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Full Length Research Paper

Characterization and comparison of three transgenic *Artemisia annua* varieties and wild-type variety in environmental release trial

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Artemisinin, a sesquiterpene isolated from *Artemsisia annua* L. (Chinese wormwood) is an increasingly popular drug against malaria. In this study, three transgenic *A. annua* varieties with increased artemisinin content were evaluated in an environmental release trial. First, because there were risks concerning genetically modified plants, the 3 transgenic varieties were compared with that of wild-type plants in regard to agronomic traits, viability and expressed material. Second, stress tolerances (salt, drought and herbicide) were evaluated under experimental conditions. The results indicated significant differences between the 3 transgenic and wild-type plants in some agronomic traits (as in plant height, crown width, stem diameter, 1000-seeds weight), and no significant differences in leaf shape. Also, the viability (germination rate, transplant survival and seeds wintering ability) from both transgenic and control plants were similar. Moreover, it also was determined that the 3 transgenic and control plants had similar stress tolerances (salt tolerance, drought tolerance, herbicide tolerance). The expression of target genes in transgenic varieties was either enhanced or suppressed. Thus, the artemisinin content was increased in transgenic varieties as expected.

Key words: Transgenic *Artemisia annua,* agronomic traits, viability, expressed materials, stress tolerances.

INTRODUCTION

Artemisinin, a sesquiterpene isolated from *Artemisia annua L.* is an effective drug against malaria. Malaria is responsible for about 500 million cases and 100 million deaths each year, mostly in Africa and Southeast Asia (Liu et al., 2006; Ro et al., 2006). Furthermore, the potential of artemisinin and its derivatives as anti-tumor natural drugs has been reported (Posner et al., 2004; Singh and Lai, 2001). Because the artemisinin content of *A. annua* is very low and the total synthesis of artemisinin is both difficult and costly, genetic engineering is an alternative to increase the content of artemisinin in *A. annua*.

Three transgenic *A. annua* varieties were generated previously through *Agrobacterium tumefaciens*-mediated transformations. One of the 3 transgenic varieties was developed using RNAi technology to suppress expression of squalene synthase (SQS), which catalyses the first step of a pathway in competition with that of artemisinin biosynthesis. The two other transgenic

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varieties were developed to enhance the expression of amorpha-4,11-diene synthase (ADS), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) and farnesyl diphosphate synthase (FPS), respectively, which are the 3 key enzymes catalyzing the steps of the artemisinin biosynthesis (Zhang et al., 2009).

Although, in recent years, many transgenic plants have been released into the environment, there are still a multitude of open questions with regard to the environmental release and commercialization of genetically modified (GM) plants. Therefore, applications for deliberate release of a new transgenic plant are required to be submitted to regulatory authorities in the European Union (EU), United Kingdom, Japan, United States and China. In the EU, applications have been submitted under the Deliberate Release Directive (90/220 and, since 2001, 2001/18). Data on genetically modified organism (GMO) field trials in the US can be found in the following website: http://www.nbiap.vt.edu/cfdocs/fieldtests1.cfm. Summaries of GMO field trials in the Organization for Economic Cooperation and Development (OECD) member countries, together with data from other countries which have been provided through United Nations Industrial Development Organization's Biosafety Information Network and Advisory Service, are presented at the following websites: http://www.olis.oecd.org/biotrack.nsf and http://binas.unido.org/binas/trials.php3. Many international consensus documents have been produced by OECD, the World Health Organization and the Food and Agriculture Organization (www.agbios.com). In China, the Ministry of Agriculture (MOA) implemented its GMO regulations in 1996 (Jia and Peng, 2002). All biosafety assessment applications related to experimental research, field trials and environmental release are required to be evaluated by the GMO Biosafety Committee before commercialization. To date, many transgenic plants have been tested in field trials, and even grown commercially; however, there is no report on the evaluation of the characteristics of transgenic *A. annua* in environmental release trial.

After the experiment of 3 transgenic *A. annua* varieties were completed, the MOA of China permitted them to be assessed for environmental release. The comparisons of the characteristics of 3 transgenic *A. annua* varieties and wild-type variety as discussed in the present study were modeled after data from the above mentioned websites. This was the first time in China that transgenic varieties used for medicine were tested in environmental release trials.

In the present study, we characterized morphological performance of one wild-type and 3 transgenic varieties. The responses of these 4 varieties to drought stress, salt stress and herbicides were compared. Persistence and survival information provided evidence for evaluating the increased fitness of transgenic lines and its ability to survive.

The 3 transgenic *A. annua* varieties were developed to increase the artemisinin content. Accordingly, the expression of the introduced genes was estimated by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and the artemisinin content was determined by high-performance liquid chromatography-evaporative light-scattering detection (HPLC-ELSD). This study will provide useful data to the environmental risks assessment for the commercialization of transgenic *A. annua* varieties.

MATERIALS AND METHODS

Plant materials

The 3 transgenic *A. annua* varieties used for this study were kindly provided by Prof. Kexuan Tang from Shanghai Jiaotong University. They were designated ANF, SQS and GFH, respectively. The *A. tumefaciens*-mediated transformation of *A. annua* collected from Chongqing (a municipality in southwest China) was established by Prof. Tang's lab (Zhang et al., 2009), and the wild-type *A. annua* plants were used as control plants. After transformation, the expression of the ADS gene was enhanced in ANF; and the expressions of FPS and HMGR genes were enhanced in GFH; in contrast, the expression of SQS gene was suppressed in SQS.

Field location

The transgenic and control plants were cultivated in White Crane field station of the Shanghai Academy of Agricultural Sciences, which is authorized by the MOA for environmental risk assessment of transgenic plants. The field station is located in the northwestern part of Shanghai City. It has a subtropical monsoon climate, distinct seasons, full sunshine with abundant rainfall, and is suitable for the growth of *A. annua* plants. There are concrete walls around the field station for isolation.

Experimental design

According to regulations for safety assessment of GMOs in China, every *A. annua* variety was planted in a rectangular plot, covering an area of about 0.3 ha (Guidelines for the Safety Assessment of Transgenic Plants, China, 2007); plot length was 100 m and width 30 m. Non-transgenic maize was planted around each plot to isolate the 4 *A. annua* varieties from each other. All plants were cultivated according to routine practices in the corresponding region, with about 1152 plants per plot (1.25 m between rows).

Transplant survival

The seedlings of 4 *A. annua* varieties were grown in pots in growth chamber for 2 months, then transplanted and grown in the field. After transplant, the number of dead seedlings was calculated.

Phenotypic characterization

Three months after transplant, the growth and morphology of transgenic and control plants were compared. The morphological comparisons included the plant height, crown width, stem diameter, and leaf length and width; leaves from different heights on plants

Table 1. Primers used for real-time RT-PCR.

Primer	Sequence (5′–3′)	Amplification length (bp)	GenBank no.
ADS-1F	AATGGGCAAATGAGGGACAC	252	AF138959
ADS-2R	TTTCAAGGCTCGATGAACTATG		
FPS-1F	TCATTGTCTATTCACCGCCG	453	AF112881
FPS-2R	CACCGCTTGGACTGCTTTGCT		
HMGR-1F	TTGTGTGCGAGGCAGTAAT	183	AF142473
HMGR-2R	CCTGACCAGTGGCTATAAAGA		
SQS-1F	ATCCCATCACAACCTCAC	451	AF405310
SQS-2R	ACAACCCTATTCCAACAAGTC		
UBC-1F	CACACTTGAGGTTGAGTCCAG	263	EF549585
UBC-2R	CATAACATTTGCGGCAGATAG		

were chosen for measuring leaf parameters to avoid variation due to environmental factors. And the dry weight of leaves was determined after 48 h in a drying oven. For seed morphology comparisons, the seeds were harvested from one spike in the year before environmental release trial. The seeds of *A. annua* are very small and difficult to collect, therefore the flowers of 30 plants were bagged and their seeds collected from the bags. The 1000-seed weight of each variety was measured based on the seeds harvested from 30 individual plants.

Seed germination

The seeds of 4 *A. annua* varieties were carefully dehusked, washed with detergent and rinsed thoroughly with distilled water. Of each variety, 100 seeds were sown in trays loaded with aseptic mixed vermiculite soil (soil : verimiculite : perlite = 7:3:1), and another 100 seeds sown in distilled water. After 7 d at 25°C, the germination rate of each variety was calculated using the equation:

Seed germination rate (%) = (number of seeds germinated/ number of full seeds) × 100.

Salt stress

NaCl was added to the nutrient solution at concentrations of 0, 0.4, 0.8, 1.2 and 1.6% (w/w). The experiments started with five-week-old plants. After 9 days, the leaf's relative water contents of stressed groups (12 plants per group) were measured:

Leaf relative water content (%) = [(Fresh weight – Dry weight)/Fresh weight] × 100 (Lai and Lui, 1988).

Peroxidase (POD) activity and free proline contents were measured according to reported methods (Hunting et al., 1959; Lei et al., 2007; Summart et al., 2010).

Drought stress

Five-week-old plants were used in this experiment. PEG 6000 was added to the nutrient solution to impose drought stress. PEG 6000 is too large to be taken up by intact plant roots (Lawlor, 1970) and enables the imposition of uniform and controllable drought stress. Of each *A. annua* variety, 12 plants were stressed with the PEG 6000 concentrations at 0, 50, 100 and 150 g/L. The free proline contents of drought stressed plants were determined as stated above.

Herbicide treatment

Two types of herbicide were purchased from Syngenta Co. (Shanghai, China): paraquat, a non-selective herbicide; and metolachlor, a selective herbicide. Paraquat was sprayed on five-week-old plants, and metolachlor was sprayed 3 days after the seeds of 4 *A. annua* varieties were sown according to instructions and plants observed later.

RNA extraction and real-time RT-PCR

RNA was isolated from the upper leaves of transgenic and control *A. annua* plants using the Plant Total RNA Isolation Kit from Tiangen Biotechnology Co. Ltd (Beijing, China). DNA contamination was removed using DNase I following the protocol provided by the manufacturer (TaKaRa). The cDNA were synthesized from the RNA samples using the Reverse Transcriptase Reagent according to the manufacturer's (Promega) instruction.

The real-time RT-PCR analysis was performed in an ABI 7500 (Applied Biosystems, Foster City, CA, USA), using the cDNAs as templates and the gene-specific primers for the analyzed gene, as listed in Table 1. The ubiquitin-conjugate enzyme (UBC) gene was used as a reference gene. SYBR Green (SYBR Premix Ex Taq; TaKaRa) was used in the Polymerase Chain Reaction (PCR) to quantify the amount of dsDNA. The relative Ct (threshold cycle value) method was used to estimate the initial amount of template present in the reaction (Liu and Saint, 2002).

Quantification of artemisinin using HPLC-ELSD

Of *A. annua* leaves, 1 to 2 g was used for quantification of artemisinin using HPLC-ELSD (Zhang et al., 2009). This was

Table 2. Morphological characteristics of transgenic *A. annua* and control plants. Each value indicates the mean ± standard error of 30 replicates.

Plants	Plant height (cm)	Crown width (cm)	Stem diameter (cm)	Leaf length[1] (mm)	Leaf width (mm)	Leaf weight (g)
ANF	110.08 ± 15.20*	82.41 ± 29.83	1.234 ± 0.22**	65–74 (72.6)	50–62 (56.8)	0.026 ± 0.004
SQS	135.15 ± 18.00*	115.26 ± 15.49**	1.925 ± 0.44*	75–90 (82.2)	55–70 (59.4)	0.023 ± 0.007
GFH	125.00 ± 13.29	96.56 ± 19.88	1.245 ± 0.23**	58–80 (70.6)	47–69 (56.8)	0.025 ± 0.005
Control	128.00 ± 13.30	90.66 ± 26.82	1.644 ± 0.20	75–88 (77.6)	55–85 (65.0)	0.027 ± 0.003

[1] Values in parentheses are mean values; *significant at $P < 0.05$, **significant at $P < 0.01$.

Table 3. Seed characteristics of 3 transgenic *A. annua* varieties and controls plants.

Plants	1000-seed weight (g)	Germination rate (aseptic soil) (%)	Germination rate (aseptic water) (%)
ANF	0.0477 ± 0.0007	88.7 ± 2.4	60.7 ± 1.4
SQS	0.0611 ± 0.0001*	90.0 ± 2.1	64.0 ± 3.2
GFH	0.0465 ± 0.0003	92.3 ± 1.7	72.0 ± 0.6*
Control plants	0.0479 ± 0.0005	90.6 ± 1.8	62.3 ± 2.5

*significant at $P < 0.05$.

repeated 3 times, each time with 3 replicates. Empower (Water's chromatography data software) was used for data analysis.

Statistical analysis

All data of 3 transgenic *A. annua* and control plants were compared and analyzed using the Independent-Samples T Test in SPSS 16.0 software.

RESULTS

ADS, SQS, HMGR and FPS are 4 key enzymes in artemisinin biosynthesis and are expressed in control as well as transgenic plants. To increase the artemisinin content, the introduced ADS, SQS, and FPS and HMGR genes were *A. tumefaciens*-mediated transformed and driven by the 35S promoter in transgenic plants ANF, SQS, and GFH, respectively (Zhang et al., 2009). Because ADS, SQS, HMGR and FPS also exist in control plants, the introduced gene products of transgenic plants will not supply novel hazardous material into the environment. In this study, the substantial equivalence between the transgenic and control plants was established on the basis of morphology, germination rate and tolerance to stresses.

To ascertain the characteristics of the transgenic compared to control plants, the plants were grown in similar and adjacent fields. The results showed that on the one hand, some agronomic characteristics of transgenic plants were significantly different from the control plants (as in plant height, crown width, stem diameter, 1000-seeds weight), while on the other hand, the 3 transgenic plants had leaf shapes, germination rates and stress tolerances virtually indistinguishable from control plants, except for the expression of introduced genes of the former, which contributed to increased artemisinin content. The following summaries provide further details of the studies.

Comparative characterizations of transgenic and control plants

Morphology

The morphological characteristics of the transgenic and control plants were examined (Table 2). The 3 transgenic *A. annua* and control plants presented significant differences in basic agronomic traits, such as plant height, crown width, and stem diameter which were significantly larger or smaller ($P < 0.05$ and $P < 0.01$, respectively). Sushil and Suchi (2005) found rich artemisinin present in *A. annua* leaves. The control and 3 transgenic plants had leaf lengths of 58 to 90 mm and widths of 47 to 85 mm. The leaves of control plants were slightly but not significantly larger than that of transgenic plants. Leaf weights were also not significantly different between 3 transgenic *A. annua* and control plants.

Seeds traits

The seeds from 30 plants of 4 *A. annua* varieties were morphologically characterized, including the weight of 1000-seeds and the rate of germination in two conditions above (Table 3). There were no significant differences in

Table 4. Relative water content of leaves treated with different NaCl concentrations (0, 0.4, 0.8, 1.2 and 1.6%).

NaCl concentration	Relative water content of leaves (%)				
Plant	**0%**	**0.4%**	**0.8%**	**1.2%**	**1.6%**
ANF	93.17 ± 0.57	90.70 ± 0.21	88.61 ± 0.32	94.31 ± 2.01	91.26 ± 1.47
SQS	94.43 ± 2.10	90.45 ± 1.05	93.35 ± 1.11	90.28 ± 0.26	87.17 ± 0.84
GFH	92.18 ± 0.56	90.78 ± 0.58	90.71 ± 1.24	89.97 ± 1.12	87.87 ± 1.00
Control plants	93.62 ± 1.02	88.33 ± 0.32	90.86 ± 0.40	88.92 ± 0.58	91.98 ± 0.51
Min. difference[a]	0.45	2.12	0.15	1.05	0.72
Max. difference[b]	1.44	2.45	2.48	5.39	4.81

[a] The minimum difference between control and the transgenic plant, [b] The maximum difference between control and the transgenic plant.

seed characteristics of the 4 varieties, except for the germination rates of GFH in distilled water, which were significantly higher than that of the other varieties.

Transplant survival

After the seedlings of 4 *A. annua* varieties grew in pots in growth chamber for 2 months, they were transplanted in field. 19 seedlings of transgenic *A. annua* ANF were dead after transplanting, 17 of SQS, 20 of GFH and 15 of control plants were dead. Almost all of the 4 varieties survived and showed no difference between them (about 1152 seedling plants of each variety transplanted).

Seed wintering ability

The seeds of *A. annua* matured in October in China. In this study, the seeds of the 4 varieties showed essentially identical wintering ability in the test field, and then germinated at the appropriate temperature (≥ 5℃) (data not shown).

Stress tolerances

A. annua is a successful invasive species due to its production of large quantities of diaspores, its extreme vigor, and its freedom of disease and pests. Due to the growth and reproductive characteristics of *A. annua*, it is necessary to evaluate whether the transgenic plants possessed altered traits or not, which could enhance their tolerances to environmental stresses, such as salt, drought, herbicide.

Salt stress

The leaf's relative water contents for different NaCl concentration treatments of the 4 varieties had a range of 87 to 94% (Table 4). As the NaCl concentration increased, the leaf's relative water content decreased slightly. With the exception of the leaf relative water content of variety ANF at 1.2% NaCl, all differences in values between control and transgenic plants were < 5. Thus, *A. annua* appeared to maintain leaf water content under salt stress, and the transgenic plants do not alter their potentials in comparison to their conventional counterpart.

Salt-stress induced oxidative stress in plants. POD plays an important role in H_2O_2 detoxification and the scavenging of soluble hydroperoxides (Otter and Polle, 1994). As the applied NaCl concentrations increased, the POD activity of ANF, SQS and control plants increased slightly in leaves (Figure 1a); however, at 1.6% NaCl, the POD activity decreased. By contrast, the POD activity of GFH decreased slightly with increased NaCl concentrations of 0.4 and 0.8%, to a maximum of 1.2% NaCl and then decreased with 1.6% NaCl. In summary, with 1.2% NaCl treatment, the POD activities of the 4 *A. annua* varieties were at their maxima and showed no significant difference between them.

Proline acts as an enzyme protectant, and stabilizes membranes and cellular structure under salt stress (Shakirova, 2007). It is generally believed that proline is a compatible solute in osmotic adjustment and plants accumulate proline under environmental stress as an adaptive trait for stress tolerance. The proline content in leaves of the 4 *A. annua* varieties increased slightly as NaCl concentration increased (Figure 1b); however, the proline content of 4 *A. annua* varieties treated with 1.2% NaCl was significantly higher than that without NaCl treatment. Proline accumulation was statistically equivalent between transgenic and control plants. By contrast, the proline content of the 4 varieties decreased slightly at 1.6% NaCl.

In summary, for different NaCl concentration treatments, the responses of the transgenic and control plants were similar. Thus, the 3 transgenic varieties do not appear to have advantages over control plants in salt tolerance.

Drought stress

Five-week-old plants of the 4 *A. annua* varieties were

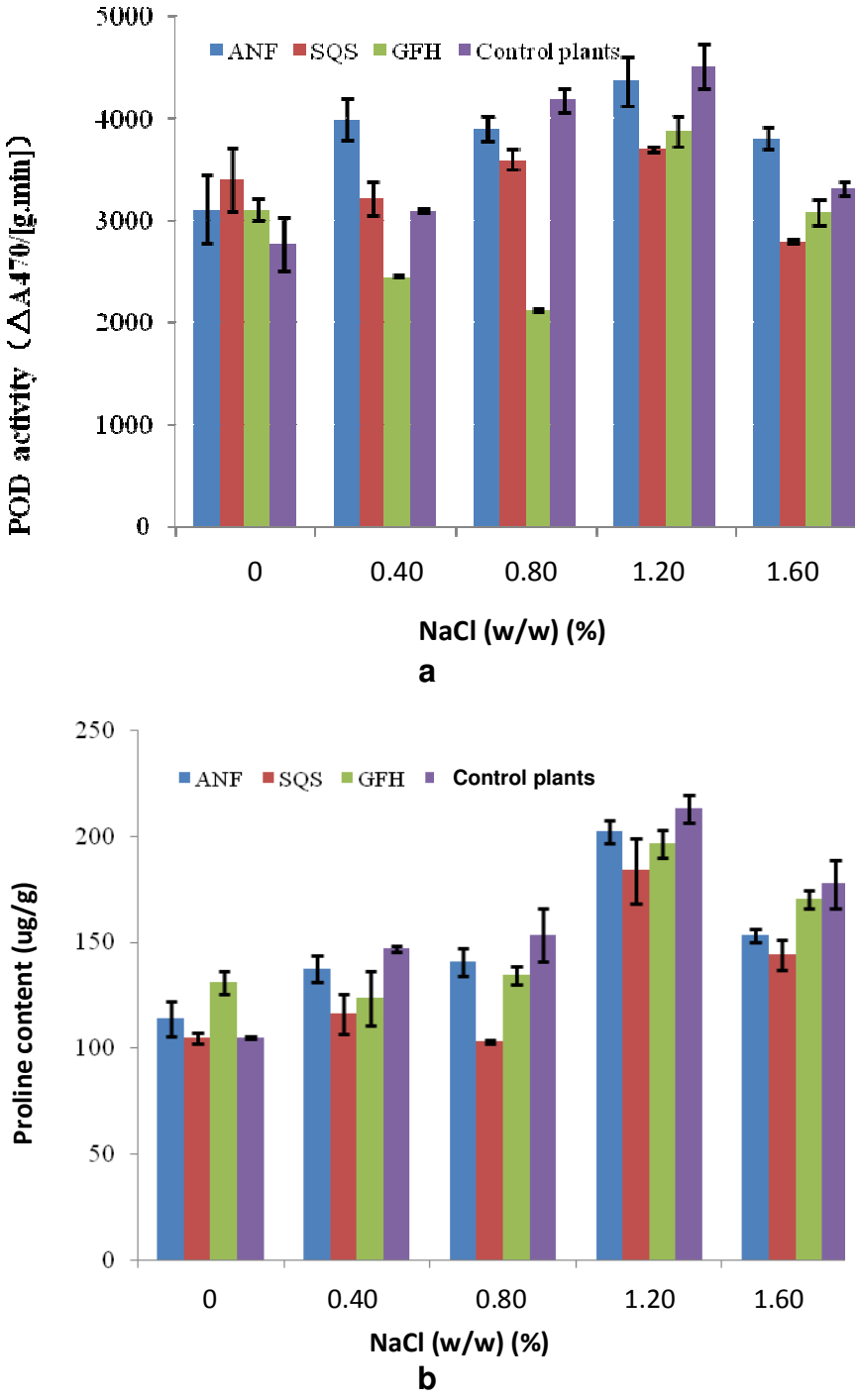

Figure 1. POD activities and proline contents in leaves of 4 *A. annua* varieties at different application of NaCl concentrations. (a) POD activities, (b) Proline contents.

treated with PEG 6000 at concentrations of 0, 100, 200 and 300 g/L. After 9 days, the relative water content, POD activity, and proline contents in leaves were measured. The results showed no significant differences between transgenic and control plants (data not shown). With higher PEG concentrations and longer treatment time, the 4 varieties were all affected but to different degrees (Figure 2). At 300 g/L of PEG 6000 treatment, both transgenic and control plants turned yellow and wilted after 15 day of treatment; however, the 4 varieties were not killed by the drought stress, but grew almost normally at 100 and 200 g/L of PEG 6000 treatment.

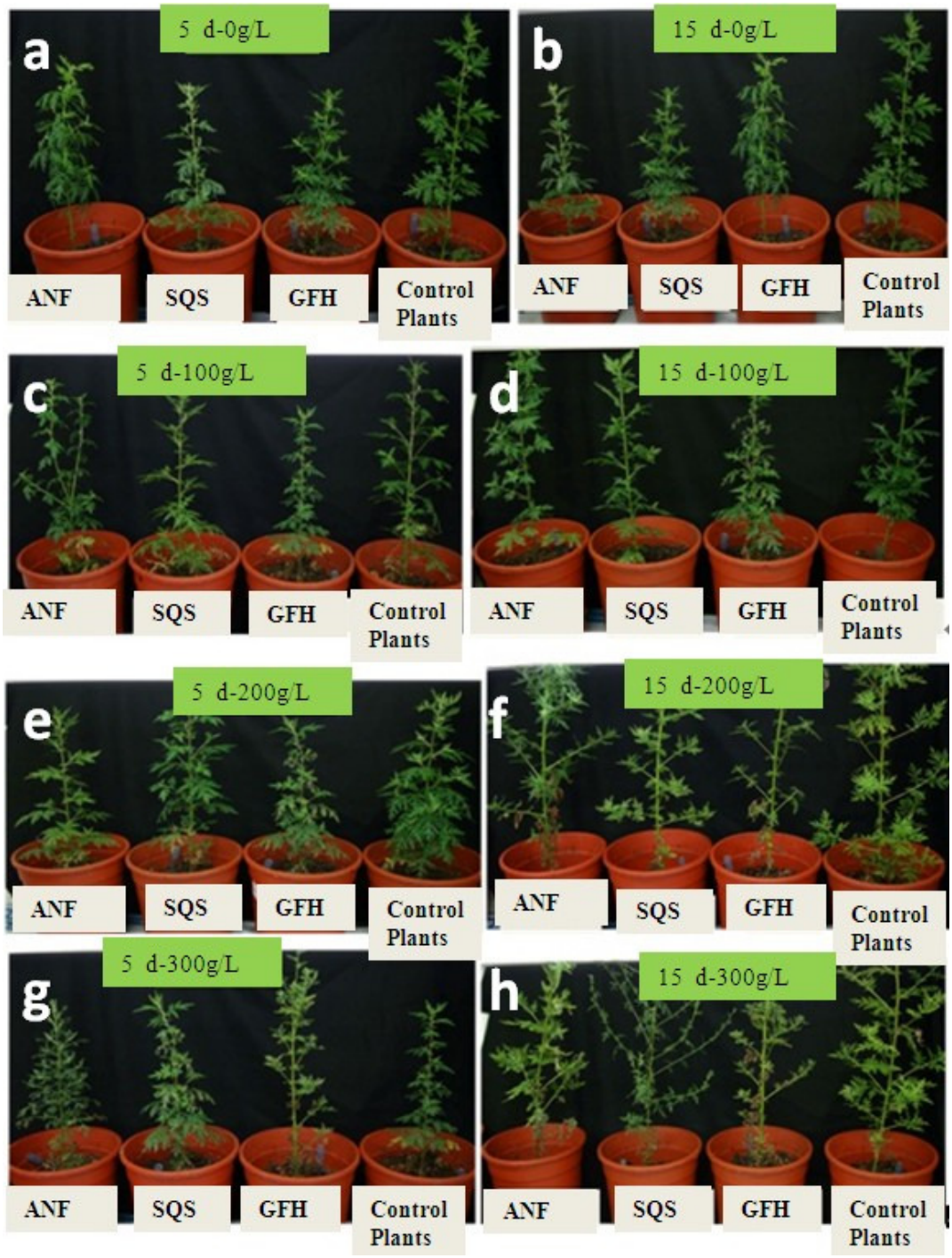

Figure 2. The growth of 3 transgenic and control varieties of *A. annua* under drought stress by application of different concentrations of PEG 6000 for different periods. (a) 0 g/L PEG 6000 for 5 days. (b) 0 g/L PEG 6000 for 15 days. (c) 100 g/L PEG 6000 for 5 days. (d) 100 g/L PEG 6000 for 15 days. (e) 200 g/L PEG 6000 for 5 days. (f) 200 g/L PEG 6000 for 15 days. (g) 300 g/L PEG 6000 for 5 day. (h) 300 g/L PEG 6000 for 15 days.

Thus *A. annua* seems to be drought tolerant, and the transgenic plants did not show enhanced drought tolerance in comparison to control plants.

Herbicide resistance

Two types of herbicides, paraquat and metolachlor, were

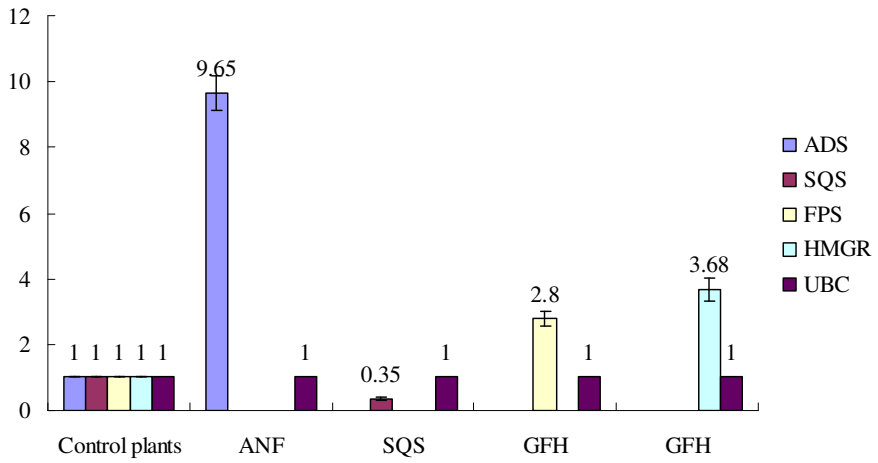

Figure 3. ADS, SQS, FPS and HMGR mRNA accumulation in transgenic plants as determined by real time RT-PCR. UBC-gene was used as the reference gene in real-time RT-PCR.

used. Paraquat is non-selective and kills a wide range of plants. After spraying paraquat, all samples of transgenic and control plants were killed in 2 h. Metolachlor is a selective herbicide and was sprayed before seed germination. No seeds of the 4 *A. annua* varieties germinated while the seeds of 4 varieties free of metolachlor did successfully and the seedlings grew well. Whether sprayed with metolachlor or not, the performance of transgenic and control plants was similar. This indicates that the transgenic and control plants had similar responses to the two common herbicides.

Expressed material

ADS, SQS, FPS and HMGR are endogenous and key enzymes in the pathway of artemisinin biosynthesis in *A. annua* (Zhang et al., 2009). The expressions of ADS and FPS and HMGR were enhanced in transgenic *A. annua* varieties ANF and GFH, respectively, while that of SQS was suppressed in SQS. It was only the content of endogenous enzymes that was increased or decreased in transgenic *A. annua* varieties. Therefore, the transgenic plants did not introduce hazardous materials into the environment in comparison to control plants.

Real-time RT-PCR (Figure 3) showed that expression of ADS, FPS and HMGR was enhanced in transgenic plants ANF and GFH (280 to 965% of control values), and that of SQS was suppressed in transgenic SQS (35% of control values). Furthermore, the 3 transgenic varieties had significantly higher artemisinin contents (the highest 14.45 mg/g DW) than control plants (9.88 mg/g DW; Figure 4).

In summary, the 3 transgenic varieties were developed to enhance the expression of endogenous enzymes, thus artemisinin content was increased in transgenic *A. annua* plants. The sequence of introduced genes (GenBank nos. AF138959, AF112881, AF142473 and AF405310) were acquired from the control plants; therefore, the expressed products were found in control as well as transgenic plants. The enhanced or suppressed expressions of target genes and increased artemisinin content were validated by real-time RT-PCR and HPLC-ELSD.

DISCUSSION

Artemisinin is an increasingly popular as an effective and safe alternative therapy against malaria. Artemisinin-based combination therapy is considered the most effective way to combat drug-resistant malaria (Olliaro and Taylor, 2004); however, the total artemisinin content of *A. annua* is very low. Moreover, the total organic synthesis is very complicated with low yields, and costly (Avery et al., 1992). In view of these problems, artemisinin production from *in vitro* plants tissue culture had been considered as an interesting alternative. The addition of naphtiphine, an inhibitor of the enzyme squalene epoxidase, to the medium can contribute to the increased artemisinin production (Woerdenbag et al., 1993). Wang and Tan (2002) demonstrated that the ratio of NO_3/NH_4 and total initial nitrogen influenced the artemisinin yield in hairy roots. (Weathers et al., 1994) proved that the addition of artemisinin precursors to the medium used for tissue cultures of *A. annua* resulted in a four-fold increase of artemisinin in the tissue and an 11-fold increase of artemisinin in the spent medium.

Moreover, genetic engineering is an approach to ensure a constant high production of artemisinin either by overexpressing the enzymes in the terpene biosynthetic pathway or by inhibiting an enzyme of another pathway competing for artmisinin precursors. In recent years,

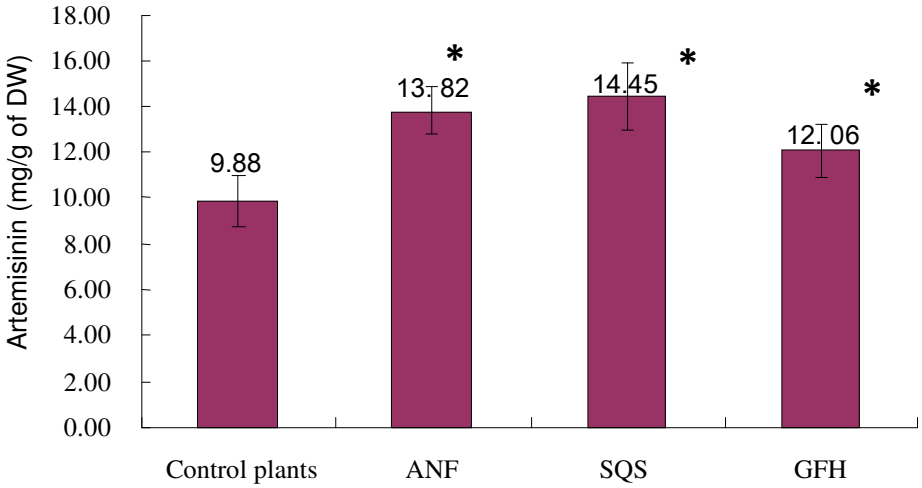

Figure 4. Artemisinin content of transgenic and control *A. annua* plants determined by HPLC-ELSD analysis. *significant at $P < 0.05$.

there has been remarkable progress in molecular regulation of artemisinin biosynthesis (Bouwmeester et al., 1999). The genes of the key enzymes involved in the biosynthesis of artemisinin, such as FPS, ADS, the genes of the enzymes relevant to the first step of a pathway in competition with that of artemisinin biosynthesis including SQS, have been cloned from *A. annua* (Chen et al., 2000; Martin et al., 2003; Liu et al., 2003). Based on previous studies, three transgenic *A. annua* varieties with significantly increased artemisinin content were previously generated (Zhang et al., 2009).

Before commercialization, transgenic plants should be assessed for environmental release. We completed the first environmental release trial for a pharmaceutical transgenic plant in China. To date, several environmental risk assessments of transgenic plants have been reported. For the most part, transgenic plants do not differ significantly from conventional plants in morphology, seed production (yield) and agronomic characteristics (Bae et al., 2008; Martha, 2006; Nair et al., 2002). However, in the present study, the morphological characteristics (Tables 2 and 3) presented significant difference between transgenic and control plants in plant height, crown width, stem diameter, 1000-seeds weight, and no significant difference was found between them in leaf shape, germinate rate, transplant survival and seeds wintering ability. Moreover, the agronomic traits of SQS showed a significant difference with control plants, which might be due to the RNAi technology. Further research is required to determine this difference. Because *A. annua* is a cross-pollinated plant, intra-specific introgression is easily detected from plants in close proximity (Bae et al., 2008). Fortunately, the artemisinin content is highest before plant flowering or during the full flowering period (El-Sohly, 1990); therefore, the best harvest time for *A. annua* with high cross-pollination rates is the period between the later vegetative growth stage and the appearance of flower buds (Sushil and Suchi, 2005). This way, gene flow between transgenic and conventional plants is avoided. In environmental release, the transgenic plants will be harvested before flowering; thus we tentatively conclude that gene flow from the 3 transgenic *A. annua* plants to conventional plants or other plant species would be rare.

In addition, although, genetic engineering transfers only short sequences of DNA, the resulting phenotype can produce an organism novel to the existing network of ecological relationships, which might lead to weediness due to acquired tolerance to selective pressure (Martha, 2006). Recipient plants (being hardy, perennial and competitive) could be high impact plants with regard to spread. Therefore, we must address concerns about the stress tolerances and the survival ability of transgenic plants. As described above (Tables 2 to 4, Figures 1 and 2), transgenic plants exhibited similar germination rate, transplant rate, seeds wintering ability and stress tolerances to control plants. Thus, in comparison to controls, transgenic plants did not show any enhanced potentials to survive. However, the data gained from the environmental release trial in Shanghai City may not be valid for other habitats with different climatic conditions. Following such a test, it is recommended to reassess environmental risks at a larger scale and in regions with different climates before commercialization (Martha, 2006).

Finally, real-time RT-PCR and HPLC-ELSD showed that the expression of target genes was either enhanced or suppressed, and the artemisinin content in transgenic plants increased significantly. Therefore, it was concluded that in the environmental release trial, the artemisinin content of 3 transgenic *A. annua* varieties increased as expected and genetic engineering was an effective means of increasing the artemisinin content of plants.

Nevertheless, the artemisinin content of these plants grown in the field was not as high as those cultivated in a growth chamber (Zhang et al., 2009). As a consequence, it would be necessary in the future to improve field cultural practices.

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