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Full Length Research Paper

Sequence analysis of β-esterase isoenzymes related to fertility changeover in TsCMS7311 of Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*)

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In order to explore the mechanism of temperature regulating fertility transformation in the thermo-sensitive male sterile of Chinese cabbage (TsCMS7311) (*Brassica rapa* L. ssp. *pekinensis*), the paper studied the related β-esterase isoenzymes from the gene expression. The β-esterase isoenzymes related to fertility changeover in TsCMS7311 were purified by one-dimensional and two-dimensional gel electrophoresis. Two polypeptides, whose molecular weights were 57.1 and 62 kD, were analyzed with Q-TOF mass spectrometry. We obtained three sequences of short peptides from the 57.1 kD polypeptide and two sequences of short peptides from the 62 kD polypeptide. The two short peptides sequences of 62 kD polypeptide were the same as that from the 57.1 kD polypeptide. This result indicated that the sequences of the two polypeptides were highly similar. By comparing the sequence with protein database of NCBI GenBank, it was found that the three short peptides were highly homologous (60 to 100%) to protein p27^{SJ}. This study would lay the theoretic foundation for the research of TsCMS7311.

Key words: Chinese cabbage, β-esterase isoenzymes, sequence analysis.

INTRODUCTION

There are wide ranging studies on photoperiod/ thermo-sensitive male-sterility so far, but not isolated a genuine gene of photoperiod/ thermo-sensitive sterility (Cao et al., 2005). Some reports indicated that esterase isoenzymes were different between a male-sterile line and its maintainer (Hu et al., 2000; Li and Qin, 2004; Zhang and Ke, 1999). The diversity and activity of esterase isoenzymes were changed along with the fertility transformation in thermo-sensitive male-sterility-Polima CMS (Tang et al., 1995; Zhang et al., 2005), therefore esterase isoenzymes may be closely associated with the fertility in Chinese cabbage and other crops.

Functional product cloning is to seek a corresponding gene studying from the gene expression, as is more efficient to get the gene function than studying from genome and transcription (Wang and Wang, 2000). The biggest challenge of functional product cloning is the isolation and purification of proteins, especially to purify enzymes present at low concentrations. The β-esterase isoenzymes had been purified with one-dimensional and two-dimensional gel electrophoresis (Zhang et al., 2008). The objective of the present study was to analyze the two polypeptides of the target β-esterase isoenzymes with Q-TOF mass spectrometry. To our knowledge, this is the first time to report the sequencing of β-esterase isoenzymes, which related with fertility expression in crops (Hu et al., 2000; Li et al., 1994; Zhang and Ke, 1999).

MATERIALS AND METHODS

Plant materials

TsCMS7311 (A) and its maintainer (B) of Chinese cabbage were

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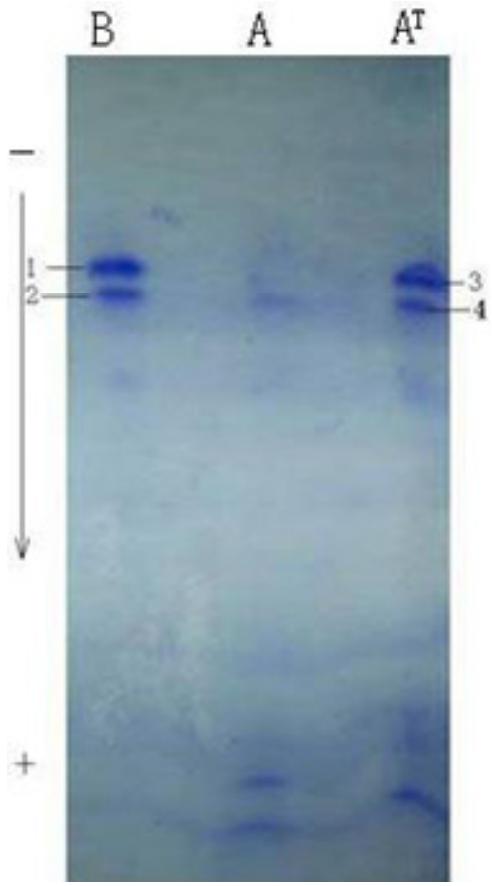

Figure 1. IEF electropherogram of β-esterase rude powder reclaimed from the little buds of B line, A line and A^T line.

bred by Chinese Cabbage Research Group of the College of Horticulture, Northwest Agriculture and Forestry University, Yangling, Shaanxi, China.

TsCMS7311 were treated with low temperature (6 - 12°C) for 3 - 9 days during the bud period, and then incubated under room temperature (about 25 °C). After 10 – 16 days, their flowers will turn to fertile for several days, and then revert to sterile again (Zhang and Hao, 2001). TsCMS7311 treated with low temperature was marked A^T.

Treatments and sampling

The seeds of lines A and B were germinated in the petri dishes for 12 h (about 25°C), and then were vernalized in 4°C refrigerator for 25 days (Hui et al., 2004). After that, the seeds were sown in porcelain tray under a 16 h photoperiod every day for 10 days and then transplanted them into the nursery pots grown under room temperature (about 25°C). Plants of line A were divided into two groups. One group was treated with 7°C for 9 days, then incubated under room temperature (about 25°C) until sampling ends (the β-esterase zymograms changed into nomal sterile line's (A))and marked A^T line; the plants in the other group of line A and line B were grown under room temperature (about 25 °C) continually. The β-esterases of 1.5 mm length buds are quite active and have a great difference between A and B lines; therefore the 1.5 mm length buds of stalk from A, A^T and B lines were chosen for the last β-esterase isoenzyme extraction.

Analysis methods

The β-esterase zymograms of A, A^T and B lines were analyzed with one-dimensional gel electrophoresis first, and then the target band region was cut out. By enriching the target sample with electro-elution and through the desalinization and vacuum freeze-drying, we finally obtained the powder of target enzyme. The powder was purified with two-dimensional gel electrophoresis (Guo et al., 2006).

The target bands in SDS-PAGE gel were cut out and submitted to the National Center of Biomedical Analysis (Beijing, China) for the analysis of polypeptides sequences by Q-TOF mass spectrometry.

The short peptide sequences obtained were blasted against Non-Redundant Protein Sequences Database using BLASTp of NCBI (www.ncbi.nih.gov).

RESULTS AND DISCUSSION

Isolation and purification of special β-esterase isoenzyme polypeptides

First, from the one-dimension electrophoresis of IEF-PAGE, we found that there were two dark blue bands separated in the lane of line A^T (bands 3 and 4) and maintainer (B) (bands 1 and 2) of TsCMS7311, which did not exist in the lane of TsCMS7311 (A) (Figure 1). Bands 1 and 3 as well as, bands 2 and 4 have the same respective pI (isoelectric point). Then the four bands were cut out and used for the second dimension SDS-PAGE electrophoresis. Five polypeptides, whose molecular weights are about 55.7, 57.1, 59.6, 60.5 and 62.0 kD were obtained from both bands 1 and 3, but only one polypeptide with the molecular weight 57.1 kD was obtained from both bands 2 and 4.

The result of second-dimensional gel electrophoresis indicated that the B and A^T lines had not only fertile phenotypes, and also similar β-esterase expression, which agreed with the previous inference. Moreover, the separation of one band from IEF-PAGE into 5 bands in the second dimension of SDS-PAGE electrophoresis indicated that the two-dimensional gel electrophoresis was effective.

Q-TOF mass spectrometry

Firstly, the polypeptide band with the molecular weight 57.1 kD was cut out from the SDS-PAGE gel, and then sent to the National Center of Biomedical Analysis (Beijing, China) for the further analysis with Q-TOF mass spectrometry. Three short peptide sequences were obtained as follows: >1: L-I-Q-V-P-S-V-A-T-S-V-A-I-P-F-N-K; >2: A-A-F-L-N-N-D-Y-T-K; >3: L-S-A-A-E-L-L-A-Y-K.

In order to find out the similarities and differences among these polypeptides, the second polypeptide band with the molecular weight 62 kD was cut out from the SDS-PAGE, and was submitted for the analysis. Two

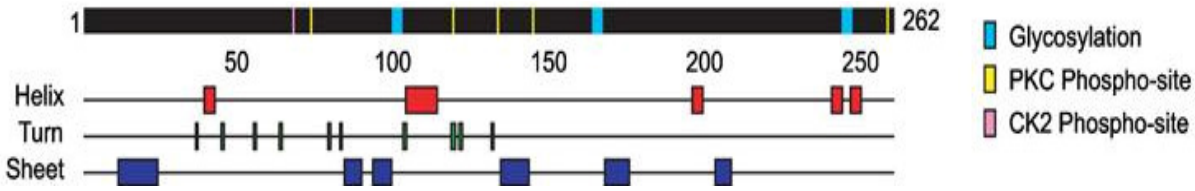

Figure 2. The secondary structure of p27^{SJ} (Sarkissian et al., 2006).

>peptide ----------AAFLNNDYT
> p27^{SJ} MADINGGGATLPQALYQTSGVLTAGFAPYIGVGSGNGKAAFLNNDYT

> peptide K----------LSAAELLAYK----------LIQVPSVAT
> p27^{SJ} KFQAGVTNKNVHWAGSDSKLSATELSTYASAKQPTWGKLIQVPSVGT

> peptide SVAIPFNK----------
> p27^{SJ} AVAIPFNKSGTAAVDLSVSELCGVFSGRITDWSGISGSGRTGAITVVYRS

> peptide ----------
> p27^{SJ} ESSGTTELFTRFLNAKCAETGTFNISTTFGTSYTGGLPAGAVSAAGSQGV

> peptide ----------
> p27^{SJ} MTALAGADGGTTYMSPDFAAPTLAGLDDATKVARVGKDVATNTAGVSP

> peptide ----------
> p27^{SJ} AAANVSAAINAVPVPASTEKP

Figure 3. The three short peptides of β-EST in Chinese cabbage are blasted to p27^{SJ}.

short peptide sequences were obtained which were amazingly identical to those of >2 and >3 above.

BLASTp

The three short peptide sequences above were blasted against the Non-Redundant Protein Sequence Database using BLASTp of NCBI. Among all homologous sequences, only a protein called p27^{SJ} (AAW57408) was extracted from a callus culture of the St. John's wort (*Hypericum perforatum*), others were all from the DING protein of *Pseudomonas aeruginosa*. Protein p27^{SJ} contained all the above three short peptide sequences, and the identities were very high (Figure 3). Short peptide sequence >2 is homologous to the 39 - 48 amino acid site of p27^{SJ}, and the identity between them was 100% (10/10); >3 is homologous to the 67 - 76 amino acid site of p27^{SJ} with 60% (6/10) identity; >1 is homologous to the 86-102 amino acid site of p27^{SJ} with 88.2% (15/17) identity. In addition, we could find that the short peptide sequences >1 and >2 were highly conserved in all these homologous sequences, which implied that the two sequences perhaps play an important roles.

According to the earlier results, the protein obtained was a new plant protein, which contains the common sequence of DING proteins possibly.

The secondary structure of p27^{SJ} and function predication of the β-esterase isoenzymes

It was represented that the protein p27^{SJ} contained 3 glycosylation-sites, 5 PKC phospho-sites and 1 CK2 phospho-site. There are 5 α-helix, 6 β-sheet and many β-turns at the 50 - 100 amino acid sites in the secondary structure of p27^{SJ} (Figure 2).

The three short peptides were located linearly in the 39 - 102 amino acid position of p27^{SJ}. Corresponding to the amino acid sequence of p27^{SJ}, there are 1 glycosylation-site, 1 PKC phospho-site, and 1 CK2 phospho-site for the two peptides at the >3 and >1 sites. We concluded that β-esterase may regulate the reversible phosphorylation of protein, which could play an important role in signal transduction of the fertility changeover.

The present report showed that several microbial proteins related to DING proteins often have some structural homology to phosphatases, and may represent phosphatase activity (Berna et al., 2002, 2008; Pantazaki et al., 2008). Recently, the DING proteins also have been detected in several higher plant species, such as tomato, potato, sweet potato, wheat and *Arabidopsis thaliana*

(Berna et al., 2002). Their functions were identified as follows: some are enzymes (oxalate oxidase, superoxide dismutase, nucleotide-sugar pyrophosphatase/ phosphodiesterase), some play a structural role in reinforcing the cell wall to resist pathogenic attack and could act as allergens, whereas others act as cell-surface receptors for molecules like auxin in *Rhizobium rhicadhesin* (Berna et al., 2002). Protein $p27^{SJ}$ is a member of the DING protein family which is an inhibitor of HIV-1 replication and transcription, and can suppress HIV-1 gene expression (Sarkissian et al., 2006). It is associated with the cell wall and sometimes secreted in the medium for *in vitro* grown cells (Perera et al., 2008).

The short peptides obtained here strongly resembled protein $p27^{SJ}$ (60 to 100%). It indicated that, the protein has the phosphatase activity and is located in the cell wall. Besides the common characteristics of the DING proteins family, it may regulate the fertility changeover in Chinese cabbage TsCMS7311. Esterases can hydrolyze many carbonic ethers and get involved in dissimilation of plant, which is related to phosphorus metabolism and fatty acid metabolism (Zhang et al., 2005). That is to say its activity and quantity predicts the level of phosphorus metabolism and fatty acid metabolism. In normal fertile plants, cell organ and cell membranes are constantly up-dating and reorganizing during the course of megasporocyte development and giving rise to tetrads of megaspores by meiosis. Phosphorus metabolism and fatty acid metabolism are quite active; therefore esterases have high activity and diversity. Whereas, in sterile plants, with weak activity and few types of esterase, the cell membrane renewal and reorganization might be hampered, thus the formation of megasporocytes is blocked, and finally normal pollen grains cannot be produced. It suggested that the esterases which short sequence obtained here might regulate the expression of TsCMS7311 fertility by affecting the build-up and functions of megasporocyte membranes.

The result of Q-TOF mass spectrometry indicated that the two single polypeptides with molecular weight 57.1 and 62 kD were very similar in the sequence of amino acids detected. These two polypeptides also have the same pI (isoelectric point) and similar molecular weights. It is known that esterases are generally dimmeric-enzyme composed by two subunits. Whether the two polypeptides come from different esterases or the different subunits of the same esterase is not clear. However, all the signs indicate that the subunits of β-esterase isoenzymes not only have similar function but also have the similar amino acid compositions.

However, the present study is just a start. Extra efforts will be needed to fully understand the real mechanism and amino acid sequence of entire polypeptide. Future tasks should involve designing primers according to the three short peptide sequences to reverse transcriptase-polymerase chain reaction (RT-PCR) and combining them with the RACE (rapid amplification of cDNA ends) technology to obtain the full-length sequence of the aimed polypeptide and to confirm its function by RNAi or transgenic technology.

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