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Molecular characterization and biological changes caused by *Agrobacterium*-mediated infiltration of PgTRX1

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Abstract In order to test the functionality of Panax ginseng thioredoxin 1 (*PgTRX1*) isolated from fermented wild ginseng roots, a transient effect on physiological activity were performed over a short time frame using the *Agrobacterium* infiltration technique. The *PgTRX1* gene isolated from fermented wild ginseng was confirmed to have a size of 579 bp, and the expression of *PgTRX1* was the highest in the sample after 6 h of fermentation. As a result of constructing this gene and confirming the infiltration reaction mediated by *Agrobacterium* in tobacco leaves, it was found that the expression of the *NbHSR203j* gene was also induced as *PgTRX1* expression increased. As a result of measuring the biological activity of the infiltration samples, the total phenol content increased by 35.45±1.84 to 49.01±1.84 μg GAE/mL compared to the control, and the total flavonoid amount of 9.52±0.41 to 9.82±0.25 μg QE/mL was slightly high. From these results, *Agrobacterium-mediated* PgTRX1 appears to be related to the hypersensitive response induction mechanism of plants and the production of secondary metabolites such as phenolic substances.

Keywords *Agrobacterium* infiltration technique · *NbHSR203j* · *Panax ginseng* thioredoxin 1 · Total phenol content · Total flavonoid amount

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Introduction

Panax ginseng C.A. Meyer, a representative medicinal plant, has been regarded as a protector with a remarkable efficacy in the treatment of and recovery from diseases. The 47 number of individuals is gradually decreasing due to difficult growing conditions and indiscriminate collection; therefore, ginseng has been investigated using biotechnological gene characterization and mass culture techniques [1]. Ginsenoside, a pharmacological substance of wild ginseng, is a substance of the triterpenoid system with a dammarane skeleton. Rg1 and Rb1 are effective for enhancing central nervous system excitement and immunity, Rf assists in pain relief, Re has anticancer effects, and Rc promotes serum protein synthesis [2,3]. Pharmacological extracts from the cultured root of ginseng have been reported to be antidiabetic, ameliorate cardiovascular disorders, improve control of the nervous system, promote brain activity, protect against cancer, increase immunity, lower cholesterol, increase antioxidant activity, prevent skin aging, and amplify whitening activity [2].

Thioredoxin 1 is a powerful antioxidant that inhibits radicals and prevents cell death by functional reduction. Thioredoxin is important regulatory elements in plant metabolism and are initially recognized as regulatory proteins for the reversible photoactivity of enzymes required for major photosynthesis, but have also been found in the cytoplasm and mitochondria. Thioredoxin 1 plays a role in reducing the activity of the Calvin cycle at night in plants. With light, electrons move from pigment 1 to ferredoxin, and thioredoxin 1 reduces the disulfate bond of other enzymes and then makes them active. In contrast, at night, thioredoxin 1 undergoes reoxidation and inactivates enzymes [4]. When *TRX1*

gene-overexpressed rice was subjected to salt stress using NaCl, transgenic plants showed better growth than the control and survived for more than 8 d. Thus, insertion of the *TRX1* gene in plants improves the homeostasis of oxidation and reduction and recovery from oxidative stress induced by hydrogen peroxide and reactive oxygen species [5].

Stable transformation is an essential tool for the functional analysis of genes by producing transgenic plants through over-expression. However, it takes too long to produce stable transformants. The success of producing stable transgenic plants requires at least 3-12 months for *Arabidopsis*, *Nicotiana* species, and *Rosa* [2,6-8]. Alternative tools include transient assays using either particle bombardment or *Agrobacterium*-mediated transformation. The advantage of transient assays is that they take less time and labor [9]. In transient assays, T-DNA in the nucleus of the host can be expressed [10].

In this study, we tested biological analysis using the expression pattern of *PgTRX1* by *Agrobacterium*-infiltration and gene expression in fermented wild ginseng. Through this analysis, the molecular operation of PgTRX1 was evaluated to reveal the functionality of the gene.

Materials and Methods

Fermentation of plant materials

A total of 1 mL of fermented microorganisms was cultured in MRS medium (MRS broth 55.25 g/L, adjusted to pH 6.2, BD Difco, Fisher Scientific, Quebec, Canada) for 24 h, then inoculated into the wild ginseng-cultured roots in a 250 mL Erlenmeyer flask for 7 weeks in a sterile laboratory. The fermentation micro-organism *Pediococcus pentosaceus* (KACC 81010BP) used in the experiment was obtained from the Microbial Bank of the National Academy of Agricultural Sciences, and was used in the experiment by stirring culture for 24 h with MRS broth adjusted to pH 6.2 in the dark at 30 °C and 200 rpm. After that, they were fermented from 1 to 6 h, washed with sterile water, then quenched in liquid nitrogen in a deep freezer (Sanyo Ultra Low JP, MDF-592) and used for experiments.

Gene isolation and genetic construction

Total RNA was extracted according to the method of Yi et al. (2004) [11]. cDNA synthesis was performed according to the manual using M-MLV RT (Moloney murine leukemia virus reverse transcriptase) ordered from Invitrogen company. The synthesized cDNA was amplified by PCR using *PgTRX1* primers to obtain a DNA fragment for sequencing. This PCR product was extracted using a gel purification kit; the base sequence was analyzed by Bionics, and then the degree of sequence similarity was analyzed on the NCBI website. *PgTRX1* with full-length was inserted into the plant expression vector pMBP1 using restriction enzymes SacI and XbaI, then transformed into *Agrobacterium* strain LBA4404 according to the method of Seong et al. (2008) [12].

Transient assay

The transient assay for *N. benthamiana* using *Agrobacterium* was performed according to Seong et al. (2013) [13]. For the functional assay of the *PgTRX1* gene, tobacco leaves were collected for total RNA extraction and cDNA synthesis after treatment for 0, 1, and 2 d, and experiments were performed according to the method of Seong et al. (2013) [13]. The cDNA product was amplified by PCR using the PgTRX1 primer, and the band was observed by electrophoresis on a 1% agarose gel (Table 1). PCR conditions were 94 °C for 5 min for the first denaturation, 1 min at 94 °C for the second denaturation, 1 min for annealing at 55, 72 °C for 1 min for extension, 30 cycles, and 72 °C for 10 min post-elongation.

Gene expression

Tobacco leaves treated by *Agro*-infiltration for 2 d were collected and the expressions of *N. benthamiana* ascorbate peroxidase (*NbAPx*) and phenylalanine ammonia lyase (*NbPAL*) were checked to determine the correlation with the expression of genes in tobacco. Since the PAL and APX genes are representative genes related to antioxidants, RT-PCR experiments were performed by selecting them. PCR conditions were 94 °C for 5 min for the first denaturation, 1 min at 94 °C for the second denaturation, 1 min for annealing at 55, 72 °C for 1 min for extension, 30 cycles, and 72 °C for 10 min post-elongation (Table 1).

DPPH assay

Samples treated by *Agro*-infiltration were collected for 1-2 days, freeze-dried for 48 h, extracted with 100% methanol, diluted to

Table 1 The primers used for RT-PCR analysis in *Nicotiana benthamiana*

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
PgTRx1	CTGCTGCTGTGCTTGAAACC	GCTGCTGGTCAACGTTGTTT
PgActin	CGTGATCTTACAGATAGGTTGATGA	AGAGAAGCTAAGATTGATCCTCC
NbActin	CAGCTCATCCGTGGAGAAGA	AGGATACGGGGAGCTAATGC
NbHSR203J	TGTGTCAGCCATGCTGATTG	CCGATAGGACCGCACGAAAC
NbAPx	GTCCATTCGGAACAATGAGG	GTGGGCACCAGATAAAGC
NbPAL	TCGAGTTGCAGCCTAAGG	TCTTCCAAATGCCTCAAGTC

100, 200, 400, 1000, and 2000 ppm, and used in the experiment. The experimental method of Blois (1958) was modified and used [14]. DPPH (α, α-diphenyl-β-picrylhydrazyl) was diluted to 0.15 mM in methanol, and 100 μL of the sample was mixed with 100 μL of 0.15 mM DPPH and reacted for 30 min at room temperature under dark conditions; absorbance was then measured at 517 nm using a UV-spectrophotometer. DPPH radical scavenging activity of each sample was expressed as the RC50 value for the control group, and ascorbic acid was used as the positive control group.

ABTS assay

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay was modified and used for the experiment according to Re et al. (1999) [15]. ABTS was diluted to 7.4 mM in tertiary distilled water, and potassium persulfate diluted to 2.6 mM in tertiary distilled water was mixed in a 1:1 ratio. After reacting, the absorbance was measured. The ABTS radical scavenging activity of each sample was expressed as the RC_{50} value for the control group, and ascorbic acid was used as the positive control group.

Total phenol content

The Folin-Ciocalteu method carried out by Taga et al. (1984) was modified and used in the experiment [16]. A total of 50 μL of Folin-Ciocalteu's reagent was mixed with 100 μL of the sample, reacted for 3 to 5 min at room temperature, and then 300 μL of 20% sodium carbonate (Na_2CO_3) diluted with tertiary distilled water was added and reacted at room temperature for 15 min. After terminating the reaction by mixing 1 mL of distilled water, centrifugation was performed at 3,000 rpm for 3 min at room temperature, and the absorbance of the supernatant was measured at 725 nm using a UV-spectrophotometer. The content of phenolic compounds was expressed as gallic acid equivalents (GAE) after preparing a calibration curve using the standard gallic acid.

Total flavonoid content

The experimental method conducted by Moreno et al. (2000) was modified and used for the experiment [17]. A total of 200 μL of a solution of 10% aluminum nitrate and 1 M potassium acetate diluted in tertiary distilled water in a ratio of 1:1 was mixed with 500 μL of each sample, reacted for 40 min at room temperature, and the absorbance was determined at 415 nm using a UV-spectrophotometer. Flavonoid content was expressed as quercetin equivalents (QE) after preparing a calibration curve using the standard quercetin.

Statistical analysis

All experiments were repeated three times and expressed as mean ± standard error (SE). All data measured at a significance level of 0.05 for statistical processing with Duncan's multiple range test of IBM SPSS Statistics Ver. 24.

Results

Alignment of amino acid sequences of PgTRX1

In order to analyze the amino acid sequence of PgTRX1, sequence identity was investigated using the blastP program on the NCBI homepage. The amino acid sequence of PgTRX1, whose gene was 579 bp, was determined by screening for similar function genes with an identity of more than 60% (Fig. 1). *AcTRX1* (GenBank: PSS16189.1) is a thioredoxin-like family gene isolated from *Actinidia chinenses* and shows 75% homology with *PgTRX1*. As a result of aligning with *PgTRX1*, *DcTRX1* (GenBank: XP_017252196.1) showed 78% identity and *AiTRXM4* (GenBank: XP_016171359.1) showed a somewhat lower 68% identity. Multiple alignments of *PgTRX1* revealed that genes with more than 90% identity were not found in the BLAST results of NCBI, even though thioredoxin genes were found in various plant species. Therefore, *PgTRX1* is expected to perform a new function different from that of the existing thioredoxin gene function.

PgTRX1* expression in fermented *Panax ginseng

The expression level of *PgTRX1* increased from 1 to 6 h of fermentation using *Pediococcus pentosaceus* (KACC 81010BP) (Fig. 2). Analysis of *APX* and *PAL* gene expression by transient overexpression of *PgTRX1* in *N. benthamiana* showed that the expression of the *NbAPX* and *NbPAL* genes was decreased compared to the control without *PgTRX1* expression for 2 d after transient infiltration (Fig. 3).

Antioxidant activities by *Agro*-infiltration of *PgTRX1*

Tobacco leaves were collected for 3 d after *PgTRX1* infiltration and analyzed for antioxidant activity. In the DPPH assay, the antioxidant levels of 612.27±1.69 to 704.07±3.49 μg/mL were not significantly different from those of the control (657.08±2.37 μg/mL) at 1-3 d of infiltration (Table 2). The antioxidant levels of ABTS were 1324.86±3.56 to 1390.64±4.56 μg/mL in the infiltrated test, which was not different from the control of 1323.61±2.63 (Table 3). Analysis of DPPH and ABTS showed that the antioxidant activity of PgTRX1 was not changed compared to that of the control in transient expression by infiltration.

Analysis of total phenol and flavonoid by infiltration of *PgTRX1*

The total phenol content of tobacco leaves by infiltration of *PgTRX1* was 45.9±1.84 to 49.01±1.84 μg GAE/mL, which was 15.93±5.54 μg GAE/mL in the control (Table 4). The total flavonoid content was 7.41±0.17 μg QE/mL in the control, but 9.52±0.41 to 9.82±0.25 μg QE/mL in the infiltration treatment. The total flavonoid content was higher in the treatments (Table 5).

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AcTRX1          MAA-VLETATFPRASGFPAT-SLAPIRSSSVCALSGGRKSVKLLEIRGLK
AiTRXM4         MATVQLQSLTLSRSSALSA--------PTTVSSISGRRESIKLPRHAGLR
DcTRX1          MAA-VLETLAIPRASTFPA------ISSPSVCSLSGHRSSIKLPQSRGLK
PgTRX1          MAAAVLETLTIPRASGFPAAASFPPIASSSVFALSGGRSSVKLPESRGLR
                **:  *:: ::.*:* :.*        ..:* ::** *.*:** .  **:

AcTRX1          IQSSYTRRSLGSSRLNSRLA--HRGARIVCEAQEIA-VEVSPITDSTWQS
AiTRXM4         LATS-TRFSGSPSRAVSRIAASRAGGRVVCETQDTAAVQVDPITDANWQS
DcTRX1          IQS--VRVTGSVS-TSSRLV--SRAGRIVSEAQDTA-VVVPSVLDATWQS
PgTRX1          IHS--ARFSGSVSLSASRLV--RRVGRIVCEAQETA-VVVPPVTDAAWQS
                : :  .* : . *   **:.     .*:*.*:*: * * * .: *: ***

AcTRX1          LVLESDFPVLIEFWAPWCGPCRMIHPVIDELAKQYIGKLKCYKVNTDECP
AiTRXM4         LVLESDTPVLVEFWAPWCGPCRMIHPIIDELAKEYAGKLKCYKLNTDESP
DcTRX1          LVLECDSPVLVEFWAPWCGPCRMIHPIIDELAKEYAGKLKCYKVNTDESP
PgTRX1          AVLESDSPVLVEFWAPWCGPCRMIHPIIDELAKEYNGKLKCYKLNTDESP
                 ***.* ***:****************:******:* *******:****.*

AcTRX1          SIATRYGIRSIPTVMIFKNGEKKDTVIGAVPKSTLTTSIEKFL----
AiTRXM4         STATRYGIRSIPTVIIFKNGEKKDAVIGAVPKTTLMTSIEKFL----
DcTRX1          SVATRYGIRSIPTVMIFKCGEKIDAIIGAVPKATLATSIDKLL----
PgTRX1          SVATRYGIRSIPTVIIFKGGEKKDAIIGAVPKTTLTSSIEKFLSTOP
                * ************:*** *** *::******:** :**:*:*
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Fig. 1 Multiple alignment of deduced amino acid sequences of *PgTRx1* compared to other plant species sequences. The GenBank and NCBI accession numbers of amino acid sequences are: *Daucus carota* subsp. sativus XP_017252196.1 (*DcTRx1*), *Actinidia chinensis* var. chinensis PPS16183.1 (*AcTRx1*), *Arachis ipaensis* XP_016171359.1 (*AiTRxm4*) and *Panax ginseng* (*PgTRx1*). Colon indicates different amino acid sequence. *The nucleotide sequence is the same part

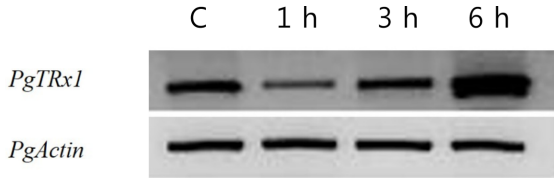

Fig. 2 Expression pattern of *PgTRX1* gene according to fermentation time of *Panax ginseng*

Discussion

The superfamily of thioredoxin (TRX) is responsible for the reflection of diverse redox-based modifications. Superfamily members have a conventional TRX region, which has emerged as a major regulator of redox-based protein signaling during plant immunity and defense-related mechanisms [18]. When saline stress was applied to the seedlings of rice (*Oryza sativa* L.) overexpressing the *TRX1* gene using NaCl, this rice showed better survival than the control for more than 8 d. It has been reported that the insertion of a heterogeneous *TRX1* gene improves oxidation and reduction homeostasis and improves the ability to adapt and recover from oxidative stress induced by hydrogen peroxide and reactive oxygen species [5]. In wild ginseng cultured roots fermented with microorganisms, the expression of *TRX1* increased with fermentation time, and total RNA extraction was not possible in the sample after 6 h. Fermentation by microorganisms is shown to be consistent reports that it causes mechanical and oxidative stress to cells by decomposing cellulose and hemicellulose on the plant cell wall [19,20].

Plant transformation is an essential method for studying genes to improve agronomic traits. To investigate gene function in a short time frame, agroinfiltration is routinely used in diverse plant species [21]. In rice leaves, *Agrobacterium*-mediated transient gene expression was checked by mechanically wounded leaves and direct incubation in *Agrobacterium* suspension [22]. In order to quickly determine the function of *PgTRX1*, we also conducted a functional study using the infiltration technique. The plant used for infiltration was *N. benthamiana*, which can be easily handled for functional studies. As a result of infiltration of *PgTRX1* through *Abrobacterium*, *APX* and *PAL* genes showed opposite expression patterns to *PgTRX1* gene expression patterns, and *HSR203j* showed similar gene expression patterns to *PgTRX1*. *HSR203j* is a plant disease-resistance gene and is known to be involved in programmed cell death. Therefore, *PgTRX1* is thought to be related to disease resistance-related mechanisms.

PAL is an important enzyme that is the starting point for biosynthesis of phenolic substances through conversion of L-phenylalanine into trans-cinnamate, and APX is known to be an important factor in preventing various oxidative stresses in plant chloroplasts [23,24]. It has been reported that the *PAL* gene is associated with various environmental stimuli and the accumulation of secondary metabolites, such as phenolic substances, in various crops [25,26]. In this study, when *PgTRX1* was transiently

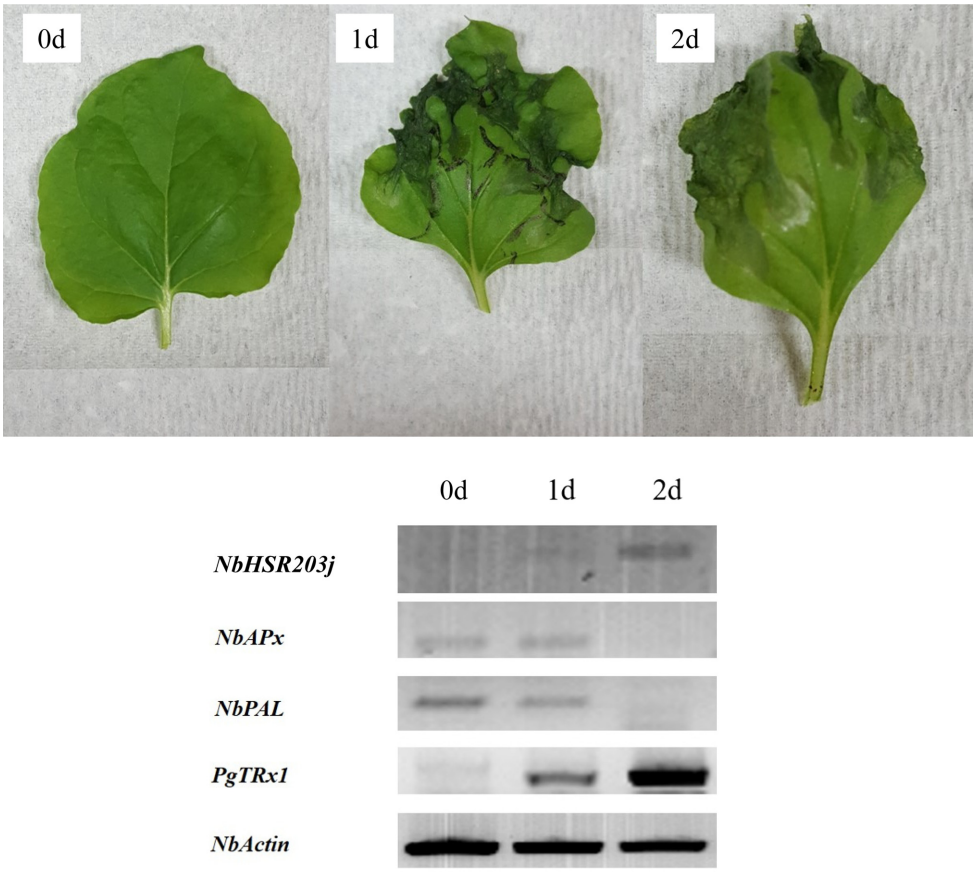

Fig. 3 *Agrobacterium*-mediated infiltration of *PgTRX1* and expression pattern of various genes involved in HR response and antioxidant activity by induction of *PgTRX1* in *Nicotiana benthamiana*

Table 2 Changes of DPPH radical scavenging activity by *Agro*-infiltration treatment period

Sample (day)	DPPH RC_{50} (μg/mL)
C	657.08±2.37[ab]
1	670.96±1.02[ab]
2	612.27±1.69[ab]
3	704.07±3.49[a]

*Concentration required for 50% reduction of DPPH at 30 min after starting the reaction.

Table 3 Changes of ABTS radical scavenging activity by *Agro*-infiltration treatment period

Sample (day)	ABTS RC_{50} (μg/mL)
C	1323.61±2.63[a]
1	1348.47±3.39[a]
2	1324.86±3.56[a]
3	1390.64±4.56[a]

*Concentration required for 50% reduction of ABTS at 10 min after starting the reaction.

Table 4 Changes in total phenolic contents by *Agro*-infiltration treatment period

Sample (day)	Total phenolic contents (μg GAE[1)]/mL)
C	15.93±5.54[d]
1	45.09±1.84[ab]
2	49.01±1.84[a]
3	35.45±1.84[c]

[1)]GAE: Gallic acid equivalent

Table 5 Changes in total flavonoid contents by *Agro*-infiltration treatment period

Sample (day)	Total flavonoid contents (μg QE[1)]/mL)
C	7.41±0.17[c]
1	9.52±0.41[ab]
2	9.82±0.25[a]
3	9.74±0.12[a]

[1)]QE: Quercetin equivalent

expressed, expression of the *PAL* and *APX* genes decreased with time. This is because of programmed cell death during the *Agrobacterium*-mediated infiltration reaction, resulting in the expression of both genes by *Agrobacterium*, and the expression of two genes is believed to decrease. Some studies have shown that these genes do not protect against stress, which is thought to be due to the complex ROS detoxification system [27,28]. In addition, the total phenol content is increased at the 3 days after infiltration with Agrobacterium, and the NbPAL gene is decreased at the 2 days in this study. This difference is considered to be a temporary tendency to increase the phenol content because the action of infiltration itself induces plant stress.

HSR203j is a gene that responds to bacteria that induces a hypersensitive response (HR) [29,30]. The transient assay medium used in this experiment to reveal the function of the *PgTRX1* gene was an *Agrobacterium*. Since *Agrobacterium* is also a type of bacteria, it is presumed that infiltration into plant leaves can cause an HR reaction. Therefore, whether *HSR203j* is induced by the expression of *PgTRX1* or whether it is due to a specific HR response to bacteria, more detailed studies are needed.

As a result of *Agrobacterium* infiltration of the *PgTRX1* gene, DPPH and ABTS activity, indicating ROS scavenging activity, showed no difference compared to the control, but there was a difference in total phenol and total flavonoid content. Compared to the control, the total phenol content of the infiltrated leaves was 2-3 times higher, and a slightly higher amount of total flavonoid was detected. It is thought that the flavonoid content slightly increased as PgTRX1 increased the total phenol content. As such, there are few studies on the correlation of the expression of antioxidant-related genes such as *APX* or *PAL* using *Agrobacterium* infiltration, but few cases reveal the association between total phenol and total flavonoid, as in this study [13,31]. The infiltration results of *PgTRX1* will of great help in functional studies involving the production of transgenic plants in the future.

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