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CLONING AND BIOINFORMATICS ANALYSIS OF CADMIUM-RESISTANT GENE *TASFT2* IN WHEAT

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Cadmium is a non-essential trace element which is highly toxic to plants. Because of its high mobility and toxicity, it has become a hot topic to study the molecular mechanism of cadmium uptake and transport by plants and to cultivate new crop varieties resistant to cadmium and low cadmium accumulation. Cd enters into the plant body, it will be absorbed by the root system and gradually transported to the above-ground part. Plants reduce toxic effects by absorbing and transporting heavy metals in different chemical forms and storing them in different organs and tissues. Under cadmium stress, plants produce a variety of physiological and biochemical mechanisms that limit cadmium absorption and transfer to reduce cadmium damage. Cadmium stress induces the expression level of metallothionine gene in gramineous crops (wheat and rice), which has a positive effect on improving plant resistance to cadmium and alleviating cadmium toxicity. It is not clear how the gene causes tolerance to heavy metals. Therefore, this experiment cloned the gene and analyzed the biological information to find the mechanism of cadmium resistance.

The full length of TaSFT2 gene was cloned by RT-PCR. The sequence analysis showed that the ORF gene was 684bp, encoding 228 amino acids, with a molecular weight of 58.542kD and an isoelectric point of 9.16. The results of evolutionary tree analysis showed that wheat TaSFT2 was closely related to maize ZmGot1/Sft2 gene and rice OsGot1/Sft2 protein.

The basic information of TaSFT2 gene can be obtained by chromosome location analysis, intron/exon analysis, ORF analysis and expression profile analysis, etc. By analyzing the basic properties of TaSFT2 protein, hydrophobicity analysis, transmembrane region prediction, signal peptide prediction and similarity prediction, the properties of gene-encoded protein can be preliminarily determined and predicted. In particular, hydrophobicity analysis and transmembrane region prediction can be used to predict whether the gene is membrane protein, which has important reference significance for determining the direction of experimental research.

Key word: *cadmium, wheat, tolerance, Cd-resistance, gene sequence analysis.*

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Introduction. Cadmium is a non-essential trace element which is highly toxic to plants. Growing in cadmium polluted areas will lead to excessive cadmium levels in agricultural products and threaten the health of animals and humans. Therefore, it has become a hot topic to study the molecular mechanism of cadmium uptake and transport by plants and to cultivate new crop varieties resistant to cadmium and low cadmium accumulation.

It was found that the toxic effect of heavy metal cadmium is related to its transport from soil to the aboveground part of plants. During the process of cadmium entering the xylem through the root cortex, through the symplast and extracellular pathways, most of it is deposited in the intercortical cells, and a few of it is transferred to the above-ground parts after arriving at the midcolumn. Studies have shown that when Cd enters into the plant body, it will be absorbed by the root system and gradually transported to the above-ground part. In paddy rice, the running rule is the highest in the root, followed by the stem, leaf sheath and leaves. In rice plant, the accumulation amount is the lowest, while most of the cadmium accumulates in the root (Li et al., 2017; Meixiu et al., 2006; Tanaka et al., 2003; 2003; Wojas et al., 2008). Other authors analyzed that only 2 % of the cadmium in soybeans moved to the stem, with the rest remaining in the roots and only a small portion of the Cd transported to the seeds. In heavy metal enriched plants, the content of Cd in stems and leaves is much higher than that in roots (Zhang et al., 2008). This indicates that cadmium accumulation is different in various crops. Therefore, the ability of cadmium transport from root to over ground is one of the important mechanisms that determine the tolerance of plants to cadmium. It was showed that in wheat cadmium was first transferred to phloem in ear and then transferred to grain, indicating that phloem transport was the main transport mode for cadmium to enter grain (Herren & Feller, 1997). At the same time, K. Tanaka with colleagues also confirmed that 90 % of cadmium in some grains was transported through phloem (Tanaka et al., 2003). Therefore, the ability of cadmium transport from xylem to phloem in spike is the main determinant of cadmium content in wheat grain, rather than cadmium content in xylem.

In wheat, cadmium in the soil is absorbed and transported to the xylem through the root system of plant, transported upward through the xylem, transferred and accumulated to the aboveground phloem, and finally enriched in wheat grains; it has undergone a series of physiological and biochemical changes (Jian et al., 2020; Song et al., 2017). The process goes through three steps: the first step is the absorption and activation of the root system, the transport of xylem, and the transfer of phloem to grains (Ma Hui et al., 2020; Ghori et al., 2019). Cadmium enters the root vascular column mainly through extracellular and symplast pathways, and metal ions from the soil migrate through extracellular spaces such as cell walls or intercellular spaces and accumulate through the cortical and endocortical tissues. The

symplast pathway is a transport type through which cadmium carrier proteins use the metabolic energy of plants to enter root cells, transfer through the symplast (intercellular ligamentum), and accumulate in the vascular column, including the transport of Ca ion channels, endocytosis, calmodulin, and cationic transporters with low affinity (Choppala et al., 2014). Secondly, cadmium in the xylem enters the duct through transporters, and in the root cytoplasm it can be transported to vacuole, mitochondria and other regional chambers, or loaded from the parenchyma cells of the root tissue into the xylem duct for transport. Then it is transported to the above-ground by transpiration and root pressure over a long distance. The third step is the transport from xylem to phloem. Crops such as wheat is transported from xylem to phloem through the stem node, and then cadmium will be transferred into grains through the phloem of cob (Abedi & Mojiri; 2020).

Plants reduce their toxic effects on plants by absorbing and transporting heavy metals in different chemical forms and storing them in different organs and tissues (Ghori et al., 2019). Under cadmium stress, plants produce a variety of physiological and biochemical mechanisms that limit cadmium absorption and transfer to reduce cadmium damage, among which cadmium transporters and their chelate related transporters play an important role in plant resistance to cadmium toxicity, and cell wall fixation and plasma membrane selective permeability also play a key role. Studies have shown that cadmium stress induces the expression level of metallothionin gene in gramineous crops (wheat and rice), which has a positive effect on improving plant resistance to cadmium and alleviating cadmium toxicity (Chen et al., 2019; Yali et al., 2017).

Various protein families involved in heavy metal transport in plants have been isolated and identified, including the P-type ATPase gene family, the ATP-binding Cassette transporter (ABC) gene family, and the Natural Resistance-Associated macrophage Protein family (NRAMP), Cation Diffusion Facilitator (CDF), H+/Cation Exchanger (CAX) family, Zrt/ irt-like Protein (ZIP) family, Oligopeptide Transporter (OPT) family for transport of tetrapeptide and pentapeptide, etc. (Ali & Nas., 2018; Huang et al., 2020).

In rice, overexpression of *OsHMA3* and interference with the silencing of *OsNRAMP5* and *OsNRAMP1* reduced the cadmium concentration in stalks and grains, and greatly reduced the absorption and accumulation capacity of Cd^{2+} in plant, indicating its involvement in the absorption and transport of Cd^{2+} (Liu et al., 2019; Russell & Soulimane, 2012; Sasaki, et al., 2014). Got1/SFT2-like protein, vesicle transport protein, the gene were involved in metal exclusion and storage, to actively pump metal ions across membranes located either in the plasma membrane (contributing to extruding metals to the cell exterior) or vesicle and vacuole membranes (creating metal storage that can be either kept in the cell or displaced). Examples of these genes include the cation diffusion facilitator transport proteins that are predicted to aid in zinc ion homeostasis and an iron permease gene predicted to transport iron ions across membranes (Takahashi et al., 2014). It was identified the gene scattered across the genome putatively involved in heavy metal tolerance. (Chiang et al., 2006). The gene encode for transmembrane transporters involved in metal exclusion and storage, immobilization, and ROS detoxification. It is not clear how the gene causes tolerance to heavy metals. Therefore, this experiment cloned the gene and analyzed the biological information to find the mechanism of cadmium resistance.

Materials and methods. Material was wheat variety Bainong 207, supplied by Henan Institute of Science and Technology. PMD-19T vector, *Escherichia coli* (*E. coli.)* and *Agrobacterium* GV3101 strains were purchased from TaKaRa biological company.

Seeds of Bainong 207 (*Triticum aestivum L*.) were disinfected with 75 % (v/v) ethanol for 1 min and 2.5 % sodium hypochlorite for 6 min, then germinated on moist filter papers. All seeds were provided by Center for Genetic Improvement of Wheat, College of Life Science and Technology, Henan Institute of Science and Technology. On the 10-th day, uniform and healthy seedlings were transplanted to 4 x 12-hole hydroponics basin under natural light and temperature at 22 ± 2 °C (day/night). The water was continuously aerated and renewed every 3 days.

Wheat genomic DNA samples were prepared using etiolated seedlings as described previously. To prepare total RNA samples from wheat of Bainonng 207 organs or seedlings, Trizon reagent (tiangen, Cat. No. 419) was used. To avoid genomic DNA contamination, total RNA samples were treated with an RNase-free DNase kit according to the manufacturer's instructions (Qiagen, http://www.qiagen.com/).

Total RNA was extracted from Bainonng 207, and the full length CDS of the homologous *Got1/Sft2* (GenBank: LOC109784566) were cloned using the primers of Got1/Sft2-F and Got1/Sft2-R. cDNA was used to design specific primers based on the conserved sequence of *Got1/Sft2* gene of wheat in GenBank. The amplification product was detected by 2.0 % agarose gel electrophoresis.

Using the SingalP4.1 and TMHMM Server v.2.0 analysis the transmembrane region. NCBI is used to sequence Got1/Sft2 gene and cloned from wheat Bainong 207 (https://blast.ncbi.nlm.nih.gov/Blast.blastn&PAGE_PE=BlastSearch&LINK_LOC=blasthome). Using Expasy online website to analyze the hydrophilic/hydrophobic amino acid sequence of the gene (http://web.expasy.org/cgi-bin/protscale/protscale.pl?1). Using Mega 5.0 to construct the evolutionary tree.

Results. *3.1. Extraction of total RNA from wheat.* The extraction quality of total RNA is the premise that determines the results of this experiment. The extraction of total RNA with high purity and integrity is an important guarantee for RT-PCR. After the extraction of RNA from wheat leaves, the total RNA quality was detected by 0.8 % agar gel electrophoresis, as shown (Fig 1.). The results showed that the extraction effect was satisfied and the integrity was good as well. The value of OD260/280 was detected between 1.7 and 2.0 by ultraviolet spectrophotometer, indicating that the RNA samples obtained in this experiment had high purity, which was used for subsequent reverse transcription experiments and amplified fragments to construct the vector.

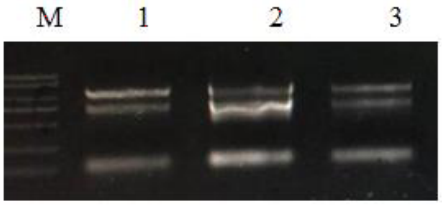
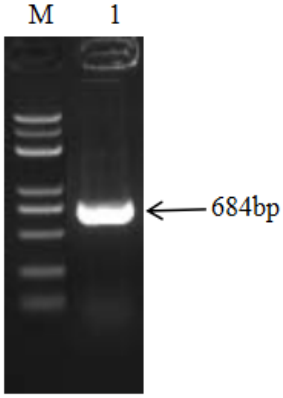


Fig 1 RNA detection by electrophoresis
M: Standard 2000+Marker; 1-5: RNA samples

3.2. Full-length cloning of wheat TaSFT2 gene. The results showed that the band with the same size as the target fragment (about 750 bp) was amplified (Fig. 2). After the strip was recovered, the plasmid was connected with pMD-19T and transformed into the competent cells of E. coli. After the successful verification by monocloning, the plasmid was extracted and named as pMD-19T-TaSFT2 plasmid and then it was sequenced.



M: Standard 2000+Marker; 1-4: Amplified band
Fig2. *TaGot1/Sft2* gene PCR amplification

The results showed that the sequence had a complete open reading frame (ORF), with a length of 684bp and encoding 228 amino acids (Fig. 2). The sequence was named TaSFT2.

3.3 The sequence analysis and bioinformatics analysis of wheat TaSFT2 gene. The physical and chemical properties of TaSFT2 protein were analyzed by Protaparam, and the molecular formula was C_{2089} H_{3496} $N_{684}O_{889}S_{191}$, the relative molecular weight was 58.542kD, and the theoretical isoelectric point pI was 9.169 (Fig. 3). Singal P 4.1 analysis showed that the sequence was a signal peptide that distinguished the transmembrane region. According to TMHMM Server v.2.0 online analysis, the *TaSFT2* protein has four distinct transmembrane regions (Fig. 4). Using Expasy online website (http://web.expasy.org/cgi-bin/protscale/protscale.pl?1), the hydrophilic/hydrophobic property of the amino acid sequence of this gene was analyzed (Fig. 5). The hydrophobic region encoded byTaSFT2 alternated with the hydrophilic region. Therefore, the *TaSFT2* protein was predicted to be hydrophilic.

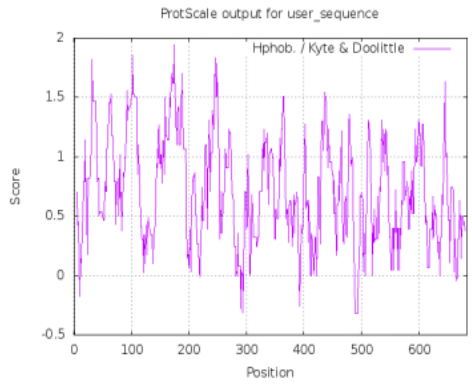


Fig3. Analysis of hydrophilicity and hydrophobicity of TaGot1/Sft2

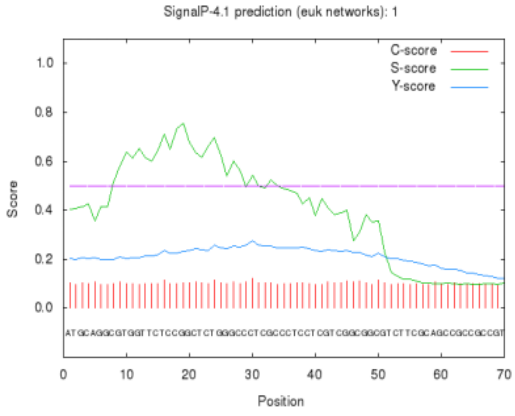


Fig4. The transmembrane region of TaGot1/Sft2

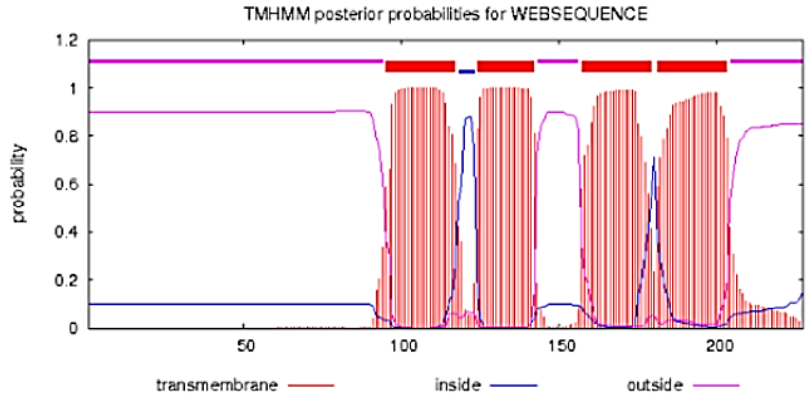


Fig5. Prediction of the transmembrane domain of TaGot1/Sft2

In order to further study the evolutionary relationship of *TaSFT2* gene in different species, the evolutionary tree of *Got1/Sft2* gene in different organismswas constructed through Clustal W comparison in MEGA5.0 and the Neighbor-joining method. The evolutionary tree was used to analyze the evolutionary relationship between *Got1/Sft2* gene in different species. As shown in Fig. 6., wheat *TaSFT2* has the closest relationship with maize *ZmGot1/Sft2* and rice *OsGot1/Sft2* proteins.

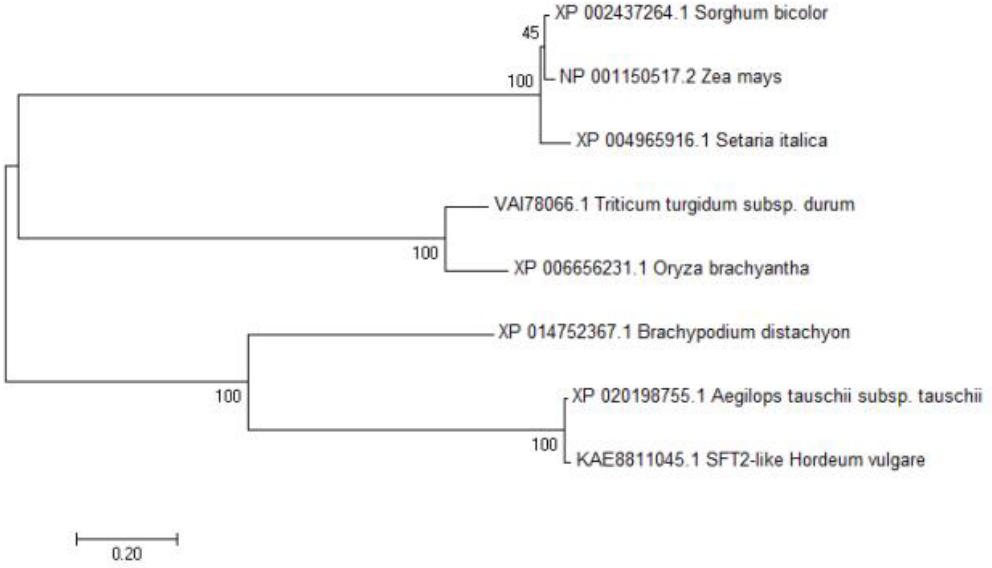


Fig6. Phylogenetic tree analysis of TaGot1/Sft2 and Got1/Sft2 protein of other species

Discussion. In this experiment, the sequence of wheat *TaSFT2* gene was successfully cloned by RT-PCR (Feeney, et al., Huai et al., 2008) . The sequence analysis showed that the ORF of the gene was 684bp in length, encoding 228 amino acids in total, with the predicted molecular weight of 58.542kD and the isoelectric point of 9.169. As Y. X. Zhu, & Y. Li (Zhu & Li, 2007) predicted the isoelectric points can be used in the separation of amino acids. In fact, in practical applications, compared with the pKa value of amino acid residues at isoelectric points of amino acids, the effect of pH on the dissociation of amino acid residues can be directly reflected in the protein properties (Bartels & Sunkar, 2005). When the pH is near the isoelectric point (pI) of the protein, the surface charge intensity and hydration ability of the protein are the lowest, and it is easier to precipitate. When pH deviates from pI value appropriately, protein solubility is better. SingalP4.1 analysis showed that the sequence was a non-secretory protein with no signal peptide sites. According to the online analysis of TMHMM Server V. 2.0, *TaSFT2* protein has four distinct trans membrane regions (Figure 3, indicating that this gene is a membrane protein. Using Expasy online website http://web.expasy.org/cgi-bin/protscale/protscale.pl?1) Hydrophilic / hydrophobic analysis of the amino acid sequence of the gene (Fig. 3–4). Hydrophobic and hydrophilic water appear alternately in *TaSFT2* encoding. In the whole peptide chain, hydrophilic amino acids are evenly distributed, with excess hydrophobic amino acids. Therefore, it is predicted that *TaSFT2* protein is hydrophilic, and the dissolution of protein in aqueous solution is the result of the interaction between protein surface charge and ions in aqueous solution, and water molecules. Too high or too low ionic strength in solution will destroy the hydration layer on the protein surface and promote protein polymerization and precipitation. Few proteins dissolve well in pure water. The dissolution of some proteins in solution requires specific helper molecules (glycerol, urea, arginine, detergent, etc.) (Liu et al., 2014; Patel et al., 2014). In order to further study the evolutionary relationship of SFT2 in different species, DNA sequences were used for developmental analysis to infer and evaluate the evolutionary

relationship of species at the molecular level, which was expressed in the form of a branching graph, namely the evolutionary tree. The evolutionary tree has multiple branches, but it is usually a binary tree. It's either a rooted or an unrooted tree. Rooted trees reflect the chronological order of tree species, while rootless trees only reflect the distance between taxa without reference to who is the ancestor. In other words, the root nodes of root trees are the nearest common ancestor of all taxa, which reflects the evolutionary relationship between taxa, while the rootless trees only reflect the taxa relationship (Whelan & Morrison, 2017). Through comparison of Clustal W in *MEGA5.0* and the neighbor-joining method, *SFT2* gene evolutionary trees of different organisms were constructed to analyze the evolutionary relationship between *SFT2* genes in different species. It was found that *TaSFT2* of wheat was closely related to *ZmSFT2* of maize and *OsSFT2* of rice.

Conclusion. The double helix structure of DNA contains the code of life, and the arrangement and change of four nucleotides contain a lot of genetic and evolutionary information. Since the Human Genome Project, data on the sequence and structure of nucleic acids (or proteins) has grown exponentially, and computers are essential to the application of such complex data. Therefore, the purpose of bioinformatics research is that people can clarify and understand the biological significance of large amounts of data through various tools such as mathematics and computer science. The basic information of *TaSFT2* gene can be obtained by chromosome location analysis, intron/exon analysis, ORF analysis and expression profile analysis, etc. By analyzing the basic properties of *TaSFT2* protein, hydrophobicity analysis, transmembrane region prediction, signal peptide prediction and similarity prediction, the properties of gene-encoded protein can be preliminarily determined and predicted. In particular, hydrophobicity analysis and transmembrane region prediction can be used to predict whether the gene is membrane protein, which has important reference significance for determining the direction of experimental research.

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КЛОНУВАННЯ ТА БІОІНФОРМАТИЧНИЙ АНАЛІЗ КАДМІЙ-СТІЙКОГО ГЕНУ TASFT2 ПШЕНИЦІ

Кадмій не належить до основних елементів живлення і є токсичним для рослин. Через його високу мобільність і токсичність актуальним питанням стало вивчення молекулярного механізму поглинання та транспорту кадмію рослинами, а також створення нових сортів сільськогосподарських культур, стійких до кадмію та здатністю до його низького накопичення. Cd потрапляє в організм рослини, поглинається кореневою системою і поступово переноситься в надземну частину. Рослини зменшують токсичну дію кадмію на власний оганізм, поглинаючи і транспортуючи важкі метали в різних хімічних формах і зберігаючи їх в різних органах і тканинах. Під впливом кадмієвого стресу рослини виробляють різні фізіологічні та біохімічні механізми, які обмежують абсорбцію і перенесення кадмію, для зниження його токсично дії. Кадмієвий стрес викликає рівень експресії гена металлотіоніна в злакових культур (пшениця і рис), що позитивно впливає на підвищення стійкості рослин до кадмію і зниження токсичності металу. Неясно, як саме ген обумовлює толерантність до важких металів. В цьому експерименті було клоновано ген і проаналізовано біологічну інформація, щодо пошуків механізму стійкості рослин до кадмію. Повну довжину гена TaSFT2 клонували за допомогою ОТ-ПЛР. Аналіз послідовності показав, що розмір гена ORF становить 684 пар основ, він кодує 228 амінокислот, з молекулярною масою 58,542 кДа і ізоелектричної точкою 9,16. Результати аналізу еволюційного дерева показали, що TaSFT2 пшениці був тісно пов'язаний з геном ZmGot1 / Sft2 кукурудзи і білком OsGot1 / Sft2 рису.

Основну інформацію про ген TaSFT2 можна отримати за допомогою аналізу місця розташування хромосом, аналізу интронов / екзонів, аналізу ORF та аналізу профілю експресії тощо. Шляхом аналізу основних властивостей білка TaSFT2, аналізу гідрофобності, прогнозування трансмембранної області, сигнального пептиду і прогнозування подібності. можна попередньо визначитий й передбачити властивості кодованого геном білку. Зокрема, аналіз гідрофобності й прогноз трансмембранної області можна використовувати для прогнозування, що має важливе значення для визначення напрямку експериментальних досліджень.

***Ключові слова**: кадмій, пшениця, толерантність, стійкість до кадмію, ген, секвенування.*

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