

Cloning and Analysis of Glutathione S-Transferase MsGSTU21 Gene in *Medicago Sativa*

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Abstract. As the perennial herb of the Leguminosae and *Medicago L.*, *Medicago sativa* is the herbage crop widely used in countries all over the world, and it is the cornerstone of the development of animal husbandry in many big powers. Under abiotic stress, plants produce excess peroxides, peroxides damage the cell membranes of plants and attack some macromolecular substances in plants. In toxicology, glutathione can be combined with cellular biological macromolecules, thereby preventing some toxic exogenous chemicals from covalently bonding to it, avoids damage to the organism, and make the body complete life activities normally to achieve the effect of interpretation. It appears that it is particularly important to help improve the stress resistance of *Medicago sativa*.

Keywords: *Medicago Sativa*, Glutathione S-Transferase MsGSTU21, Peroxides.

1. Introduction

Glutathione S-transferase (GST) is a common cytoplasmic dimer protein molecule in organisms, which is inside the cytosol and plays a very important role in the interaction of glutathione. As a very important enzyme in glutathione ascorbate, it can maintain the circulation of glutathione ascorbate system. In toxicology, glutathione can be combined with cellular biological macromolecules, thereby preventing some toxic exogenous chemicals from covalently bonding to it, avoids damage to the organism, and make the body complete life activities normally to achieve the effect of interpretation.

The gene chip experiment of *Medicago sativa* in the early stage showed that when *Medicago sativa* was under aluminum stress, the expression of a glutathione S-transferase gene (chip probe number: A_27_P104331) in plants rise sharply, since living organisms will instinctively highly express genes that can resist the stress when they are under stress, therefore, it can be inferred that *Medicago sativa* increases the expression of GST gene in order to resist aluminum stress. This research cloned the GST gene and analyzed the protein structure and basic attributes encoded by the gene.

2. Experimental Method

2.1 Material

2.1 Plant material

The *Medicago sativa* "WL525" as the experimental material, it was provided by Beijing Zhengdao Ecological Technology Co., Ltd.

2.2 Bacterial strains

Gene cloning vector: pMD18-T, purchased from Takara Biomedical Technology (Beijing) Co., Ltd (Beijing). *Escherichia coli* competence: DH5 α , purchased from Shanghai Weidi Biotechnology Co., Ltd. *Agrobacterium tumefaciens*: GV3101, purchased from Shanghai Weidi Biotechnology Co., Ltd.

2.3 Plasmid vector

pHB-YFP, preserved in the laboratory of the research group.

2.4 Main reagents

The enzymes involved in this experiment: Easy-Taq (Takara Biomedical Technology (Beijing) Co., Ltd. (Beijing)), 10×Flycut Buffer (Tiangen Biotech) (Beijing), 2×Taq Plus Master Mix II (Nanjing Vazyme Biotech Co., Ltd).

The test kits involved in this experiment: RNA extraction (Beijing TransGen Biotech), DNA extraction (Nanjing Vazyme Biotech Co., Ltd), plasmid extraction (Tiangen Biotech).

The inorganic compounds involved in this experiment were purchased from China National Pharmaceutical Group Co., Ltd. (Sinopharm) and Shanghai Sangon Biotech.

2.5 Experimental equipment

Clean workbench, nucleic acid electrophoresis apparatus, constant temperature shaker (Shanghai Yiheng), microplate reader (Thermo), constant temperature shaking metal bath (Hangzhou Risen), balance (Sartorius), pH meter (Mettler Toledo), pipette (Eppendorf), low temperature centrifuge (Eppendorf), mold box (Shanghai Dengsheng), autoclave (Sanyo), water purifier (Shanghai Moore Company), normal temperature centrifuge (Eppendorf), PCR instrument (Eppendorf).

3. Experimental Steps

3.1 Germination and hydroponics of *Medicago sativa* seeds

Take right amount of alfalfa WL525 seeds and wrap them with soft gauze, gently rub under running water until the coating is completely washed, use absorbent paper absorb the water, and spread two layers of filter paper in 30cm × 40cm iron plate, spread washed seeds evenly on the filter paper, soak them with distilled water, it is proper to form water film around the seeds, cover them with preservative film, and germinate for 6 days.

Each 5 seedling in good growth condition are gently wrapped with sponge strips and are transferred to the foam floating plate with 9 holes evenly distributed, and quickly placed in the plastic bucket containing 2L 1/2 Hoagland nutrient solution to continue to cultivate in the culture room for 5 days. replace the nutrient solution every 2 days.

3.2 Aluminum treatment of *Medicago sativa*

Transfer the *Medicago sativa* seedlings with the same growth state to 1/2 Hoagland's (pH5.8) nutrient solution, change the nutrient solution every 2 days until 4 true leaves grow (about 10d days; replace the nutrient solution with 1/2 Hoagland's (pH 4.5) and grow for 2 days; then transfer 1/2 Hoagland's (pH4.5), 1/2 Hoagland's+100uM AlCl₃ (pH 4.5) nutrient solution, carry out aluminum treatment, sample at 0h, 1h, 3h, 6h, 12h, 24h, respectively, each 0.1g leaf is a sample, they are temporarily placed in liquid nitrogen, and are transferred to -80°C refrigerator for extracting RNA.

3.3 Extraction of plant tissue RNA of *Medicago sativa*

RNA extraction kit of TransGen Biotech is used to extract total RNA of plant. The steps are as follows: grind the sample and place it on ice, add 1mL of TransZol Up, mix evenly and stand for 5 min, add 0.2 mL of trichloromethane, mix evenly and stand for 3 minutes, centrifuge at 1000× g for 15 minutes (4°C), add 600 uL of absolute alcohol, after mix evenly, transfer them to a spin column, centrifuge at 12,000xg for 30 seconds, add 500 uL of CB9, centrifuge at room temperature for 30 seconds, add 500 uL of CB9, centrifuge at room temperature for 30 seconds, add 500 uL of WB9, centrifuge at room temperature for 30 seconds, centrifuge at 12,000 xg for 2 minutes, open the lid and dry it at room temperature for 5 minutes to completely remove residual ethanol, add 60 uL of distilled water, centrifuge at 12,000 xg for 2 minutes, and preserve at -20°C.

After extraction, immediately put it on ice for later use, then use 1% agarose gel electrophoresis and nucleic acid concentration analyzer detect the purity and concentration of the extracted RNA, the ratio of A260 and A280 is 1.8-2.0, then it can be used for the next step.

3.4 Primer design of MsGSTU8

Glutathione S-transferase (GST) uses glutathione to remove reactive oxygen species (ROS) generated by abiotic stress. Based on gene chip result of *Medicago sativa* in the laboratory under aluminum stress in the early stage, the overexpression of a glutathione S-transferase family gene under aluminum stress was found, and it was named MsGSTU8.

Because the homology between *Medicago sativa* MsGSTU21 and *Medicago truncatula* MtGST21 is higher, the gene sequence of *Medicago truncatula* MtGST21 is searched through National Center for Biotechnology Information, Primer Premier 5.0 is used to design specific primers based on the gene sequence of *Medicago truncatula* MsGST21, T_m values of the upstream and downstream primers are determined, the upstream and downstream primers are named MsGSTU21-F and MsGSTU21-R, respectively.


MsGSTU8-F: 5'-ATGGCTACAAATCAAGAACATGTGA-3'

MsGSTU8-R: 5'-ACTAGTCTTTGAAGCAGCAAGAAGG-3'

3.5 PCR reaction

The cDNA of *Medicago sativa* obtained in 2.3 is diluted 10 times as the template, MsGSTU21-F and MsGSTU21-R as upstream and downstream primers for PCR amplification, the amplification process is as follows:

94°C 8 minutes
94°C 40 seconds
58°C 40 seconds
72°C 40 seconds



30 cycles

Extend at 72°C for 10 minutes and then preserve at 4°C.

3.6 Detection of agarose gel electrophoresis

20 μL of the PCR product obtained and 5 μL of 2K plus Maker are spotted in the sample hole of 1% agarose gel, respectively, the current is 400 A, the voltage is 120 V, and electrophoresis is 30 minutes.

3.7 Extraction of agarose gel DNA

After agarose gel electrophoresis ended, the correct product band is determined in accordance with the size of the target gene fragments, cut the colloid under ultraviolet light, and place it in the 1.5 mL of tube for reserve. the DNA extraction test kits (Tiangen) is used to extract DNA of gel. Add 700 μL of Buffer GDP, heat to dissolve, transfer it to adsorption column, centrifuge at 12,000×g for 30 seconds, add 300 μL of Buffer GDP, centrifuge at 12,000×g for 30 seconds, add 700 μL of Buffer GW, centrifuge, add 700 μL of Buffer GW, centrifuge, empty centrifuge for 2 minutes, 20-30 μLHO, centrifuge at 12,000×g for 1 min, and preserve at -20°C.

3.8 Construction of PMD18-T cloning vector of MsGSTU8 gene

After mixing 4 μL of gel recovery product, 1 μL of pPMD18-T Vector and 5 μL of Solution evenly, it is placed in the low temperature and constant temperature reaction bath at 16°C overnight.

3.9 MsGSTU8-PMD18-T transform escherichia coli DH5α

Take out 100 μL of the DH5α bacterial strain from the -80°C refrigerator and quickly transfer it to ice to thaw, add 10 uL of the ligation product (MsGSTU21-PMD18-T vector) obtained in the previous

step to the clean workbench, blow gently and mix evenly with pipette, stand on ice for 20 min, heat shock in metal bath at 42 °C for 90 second, quickly ice bath for 3 minute after heat shock, add 700 μ L of LB liquid medium at room temperature, incubate at 37 °C shaker at 200 rpm, after the incubation, absorb 50 μ L of coating onto the plate containing LB+Amp, invert it overnight at 37C until 1-2 mm of plump single colony has grown. Select 6 single colonies into 15 mL of centrifuge tube containing 5 mL of LB medium, and continue to cultivate them in 37C 250 rpm shaker for 8-10 h until OD comes to 0.6.

3.10 PCR identification of bacterial solution

Six tubes of bacterial solution obtained in the previous step is taken as templates, and the upstream and downstream primers (MsGSTU8-F, MsGSTU8-R) of MsGSTU21 are used for PCR. 20 μ L of the obtained PCR product and 5 μ L of 2K plus Maker are spotted in the sample hole of 1% agarose gel, the current is 400A, the voltage is 120V, and electrophoresis is 30 minutes. The bacterial solution with bright bands in the 6 tubes of bacterial solution is sent to the company for sequencing (Sangon Biotech Co., Ltd.).

4. Experimental Results and Analysis

4.1 MsGSTU21 gene cloning

WL525 seedlings of Medicago Sativa is treated with aluminum stress, RNA is extracted, cDNA is reverse transcribed, primers is designed, MsGSTU21 is cloned by PCR, and about 500bp of DNA fragment is obtained by electrophoresis (Fig.1).

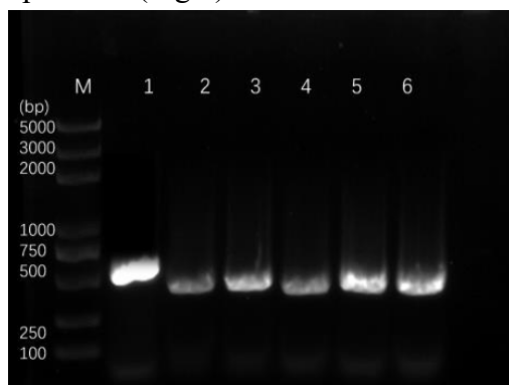


Fig. 1 gel electrophoresis bands of MsGSTU21, 1-6 represent the amplified bands with different T_m values

The PCR product is recovered and connected to PMD18-T vector to transform escherichia coli, identify whether the connection is successful by bacterial liquid PCR (Fig.2), positive clones are sent to Sangon Company for sequencing.

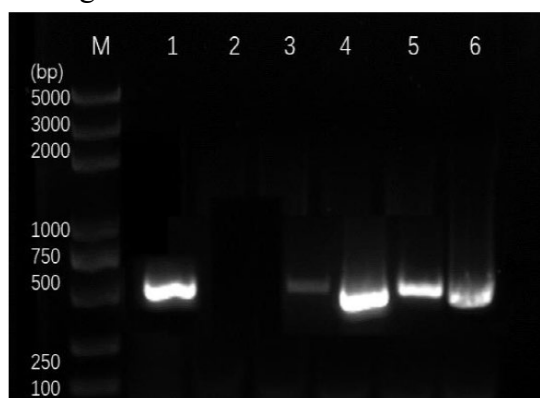


Fig. 2 PCR gel electrophoresis of MsGSTU21 gene bacterial solution, 1-6 represent different monoclonal band

4.2 Bioinformatics analysis of MsGSTU21 sequence

Sequencing results confirmed that the obtained 675bp of DNA fragment contained complete ORF. The encoded amino acid was predicted with DNAMAN analysis software, it was found that this gene is highly similar to MtGSTU21 in *Medicago truncatula*, it was named MsGSTU21. The analysis of amino acid showed that the amino acid sequence encoded by the gene contained two conserved domains: GST_N_Tau and GST_C_Tau (Fig.3). DNAMAN8.0 software was used, the amino acid sequence encoded by the cloned MtGSTU21 was compared with the GST family members of seven plant protein sequences with high homology (*Medicago truncatula* MtGSTU21, *Arabidopsis thaliana* AtGSTU8, maize ZmGST23, rice OsGST23, soybean GmGSTU22, chickpea CaGST, mung bean VrGST) were compared, a total of 226 amino acid residues were compared (Fig.4), and the conserved amino acid residues were as high as 72.01%. The consistency with MtGSTU21 (XP_003623202.3) of *Medicago truncatula* was the highest (99.11%) (Fig. 5). The analysis of the evolutionary relationship of GST proteins showed that MtGSTU21 was the closest relative to MtGSTU21 of *Medicago truncatula*, the next was Fabaceae, the last was *Arabidopsis thaliana*. MtGSTU21 was the most distantly related to monocotyledon GST proteins (Fig. 5).

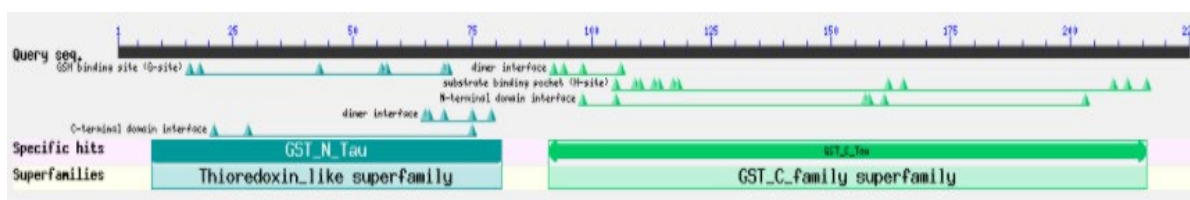


Fig.3 Conserved domain of MtGSTU21

msgstu21	MATNCEHVKLLGATGSEFFVGRVQIALKLGIEYEFVEENLA.TKSECLLRKYNEVHKVVPVFEVHNEKPISESLVILEYIDEVWVQ.	83
atgstu8	.MNQEEHVKLLGLWGSFFSHRVEMVLRKLGIPYIYEIEDVYGNRSEMLLKYNEIHKVVPVLIHNGRSIAESLIVVEYIEDTQKTT	84
zmgst23	.MAEKGVKVLGMWASEMVIRVEWALRLKGVVEYVDELLA.NKSEALLRHNEVTKKVPVLIHNGKPVAEETIIVVEYIDEVWVKG	82
osgst23	.MAADKGVKVFEMWASEMATRVEWALRLKGVVDEYVDELLA.NKSEALLRHNEVTKKVPVLIHNGKPLAESTVIVVEYIDEAWKHG	83
gmgstu22	MATNCEHVSLIGVVGSEFFVGRVQIALKLGICQYKFEENLV.NKSEALLKYNEVHKVVPVFEVHNEKPIAESLIVVEYIDEVWVKN.	83
cagst	MATNCEHVKLLGIVGSEFFVGRVQIALKFKIYKFELEQLNF.DKSEALLKYNEVHKVVPVFEVHNEQPISESLVILEYIDEVWVQ.	83
vrgst	MGSSCEEVILLGIISAFVGRVKIALKLGVEYKYVEENLG.NKSECLLRKYNEVHKVVPVFEVHNGKPLAESLIVVYIDEVWVNN.	83
mtgstu21	MATNCEHVKLLGATGSEFFVGRVQIALKLGIEYEFVEENLA.TKSECLLRKYNEVHKVVPVFEVHNEKPISESLVILEYIDEVWVQ.	83
Consensus	v g s r v l k y s l l n p k k v p v h e s i y i w	
mmsgstu8	NEILPDSYHQRADARFWSKEIDDKIVSASFKSVFSLD.EKEREKNIEPATEAHFLBNELK.DKYFGGEEFNFDIAAVVFAFW	166
atgstu8	HTILPCDEYERAMARFWAKYVDEKVMVLAVKACWGPE..SEREKVKEAYEGPKCLBKLGDKLFEGGETIGFVDIAADFIGYWL	167
zmgst23	YEIMPGDEYERACARFWAREAEIKCNAALYPIFATG..EAQRRAVHEACCCPKTLBTALEGKRFFGGDAVGYLDIVVWGFHAWL	165
osgst23	YEIMPSDEFDRACARFWAREAEIKCNAALYPIFMTTG..EEQKRLVHEACCCPKTLBTALEGKRFFGGDAFGYLDIVVWGFHAWL	166
gmgstu22	NEILPDSYHQRADARFWSKEIDDKIGGAVWRKSVFTVD.EKEREKNVDESLEAIQFLESEIKGKRFEGGEEFGMVDIAAIFIAFW	167
cagst	NEILPDSYHQRADARFWAKEIDDKIVSSSFRAVFTVDEKEREKNVDESSEAKILENELK.DKRFEGGEEFGVDIAAVFIAFWI	167
vrgst	NEILXSDYHQRADARFWSKEIDDKIVGASWRSVFTVD.EKEREKNIAETYESIQFLENEIKKRFEGGEEGLVDIAAVVFAFWI	167
mtgstu21	NEILPDSYHQRADARFWSKEIDDKIVSTSFASVFSLD.EKEREKNIEPATEAHFLBNELK.DKYFGGEEFNFDIAAVVFAFW	166
Consensus	i dp ra arfw k k e l le fgg di w	
mmsgstu8	PLVCDITELQLFTAEKFEKLYNWSQEFLDHPVVKETLPEREPLFFFGGRYESLLAASK	225
atgstu8	GIFQEASGVTIIMTAEFFPKLQRWSEDFVGNNFIREVLEPKKLVAVLRAMFGSVTSN..	224
zmgst23	PVIEEVTGASVVTHEEELPLMKAFGRFLALDVVRAALHRRDLILANKARREQLLSA..	222
osgst23	PVIEEACGVEVVITDDELPLMKAFDRVLAVDAVAVLPRDKLVAVNARREQLLSA..	223
gmgstu22	PMVCEIAGLELFTSRKFEKLYNWSQEFMSHPVVKETLPERDPLFFFGGRYESLLADSK	226
cagst	PLVCDITGLQLFTAEKRYPKLHNWSQEFLNHPVVKENLPEREPLFFFGGRYESLLAASK	226
vrgst	PLICEIAGLELFTSRKFFNLYRWSQEFLNHPVVKESLPERDPLVFFFGGRYESLIFSSK.	225
mtgstu21	PLVCDITELQLFTAEKFEKLYNWSQEFLDHPVVKETLPEREPLFFFGGRYESLLAASK	225
Consensus	t e p w k l p a k	

Fig. 4 GSTs sequence comparison of multiple species

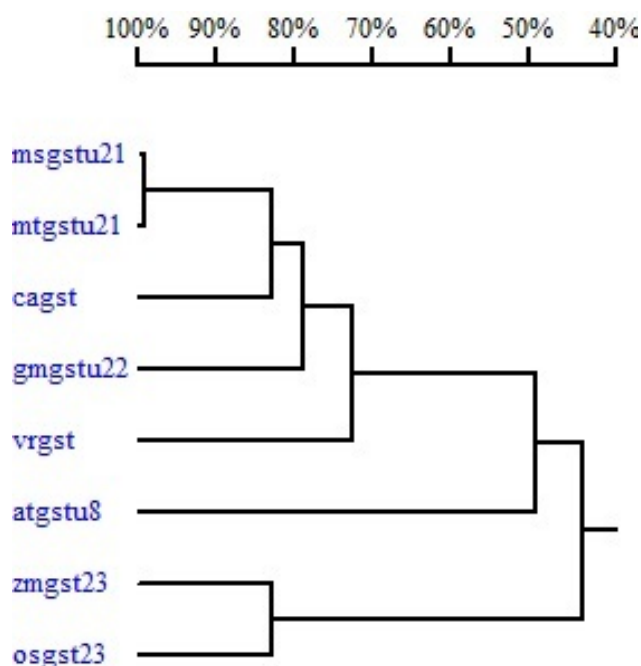


Fig. 5 Analysis of the GSTs evolutionary tree

According to SOPMA software analysis of Hunault, the secondary structure of amino acid of MsGSTU21 protein is composed of 55.6% alpha helix, 12.44% extended strand and 27.11% random coil. (Fig.6)

Sequence length :	225
SOPMA :	
Alpha helix (Hh) :	125 is 55.56%
3 ₁₀ helix (Gg) :	0 is 0.00%
Pi helix (Ii) :	0 is 0.00%
Beta bridge (Bb) :	0 is 0.00%
Extended strand (Ee) :	28 is 12.44%
Beta turn (Tt) :	11 is 4.89%
Bend region (Ss) :	0 is 0.00%
Random coil (Cc) :	61 is 27.11%
Ambiguous states (?) :	0 is 0.00%
Other states :	0 is 0.00%

Fig. 6 Prediction of the secondary structure of MtGSTU21

4.3 Prediction of protein structure of MsGSTU21

ExPASy - ProtParam Tool online analysis was used, the results show that MsGSTU21 encoded 225 amino acids, the molecular formula was C 1215 H 1853 N 297 O 339 S 2, the relative molecular mass of the protein was 2.6kDa, the theoretical isoelectric point (pI) was 5.37, the unstable coefficient was 33.61, it was the stable protein; the grand average of hydropathicity (GRAVY) was -0.235, it showed that MsGSTU21 was the hydrophilic protein. I-TASSE was used to predict the 3D structure of the protein, it was found that the protein encoded by MsGSTU21 had the typical features of the typical GST Tau subclass, the N-terminal contained several α -helix and several small β -sheet, the C-terminal had 5 α -helix and contained structure of Linker (Fig.7). It was further confirmed that the gene belongs to the Tau subfamily.

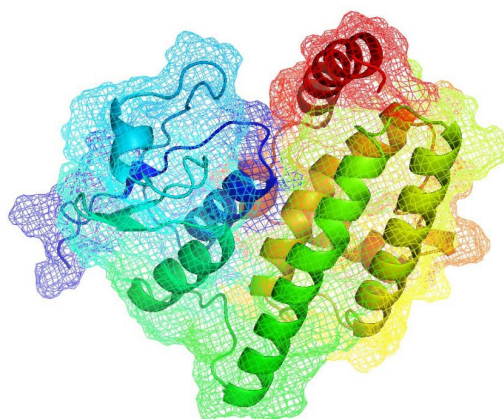


Fig. 7 prediction of the 3D structure of MsGSTU21
(C→N terminal is the color change of the rainbow)

5. Discussion

GSTs are a multifunctional superfamily, which play a very important role in plant detoxification and resisting adversity [2-5]. According to the protein structure and biology, GSTs of plant can be divided into 8 subgroups: Phi(F), Tau(U), Theta(T), Zeta(Z), Lambda(L), DHAR (Dehydroascorbate reductase), TCHQD (Tetrachlorohydroquinone dehalopgenase) and microsomal GSTs. The first seven subgroups are water-soluble proteins, while the eighth subgroup is microsomes and belongs to the family protein of MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism), and it is the fat-soluble protein. The GSTs of microsome and the first seven subgroups of GSTs are large in sequence differences, but have the same enzymatic activity depending on glutathione (GSH) substrate. Phi, Tau, Lambda and DHAR are special in plants. Especially Phi and Tau, they have the most species in plants.

The protein encoded by the MsGSTU21 gene cloned in this paper belongs to the GST Tau subfamily. This type of GSTs is widely distributed in various plants, they are regulated by hormone and have various biology, they can be used as enzymes to participate in the removal of oxygen free radicals, and they can also be used as connexin protein to bond biochemical substances to reduce the damage of harmful substances to plants, it is an important plant anti-stress protein. The expression of MsGSTU21 gene rises under adversity stress, it is speculated that this gene is involved in plant anti-adversity response and has theanti-adversity function, and it is necessary to further study.

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