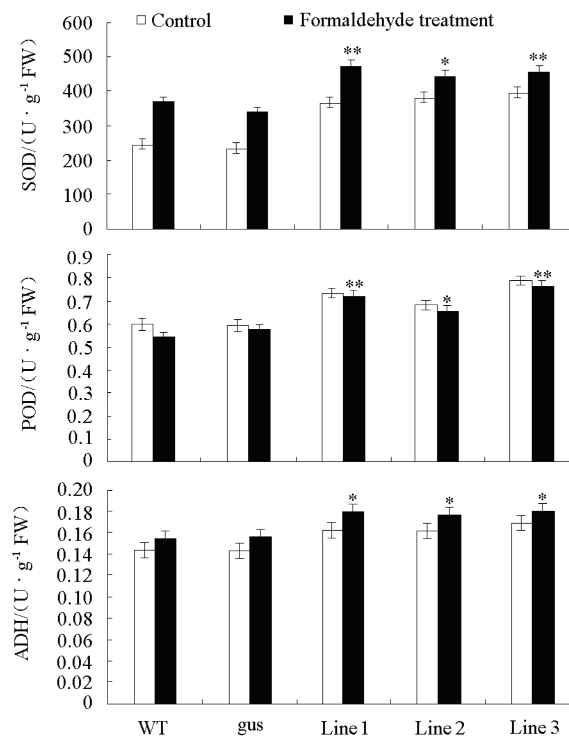

Fig. 2 Changes in the enzymatic activities of SOD, POD and ADH in wild-type, *gus*-transgenic and *cyp2e1*-transgenic *Petunia hybrida* treated with formaldehyde

WT: Wild-type plantlets; *gus*: *gus*-transgenic plantlets; Line 1, Line 2 and Line 3: Three different *cyp2e1*-transgenic plantlet lines. * and ** indicate significant difference between the transgenic lines and wild-type plants at $P < 0.05$ and $P < 0.01$, respectively, based on the *t*-test.

Table 2 Changes in total glutathione contents in *Petunia hybrida* treated with formaldehyde

Formaldehyde treatment/(mg · L^{-1})	Sample	Line	Total glutathione content/(μmol · g^{-1} FW)
0	Wild-type		132.818 ± 6.64 a
	gus-transgenic		114.427 ± 5.72 b
	cyp2e1-transgenic	Line 1	76.983 ± 3.85 c
		Line 2	93.020 ± 4.65 c
		Line 3	85.002 ± 4.25 c
50	Wild-type		112.588 ± 5.63 a
	gus-transgenic		93.737 ± 4.69 b
	cyp2e1-transgenic	Line 1	44.542 ± 2.23 c
		Line 2	40.864 ± 2.04 c
		Line 3	50.519 ± 2.53 c

Note: Different letters in the same column for the same treatment indicate significant difference among treatments at the 0.05 level.

Table 3 Contents of IAA, ZR, ABA and GA plant hormones

Formaldehyde treatment/ (mg · L^{-1})	Sample	Line	IAA/(pmol · g^{-1} FW)	ZR/(ng · g^{-1} FW)	ABA/(μg · g^{-1} FW)	GA/(μg · g^{-1} FW)
0	Wild-type		0.194 ± 0.01 c	133.952 ± 6.70 b	1.667 ± 0.08 b	2097.535 ± 104.88 c
	gus-transgene		0.225 ± 0.01 c	122.208 ± 6.11 b	1.756 ± 0.09 b	1999.706 ± 99.99 c
	cyp2e1-transgene	Line 1	0.239 ± 0.01 b	188.701 ± 9.44 a	1.894 ± 0.09 a	2093.912 ± 104.69 b
		Line 2	0.241 ± 0.01 b	179.547 ± 8.97 a	1.889 ± 0.09 a	2086.665 ± 104.33 b
		Line 3	0.263 ± 0.01 a	198.200 ± 9.91 a	1.879 ± 0.09 a	2307.687 ± 115.38 a
50	Wild-type		0.216 ± 0.01 b	142.933 ± 7.15 b	1.775 ± 0.08 a	1927.240 ± 96.36 c
	gus-transgene		0.242 ± 0.01 a	154.677 ± 7.73 b	1.827 ± 0.09 a	1811.295 ± 90.56 c
	cyp2e1-transgene	Line 1	0.203 ± 0.01 c	177.648 ± 8.88 a	1.679 ± 0.08 a	2209.858 ± 110.49 b
		Line 2	0.191 ± 0.01 c	172.984 ± 8.64 a	1.745 ± 0.09 a	2195.364 ± 109.77 b
		Line 3	0.176 ± 0.01 d	164.694 ± 8.23 a	1.664 ± 0.08 a	2517.838 ± 125.89 a

Note: Different letters in the same column for the same treatment indicate significant difference among treatments at the 0.05 level.

3.3. Changes in glutathione contents in cyp2e1-transgenic petunia plants

Before the formaldehyde treatment, the total glutathione content in the *cyp2e1*-transgenic petunia plants was lower than in the wild-type plants (Table 2). Under formaldehyde stress, the glutathione contents in petunia plants of all three groups decreased but the decreasing trend in *cyp2e1*-transgenic plants was more significant ($P < 0.01$).

3.4. Changes in the plant hormone contents in cyp2e1-transgenic petunia plants

Formaldehyde stress caused changes in plant hormone contents. Compared with untreated samples, under the formaldehyde stress at 50 mg · L^{-1}, the contents of IAA, ZR and ABA in *cyp2e1*-transgenic petunia plants displayed decreasing trends, whereas the GA content displayed an increasing trend. In contrast, in wild-type and *gus*-transgenic petunia plants, the contents of IAA, ZR and ABA displayed increasing trends, whereas the GA content displayed a decreasing trend ($P < 0.05$) (Table 3).

3.5. Changes in formaldehyde content remaining in the treatment solution

In the cultivation medium containing formaldehyde, within the first 8 h, the formaldehyde content remaining in the solution of *cyp2e1*-transgenic petunia plants was not different from that in the solution of wild-type plants. However, after prolonged treatment up to 72 h, the formaldehyde content in the treatment solution of *cyp2e1*-transgenic plants was close to zero, whereas the formaldehyde contents remaining in the treatment solutions of wild-type and *gus*-transgenic plants were close to the half of the original contents (Fig. 3).

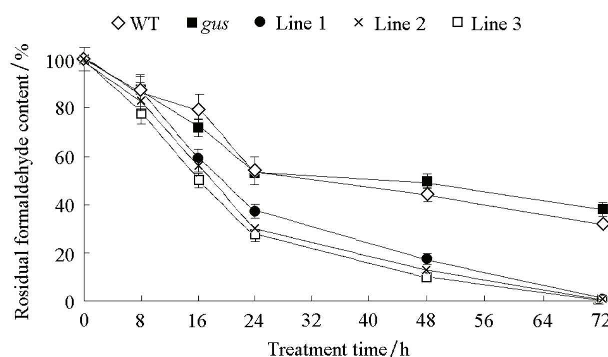

Fig. 3 Changes in the residual formaldehyde content in the treatment solution

WT: Wild-type plantlet; *gus*: *gus*-transgenic plantlet; Line 1, line 2, line 3: Three different *cyp2e1*-transgenic plantlets.

4. Discussion

Cytochrome P450 CYP2E1 is mainly present in hepatocytes in the livers of mammals, where it participates in activating and / or inactivating many pre-toxicants, pro-carcinogenic substances and some medicines. It mediates the response to oxidative stress induced by oxidative free radicals (Liu and Yue, 2010). It has been shown that the trichloroethylene and ethylene dibromide metabolizing capabilities of transgenic tobacco (*Nicotiana tabacum*) harboring the *cyp2e1* gene were significantly increased. In plants, the mammalian CYP2E1 enzyme was capable of degrading trichloroethylene into chloral and trichloroethanol and degrading 1,2-dibromoethane into intermediate substances, bromoacetaldehyde, and bromide ions (Doty et al., 2000). The results of the present study demonstrated that under formaldehyde stress, the MDA content in *cyp2e1*-transgenic petunia plants was significantly lower than in wild-type and *gus*-transgenic petunia plants, which ameliorated the damage to cellular membranes caused by formaldehyde stress and maintained the integrity/intactness of cellular membranes. The enzymes SOD and POD are anti-oxidant enzymes that remove excess oxidative free radicals within cells (Barreto et al., 2014). The activities of both SOD and POD in *cyp2e1*-transgenic petunia plants were increased, which helped to protect the structural integrity of the cells. It has been reported that plants themselves possess a dehydrogenase that metabolizes endogenous formaldehyde (Sakamoto et al., 2002). This dehydrogenase is glutathione-dependent alcohol dehydrogenase (ADH), which catalyzes the reaction HCHO + glutathione + NAD^+ → S-formyl glutathione + NADH + H^+ (Thompson et al., 2010). Compared with wild-type and *gus*-transgenic plants, the ADH activity in *cyp2e1*-transgenic petunia plants was slightly increased. Under formaldehyde stress, the ADH activity was slightly higher in *cyp2e1*-transgenic plants than in wild-type and *gus*-transgenic plants, indicating that the endogenous ADH is involved in the process of metabolizing xenobiotic formaldehyde. Additionally, under formaldehyde stress, the *cyp2e1*-transgenic petunia plants consumed more glutathione. This is probably because more ADH was involved in metabolizing formaldehyde in the *cyp2e1*-transgenic plants and ADH needs glutathione to perform this role (Thompson et al., 2010). Glutathione itself also possesses an anti-oxidant function (Choe et al., 2013). After being treated with cultivation solution containing formaldehyde for 72 h, the formaldehyde content remaining in the solution of *cyp2e1*-transgenic petunia plants was close to zero, indicating that the *cyp2e1* gene may induce the enzymatic system involved in metabolizing formaldehyde that has already entered the cells instead of resisting the formaldehyde outside the cellular membranes. However, the results of this study still cannot clearly explain whether the formaldehyde that entered the cells was completely degraded or combined with other cellular substances to form complexes. Moreover, compared with wild-type plants, the ADH activity in the *cyp2e1*-transgenic petunia was not significantly higher but the *cyp2e1*-transgenic plants still grew normally even under the formaldehyde stress at 50 mg · L^{-1} and their resistance to formaldehyde was significantly enhanced (Zhang et al., 2011). Kang et al. (2010) reported that after being treated with trichloroethylene, more genes were up-regulated and/or down-regulated in *cyp2e1*-transgenic poplar plants than in wild-type plants. They proposed that the up- and/or down-regulated genes were involved in the process of metabolizing trichloroethylene. Whether similar up- and/or down-regulation of genes occurs in *cyp2e1*-transgenic petunia plants under formaldehyde stress and whether these genes are involved in metabolizing xenobiotic formaldehyde remains to be further explored.

Under environmental stresses, changes in the balance of plant hormones can lead to morphological and physiological changes related to the corresponding responses to these stresses (Hey et al., 2010). After being treated with formaldehyde, the IAA, ZR, and ABA contents in the *cyp2e1*-transgenic petunia plants exhibited decreasing trends, whereas the GA content exhibited an increasing trend. In contrast, the opposite changes were observed in wild-type and *gus*-transgenic plants, i.e. the IAA, ZR and ABA contents all displayed increasing trends whereas the GA content displayed a decreasing trend. These results indicate that the *cyp2e1* gene influences multiple signal transduction pathways mediated by plant hormones, which in turn confers resistance to external stresses such as formaldehyde stress. However, the cellular and molecular mechanisms underlying the changes in plant hormone-mediated pathways in *cyp2e1*-transgenic petunia plants under environmental stresses remain to be further elucidated.

Acknowledgments

The work was supported by the Hangzhou Science and Technology Development Plan (Grant No. 20140432B05) and the New-shoot Talents Program of Zhejiang Province (Grant No. 2013R421008).

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