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Table: 1, Figs : 6

Soybean autotoxicity: Effects of *m*-hydroxy-phenylacetic acid on cell ultrastructural changes and gene expression in soybean roots

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ABSTRACT

The application of *m*-hydroxy-phenylacetic acid (MHPA), significantly inhibited the radicle growth and root tips became thicker and brownish in colour than untreated control. Transmission electron microscopy analysis showed that MHPA adversely affected the soybean root tip, leading to sparse cytoplasm and fewer organelles in root meristematic cell, cell vacuolation and poorly developed irregular amyloplasts in root columella cell. A total of 33 differentially displayed cDNA fragments were found between the MHPA treated and control samples and 6 differential displayed cDNA fragments were observed after the subsequent DDRT-PCR reactions on extracted RNA. Results from RT-PCR confirmed two differential expressions, (i). one down-regulated gene (S385) and (ii). one up-regulated gene (S452), these expressed approximate half-fold and 5-folds over the control plants, respectively.

Key words: Autotoxicity, differential gene expression, *Glycine max* L. Merr., *m*-hydroxy-phenylacetic acid (MHPA), phenolic acids, radicle, real-time RT-PCR, soybean, ultrastructure.

INTRODUCTION

The chemical influence of one plant on the development of individuals of same species is called Autotoxicity and plays major ecological role (5,17). It occurs in natural, semi-natural, agricultural communities [fruit trees, perennial forages and cucurbit crops (5,11,30)] and its negative effects varied with varieties and growth season (16). It reduces the establishment of individual plants of parent species, to alleviate the intraspecific competition, especially in deficient growth resources and maintains a sustainable seed bank in its habitat. The study of MHPA gave an insight into the understanding of soybean autotoxicity. In north-eastern China, soybean is the major crop and continuously cropped in > 10 million ha. In continuous monoculture of soybean, there is poor growth, low yield, poor quality and high incidence of plant diseases (15). Autotoxicity with other factors reduces the yields in continuous soybean cultivation (15). After pre-fumigation with methyl bromide, the yield in continuous-soybean was still lower than control, where soybean was rotated with maize 2-years' interval (21). Thus other constraints excluding the

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biotic factors, such as autotoxic chemicals played major role in reducing the soybean yield. In pot experiment, autotoxicity caused > 30% reduction in growth of continuous soybean (22). Besides in hydroponic culture system, the addition of activated carbon decreased the magnitude of biomass reduction in continuous soybean, indicating that autotoxic substances in soybean root exudates were eliminated by carbon adsorption, because they caused poor growth and low yield of soybean in continuous-soybean cultivation (31). Several allelopathic compounds [vanllic, p-coumaric, feruilc acid and p-hydroxy-phenylacetic acid (PHPA)] are present either in decomposed root residue or soil solutions or both (20). Similarly, 2-hydroxyphenylacetic acid and PHPA was detected in rice roots exudates by GC/MS/MS (23).

Although autoxicity had been suggested to be involved in the growth inhibition of continuous cultivated soybean but the mechanism of action of allelochemicals is not known. The effects of allelochemicals on peroxidase and phenylalanine ammonia-lyase activities could be some of the many mechanisms to influence the soybean growth (7,10). We had found that the expression of low weight molecular proteins 16.1kD and 20.2kD was inhibited in the presence of PHPA and its isomer MHPA (20). Since MHPA strongly inhibited the plant growth, it was chosen as model compound to further investigate the mechanism of phenolic acids autotoxicity on soybean growth.

This study aimed (i). to determine the effects of MHPA on the cell ultra-structure of soybean root tip, (ii). to explore the differential expressions of the specific genes in soybean roots treated or not with MHPA and (iii). to isolate and identify the comparative expressed sequence tags involved.

METHODS AND MATERIALS

The seeds of *Glycine max* L. Hefeng-25 variety (poor growth and reduced yield in continuous monoculture), were provided by Soybean Research Institute, Heilongjiang Academy of Agricultural Sciences, Harbin, China. m-Hydroxy-phenylacetic acid (MHPA) was purchased from Sigma-Aldrich Company, Harbin. Soybean seeds were surface-sterilized with 3 % H_2O_2 and thoroughly rinsed with tap water and distilled water. Ten ml MHPA solutions of various concentrations (0, 60, 120 or 300 mg. L^{-1}) were added as per treatment into the autoclaved Petri dishes (9 cm dia.) with two-layers of filter papers at the bottom. Ten soybean seeds were sown randomly in each Petri dish and these were kept in dark in a growth chamber at 25°C for 82 h. Root tips of seedlings treated with 0,60 and 300 mg.L^{-1} of MHPA were used for transmission electron microscopy (TEM) studies. Roots of seedlings with 0 and 120 mg.L^{-1} MHPA treatments were sampled and kept at -70 °C for differential display RT-PCR assay. The germination assay was replicated four times and three of them were used for total RNA extraction as described below.

Transmission electron microscopy

The study of MHPA gave an insight into the understanding of soybean autotoxicity. Samples (0.5-cm taproot tips) were fixed with 0, 60 and 300 mg.L^{-1} of MHPA and subjected to TEM studies. Samples were fixed in 2.5% glutaraldehyde solution buffered with 0.1 M phosphate (pH 7.4) at 4°C for 24 h and postfixed in 1% osmium tetroxide in phosphate buffer (pH 7.4) for 3 h. After fixation, the samples were rinsed

several times with phosphate buffer, dehydrated in gradient acetone solutions and embedded in Epon812 resin. Ultrathin sections obtained in a LKB-V ultramicrotome were stained with uranyl acetate (2.5%) and lead citrate and examined in a Philips EM400ST transmission electron microscope.

RNA Extraction

Total RNA was extracted from the roots using TRIzol kit (In Vitrogen) as recommended by manufacturer. RNA extracts were mixed with DNase I (RNase free) and incubated for 30 min at 37 °C to digest the remaining genomic DNA. DNase I was removed by adding DNase inactivation reagent followed by centrifugation at 14000 g for 1 min and the supernatant was transferred to a new tube and stored at -70°C. The concentration of isolated RNA was measured with Biophotometer. The integrity and quantity of total RNA was checked on 1% denaturing agarose gel.

Differential Display Reverse Transcript (DDRT-PCR)

Total RNA was used for cDNA synthesis. The first strand cDNA synthesis was primed with 3' anchored poly(dT) primer AAGCTTTTTTTTTTG, AAGCTTTTTTTTTTA and AAGCTTTTTTTTTTC. cDNA synthesis was done using reverse transcriptase AMV (Promega) as per the manufacture's instruction and the reaction was run at 42°C for 1 h, 95°C for 5 min and the cDNA was kept at 4°C before use. Second strand cDNA synthesis from MHPA treated and control root samples were conducted. A total of 78 primer pairs (26 arbitrary primers vs. 3 anchored poly(dT) primers (Table 1) were used to perform PCR in a 20 μL reaction mixture (Liang, Averboukh 1992). Reactions were performed on a MJ DNA engine PTC-100 PCR machine (MJ Research, Reno, USA) as under: 94°C for 3 min then 40 cycles of 94°C for 30 s, 40°C for 60 s, 72°C for 45 s and a finally extension at 72°C for 10 min.

Analysis of amplified products

Amplified PCR products (10μL) were separated in 8% denaturing PAGE gel and the bands were visualized by silver staining. The differentially expressed bands between the control and treated samples were excised from the gels and reamplified using the original primer pair. Then selected amplified DNA fragments were excised, purified and lignated directly to pMD18-T vector. The recombined clones were sequenced using an automated DNA sequencer (Shanghai Sangon, China).

Real time PCR

Quantitative RT-PCR was used to confirm the differential expression of DNA fragments isolated from the control and MHPA treated samples. Based on the results of DNA sequencing, the specific primers were re-designed for fragment S385 (forward, 5´-GTCCCCGCCATCTTTTATTC-3´; reverse, 5´-CTCCACTACCACCGCCTTTA-3´) and fragment S452 (forward, 5´-TTTGAAAGTAATGAATAGCACAGT-3´; reverse, 5´-GGCAGGTGGACAATAAACA-3´). A house-keeping gene (209 bp 18S RNA) present at a constant amount in all samples, was used as the reference gene for quantification. The primers for 18S RNA were: 5´-CCTTGCTTGTTGCTTTACTAAATAG-3´(forward); and 5´-ATGCACCTTTTCGTTTGTTTCGGAG-3´(reverse). Quantitative RT-PCR was performed in the DNA Engine OPTICONTM quantitative PCR (MJ Research, Reno,

USA). The intercalation dye SYBRGreen I was used as a fluorescent reporter. The PCR reaction was performed in a 25 μL mixture with an initial step of 94°C for 4 minutes and then 35 cycles of 94 °C for 30 s, 53°C for 45 s, 72 °C for 45 s and a final step at 72°C for 3 min. The fluorescent signals were recorded at 75 °C.

Table 1. List of 26 Arbitrary primers and 3 Anchored Poly (dT) primers

No.	Arbitrary primers	No.	Arbitrary primers	No.	Arbitrary primers
1	5'd(TACAACGAGG)3'	10	5'd(GATCAAGTCC)3'	19	5'd(TACCTAAGCG)3'
2	5'd(TGGATTGGTC)3'	11	5'd(GATCCAGTAC)3'	20	5'd(CTGCTTGATG)3'
3	5'd(CTTTCTACCC)3'	12	5'd(GATCACGTAC)3'	21	5'd(GTTTTCGCAG)3'
4	5'd(TTTTGGCTCC)3'	13	5'd(GATCTGACAC)3'	22	5'd(GATCATGGTC)3'
5	5'd(GGAACCAATC)3'	14	5'd(GATCTCAGAC)3'	23	5'd(GATCATAGCG)3'
6	5'd(AAACTCCGTC)3'	15	5'd(GATCATAGCC)3'	24	5'd(GATCTAAGGC)3'
7	5'd(TCGATACAGG)3'	16	5'd(GATCAATCGC)3'	25	5'd(GGTACATTGG)3'
8	5'd(TGGTAAAGGG)3'	17	5'd(GATCTAACCG)3'	26	5'd(GATCTGACTG)3'
9	5'd(TCGGTCATAG)3'	18	5'd(GATCGCATTG)3'		
			Anchored poly (dT) primers		
1	5'd(AAGCTTTTTTTT TTG)3'	2	5'd(AAGCTTTTTTTT TTA)3'	3	5'd(AAGCTTTTTTTT TTC)3'

Sequence homology comparison

The above sequences were submitted to GenBank. For similarity analysis, the nucleotide sequences of the selected cDNA clones were compared with entries in the NCBI GeneBank databases (NCBI, NIH,Washington, DC, U.S.A.).

RESULTS

Effects of MHPA on root growth

Poor root development of 82-h-old soybean seedlings was observed in the presence of MHPA (Fig. 1), especially at 300 mg.L^{-1}. These roots were brownish and had thicker and shorter radicle than the untreated control.

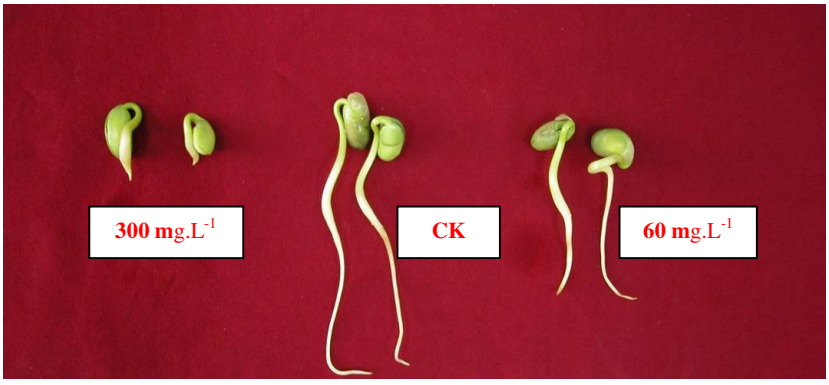

Figure 1 Effects of MHPA at the level of 0 mg·L^{-1} (CK), 60 mg·$kg^{-1}$and 300 mg L^{-1} on the 48-h-old *Glycine max* L. Merr. seedlings.

Transmission Electron Microscopy Analyses

In control seedlings, the cells in statolith zone of root tip were well organized with dense cytoplasm and rich organelles. In particular, the typical statolith amyloplasts packed with starch granules was developed regularly (Fig. 2A). However, in the presence of MHPA, poorly developed cells in stolith zone were observed via the transmission electron microscopy. For example, both at 60 $mg.L^{-1}$ and 300 $mg.L^{-1}$, irregular nucleus shape and irregularly organized amyloplasts containing more loosely linked components of starch granules were observed (Fig. 2B-C). Moreover, serious vacuolation and plasmalemma invagination was observed in the MHPA treated cells, especially at 300 $mg.L^{-1}$ (Fig. 2C). In addition, fewer organelles, irregular mitochondria and sparse cytoplasm were also observed under the MHPA stress.

The meristematic cells in untreated soybean root tips were well organized with the normal developed organelles, well defined nucleolus and nucleus (Fig. 3A). The amounts of mitochondria and Golgi apparatus and rough endoplasmic reticulum were decreased after the treatment with 60 $mg.L^{-1}$ MHPA (Fig. 3B). At high concentration (300 $mg.L^{-1}$), the organelles in the meristematic cells became quite rare. Cytoplasm was seriously agglutinated and nuclear envelope was not distinct. The nucleus to nucleolus ratio was substantially higher than other treatments, with pronounced dispersion of nucleolus (Fig. 3C)

DDRT-PCR

DDRT-PCR partitions transcripted into non-overlapping sets by synthesizing cDNA and amplifying their terminal parts with primer pairs consisting of an anchored poly (dT)-primer and a random primer. Three anchored poly (dT) primers and 26 random primers (see Table 1) were employed, resulting in 78 primer combinations. A total of differentially displayed 33 cDNA fragments were detected between the MHPA treated and control samples taken from one of the three experiments. To decrease the ‘‘false positive’’ rate of mRNA differential display, we repeated another two sets of DDRT-PCR reactions using RNAs extracted from the other two independent experiments and 6 of the 33 differentially displayed cDNA fragments were confirmed. These 6 cDNA fragments were cloned and their sequences were determined for later use. The differentially displayed fragments S385 and S452 were amplified and shown in Fig. 4.

Sequence analysis

Sequence analysis was performed for cDNAs of S385 and S452. The sequences of the 2 differentially expressed cDNAs were shown below and their sequence data can be assessed in the EMBL/GenBank data libraries under accession numbers AY750901 (*Glycine max* clone S385, 332bp) and AY750904 (*Glycine max* clone S452, 327bp)(Fig. 5). By homology comparison, the two cDNAs were found to have high similarity with sequences related to water and cold stress.

Real time RT-PCR

To confirm the effect of MHPA on the expression of above corresponding genes, quantitative real time RT-PCR was performed. Results showed that the expression of one gene (designated as S385) was down-regulated by about 50% and the other gene (S452) was up–regulated by over 5 folds in the presence of MHPA (120 $mg.L^{-1}$) (Fig. 6A-B).

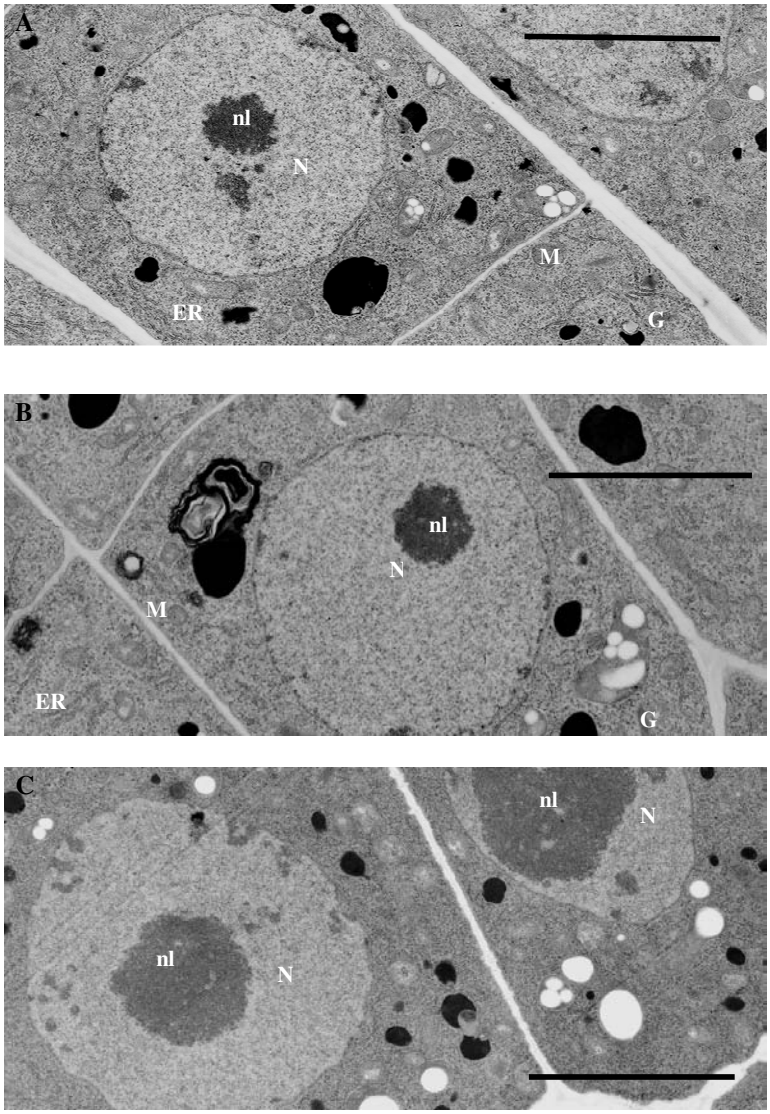

Figure 2A-C. Transmission electron microscopy of *Glycine max* L. Merr. meristematic root cells. Bar=5μm. **A:** Control cells were normal with well-defined nucleus (N), nucleolus (nl), rich mitochondria (M), Golgi apparatus (G) rough endoplasmic reticulum (ER) and cellular organelles are distinct. **B**: Cells treated with 60 $mg.L^{-1}$ MHPA. The amount of mitochondria and Golgi apparatus and rough endoplasmic organelles were decreased. **C:** Cells treated with 300 $mg.L^{-1}$ MHPA. The number of cell organelles was rare and cytoplasm showed seriously agglutinated and dispersal of nucleolus. Ratio of nucleolus: nucleus was markedly high than other treatments.

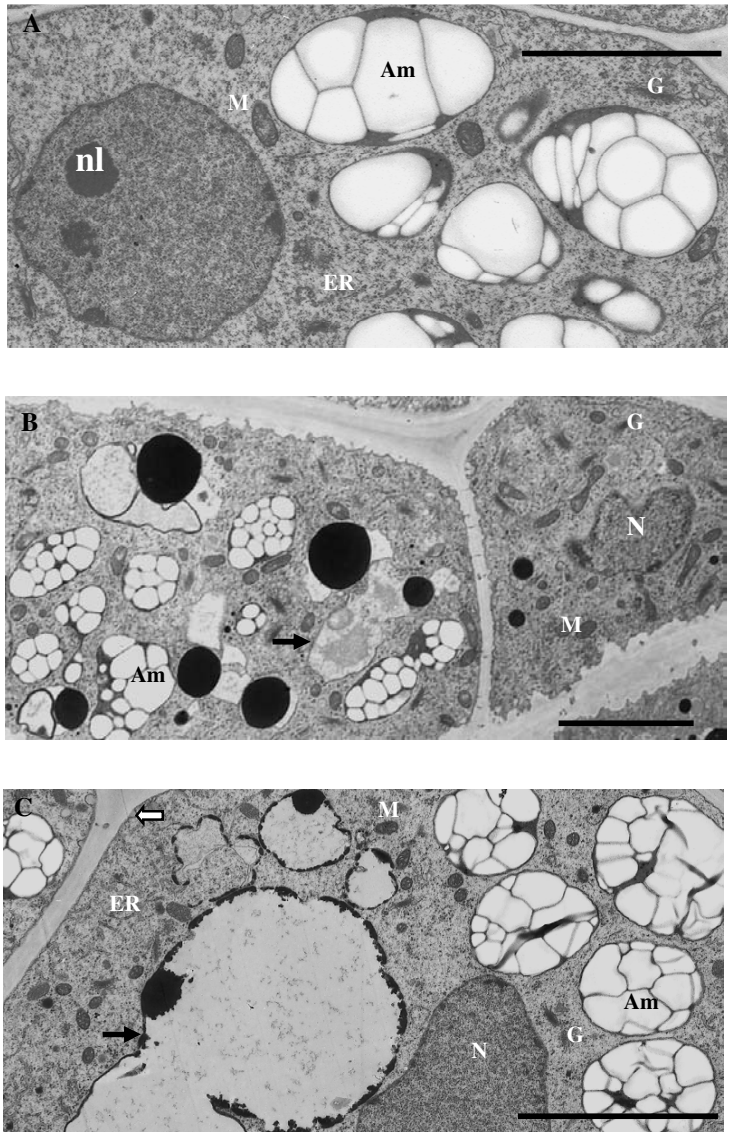

Figure 3A-C. Transmission electron microscopy of *Glycine max* L. Merr. root columella cells. Bar=5µm. **A:** control cells showing dense cytoplasm clear boundary with nuclei zone. **B:** Root columella cells exposed to 60 mg.L^{-1} MHPA showing morphological abnormalities with slight vacuolation (small arrows), poor developed amyloplasts, sparse cytoplasm and irregular nucleus shape. **C:** Root columella cells exposed to 300 mg.L^{-1} MHPA showing thickening walls less cytoplasm, serious vacuolation (small arrows), plasmalemma invagination (white arrows), irregular nucleus shape and loosely linked amyloplasts with more components of irregularly organized starch granules. N, nucleus; nl, nucleolus； M, rich mitochondria; G, Golgi apparatus； ER, endoplasmic reticulum.

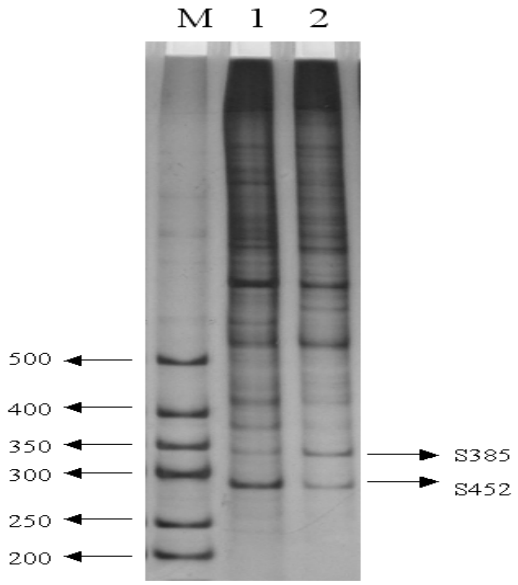

Figure 4. Changes in intensity of DDRT-PCR bands from mRNA of soybean roots after treatment with MHPA. Two differentially displayed cDNA fragments (S385 and S452) were amplified from the isolated total RNAs by the corresponding primer combination. M: Markers; Lane 1: Pattern of amplified cDNA fragments from root treated with 120 mg.L^{-1} MHPA; Lane 2: Pattern of amplified cDNA fragments from control roots. One fragment (S452) showed down-regulation of corresponding gene and the other (S385) showed up-regulation of corresponding gene in MHPA treated sample.

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S452 TGATACAAATGAAATTGTGTATTTCAAATCAATTACCAAATCTAGAGACTACATACAC 58
BG838199 **********C********************************************** 58
CX707144 ************************************************************58

S452 AAGCTAGTAAGCTACACACTCCACTCTCGCTTAAGCTAAGAACAAAAGGGGTTTTAAA 116
BG838199 AAGCTAATAAGCTACACACTCCACTCTCGCTTAAGCTAAGAACAATAGGGGTTTTAAA 116
CX707144 AAGC-AGTAAGCTACACACTCCACTCTCGCTTAAGCTAAGAACAAAAGGGGTTTTAAA 115

S452 AACAGAACATAAGAATATGACAACATTGGC---GATATATTAAAGAAACACTAATATC 171
BG838199 *G**************************ACAAC************************  174
CX707144 ************************************************************  170

S452 TGAGTTGAAAGACAGGAACGCCCATGACATTTGTTTATTGTCCACCTGCCACAAAAAA 229
BG838199 ***************A*****************************-----*A***GTT  227
CX707144 ***************A*****************************-----*****GTT  223

S452 AAAAGCT--T-----                                    237
BG838199 ***G*A*GA*GCACT                                242
CX707144 ***G*G*GA*-CACT                                237
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Figure 5. Alignment of nucleotide sequence acid of S452 (accession No.AY750904) with other two most similar ESTs: (i). accession No.CX707144: Obtained from the soybean root tip soybean under water stress and (ii). Accession No. BG838199: Obtained from cold-stressed *Glycine clandestine* seedlings.

DISCUSSION

In natural plant communities, autotoxins from decaying shoots of spotted knapweed (*Centaurea maculosa*) and common rush (*Juncus effusus*) inhibits their own germination and early growth of seedlings (8,17). The study of MHPA gave an insight into the understanding of soybean autotoxicity. As per our previous study (20) the MHPA applied to germinating soybean seedlings, drastically reduced the radicle growth depending on MHPA concentration (Fig.1). Root membrane is primary site of action for phenolic acids (13) and subsequently influences other physiological and biochemical processes (3). When exposed to allelochemicals, lignification of cell wall occurs (9,26) and ethylene inhibits the growth (18). The allelochemicals adversely affects the root growth and root tip ultrastructure (6). Unlike the findings of Burgos *et al*. (4) that the number of amyloplasts of cucumber seedling roots was greatly reduced in response to 2(3H)-benzoxazolinone BOA and 2,4-dihydroxy-1, 4(2H)-benzoxazin-3-one DIBOA, the number of amyloplasts in the MHPA-treated germinated soybean seedlings declined drastically, whereas, their shape and organization was negatively affected (Fig. 2B-C), suggesting that the function for gravity perception might be impaired due to deformed amyloplasts. In addition, BOA and DIBOA increases the cytoplasmic vacuolation and reduces the number of mitochondria (4) and the drastic degradation of cell (Fig. 3 B-C). Likewise, cell vacuolization occurs in the roots of mustard (*Brassica juncea* L.) grown in soil treated with benzoic acid (12). The cell vacuolization also occurs in the presence of other substances (33) or abiotic stress (29). The major dispersion of nucleolus and disruption of nuclear envelope of soybean root meristemic cell was observed at higher MHPA concentration (Fig. 2C). It was consistent with the irreversible ultrastructural damage and subsequent death of the *Azolla imbricata* in the presence of Hg^{2+} and Cd^{2+} (24). In this study the irregular ultrastructure of meristemic zone cells and root columella cells showed the cellular damage due to MHPA treatment. High MHPA concentrations adversely affected the persistence and the cell damage was irreversible and may cause cell death.

Currently the molecular technology is being applied to allelopathy to understand its mechanisms (1,27,32). In addition, the damage of cellular structures and impairment of membrane functions, heat and other abiotic stresses can also alter the pattern of gene expression (28,34). However, the studies of phenolic acid related to the changes in patterns of gene expression were rarely reported. In this study, two differentially expressed genes were detected (Fig. 4), including one up-regulated and one down-regulated gene, of which transcripts were examined by quantitative RT-PCR using plant tissues of the MHPA-treated and the control plants (Fig. 6A-B). Comparison with EST databases indicated that more than 10 and 60 ESTs were identical (over 90%) to S385 and S452, respectively and most of them from *Glycine max* genome systems clones. Moreover most of these ESTs were related to abiotic stress (drought, water and cold) suggesting that the two differentially expressed genes identified in our study were not specific genes in response to MHPA stress. For example, S452 (accession No.AY750904) was similar to the EST (CX707144) (94.9%) obtained from the soybean root tip under water stress and similar to EST (Accession No. BG838199) (92.8%) obtained from cold-stressed *Glycine clandestine* seedlings (Fig. 5). Since we did not find the known gene which matched these two ESTs, further research needs to be done to obtain the full length cDNA and then analyze

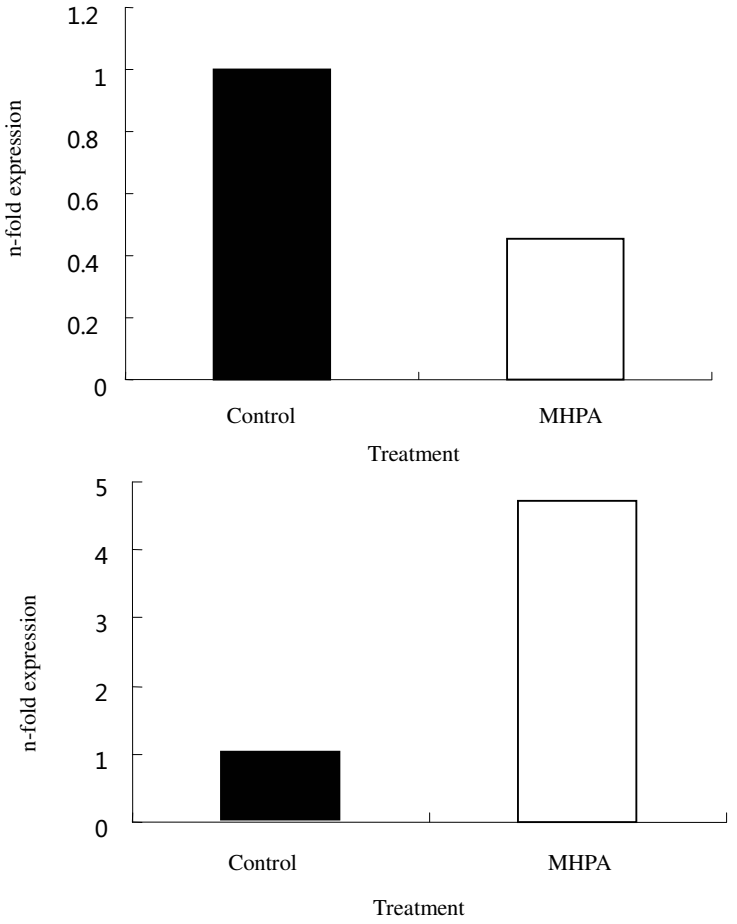

Figure 6A-B. Expression analysis of *S385* and *S452* in MHPA-treated and control (without MHPA) root tip detected by real time RT-PCR. **A:** In MHPA treatment, the expression of *S385* was down-regulated. **B:** In MHPA treatment, the expression of S452 was up-regulated. 1.

their functions. Since phenolic acids are common active groups, we hypothesize that as with other phenolic acids, MHPA might influence the plant via the similar mechanisms depending on the phenolic acids *per se* and the plant species. In response to the exogenous MeJA and MeSA, rice gene transcription of phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H), the two key enzymes in the phenylpropanoid pathway were enhanced and the amounts of phenolic acids in the rice leaves were increased (2). Similarly, Bais *et al.* (2003) reported that *Arabidopsis thaliana* treated with the flavonol (–)-catechin, the genome-wide changes in gene expression were triggered in the root

meristemic tissue, including related to the phenylpropanoid as well as oxidative stress and signal transduction mediated oxidative stress. In addition, low (±)-catechin concentrations causes the up-regulation of pathogenesis-related genes such as PR1 via the SA/NPR1-dependent pathway, thereby enhancing the cell division and cell expansion (19).

CONCLUSIONS

These studies showed that the high level of MHPA (300 $mg.L^{-1}$) substantially affected the ultrastructure of root tip cells. Two differential expressed genes were confirmed in the presence of MHPA. Further studies are necessary to determine whether these two genes were implicated in the poor development of continuous monoculture of soybean crop.

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