Subcellular Localization of BoaZDS Gene in Chinese kale

Qiao Yuan¹, a, Wenli Huang¹, b, Yue Jian¹, c, Hao Zheng¹, d, Min Jiang¹, e, Jiaqi Chang², f, Fen Zhang¹, g, and Bo Sun¹, *

¹College of Horticulture, Sichuan Agricultural University, Chengdu, China
²College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, China

*Corresponding author e-mail: bsun@sicau.edu.cn, a1729759838@qq.com, b1871850825@qq.com, c2523865696@qq.com, d2673468696@qq.com, e1754425081@qq.com, f627018572@qq.com, gzhangf_12@163.com

Abstract. The experiment used the 'Sijicutiao' mustard sterile seedlings as the material, firstly constructed the BoaZDS subcellular localization vector with the cloned BoaZDS gene, and extracted the plasmid, then separated the Chinese kale leaf protoplast by enzymatic hydrolysis. After purification, the BoaZDS gene was transiently transformed, and then the position of the BoaZDS gene was observed under a fluorescence microscope. The results showed that the BoaZDS gene was localized in the chloroplast, which was consistent with the predicted results of bioinformatics software. The results provide a basis for studying the function of the ZDS gene in Chinese kale.

1. Introduction
Chinese kale (Brassica oleracea var. alboglabra) is a biennial herb of the genus Brassica, native to China [1]. It mainly uses flower buds and young leaves as edible organs, and is widely cultivated in Guangdong, Fujian and other provinces [2]. Because it is rich in vitamin C, carotenoids, glucosinolates, chlorophyll and total phenols and other nutrients and is loved [3].

Z-carotene desaturase (ZDS) is an important enzyme in the biosynthesis of carotenoids, catalyzing 7, 9, 9'-tetra-cis-lycopene to form 7, 9, 7', 9'-tetra-s-lycopene. The gene encoding the ZDS protein has been isolated in a variety of plants, including Arabidopsis, tomato and strawberry. Overexpression of the ZDS gene in tobacco resulted in an increase in the accumulation of β-carotene on flowers and leaves respectively by 49% and 91% [4]. After rice ZDS gene deficiency, carotenoid biosynthesis is impaired, which can cause photo oxidation and ABA-deficient phenotype. The ABA content in the mutant is lower than that of wild type, and it causes panicle germination and whitening phenotype [5].

Plant protoplasts refer to naked cells with viability that are removed from the cell wall and surrounded only by the plasma membrane [6], which have pluripotency, can dedifferentiate under appropriate culture conditions, and then proliferate or regenerate various organs. The protoplasts that remove cell walls have the characteristics that are not found in common plant cells: (1) Protoplasts can directly ingest foreign DNA, plasmids, viruses, organelles, etc., and are ideal receptor materials for genetic transformation [7]; (2) Using Prokaryotic expression of protoplasts can be used to study subcellular localization of proteins, gene and promoter activities, protein-protein interactions and signal transduction [8]; (3) Using protoplast fusion techniques to induce different species protoplast can break the distant hybrid incompatibility disorder and achieve distant genetic recombination [9].
2. Materials and methods

2.1. Plant materials
'Sijicutiao' is the Chinese kale variety used.

2.2. BoaZDS subcellular localization prediction in Chinese kale
The WoLF PSORT software was used to predict the subcellular localization of BoaZDS. The WoLF PSORT was based on sorting signal, amino acid composition and functional motifs to convert the protein amino acid sequence into numerical localization features, which were then used for prediction.

2.3. Subcellular localization vector construction
The obtained BoaZDS gene sequence and the green fluorescent protein-containing expression vector pC2300-35S-eGPF sequence were subjected to restriction enzyme digestion analysis, and restriction endonucleases BamHI and SalI were selected. The primer BoaZDS-GFP containing the restriction site was designed. PCR amplification was carried out with high-fidelity enzyme, and the vector pC2300-35S-eGPF and the amplified product were double-digested with BamHI and SalI, respectively, and purified by a gel recovery kit. The C-terminus of the BoaZDS sequence was separately the eGFP of pC2300-35S-eGFP was fused with T4 DNA ligase to obtain the recombinant plasmid pC2300-35S-BoaZDS-eGFP, and the recombinant plasmid was identified by double enzyme digestion, bacterial liquid PCR detection and sequencing.

2.4. Plasmid extraction
Prepare plasmid DNA by medium-sized SDS alkaline lysis method. The specific steps are as follows:
Centrifuge 20 ml of the bacterial fluid at 2000 xg for 10 min at room temperature, pour off the supernatant; add 200 μl of alkaline lysate I, mix by shaking; add 400 μl of alkaline lysate II (currently available). Quickly and gently invert the top and bottom 5 times and place on ice; add 300 μl of ice-cold alkaline lysate III, gently invert 5 times, place on ice for 4 min; centrifuge at 13,000 rpm for 5 min at 4°C, take 700 ml supernatant to a new centrifuge tube; add an equal volume of phenol: chloroform (25:24), shake vigorously and shake, centrifuge at 13000 rpm for 2 min at 4°C; take 600 μl of supernatant in a new centrifuge tube, add Equal volume of isopropanol and mix, stand at room temperature for 2 min, centrifuge at room temperature 13000 rpm for 5 min; discard the supernatant, retain the nucleic acid precipitate, wash the pellet 2-3 times with 75% ethanol; blot dry alcohol, dry at room temperature 10-15 Min; dissolve the nucleic acid with RNase-containing TE and store at-20°C until use.

2.5. Protoplast preparation
The isolation and purification process of Chinese kale protoplasts was carried out according to the method of Sun et al. [10]. Take the Chinese kale mustard green leaves and cut into filaments of 1-2 mm for 9 h with 2.0% (w/v) cellulase and 0.1% (w/v) pectolase in a solution containing 0.6 M mannitol. The optimal nylon membrane mesh and centrifugal speed were 400 mesh and 179 ×g. The obtained protoplasts can be used in the next transformation experiment.

2.6. Protoplast transformation
Following a slight adjustment of the Arabidopsis thaliana protoplast transformation, the recombinant plasmid pC2300-35S-BoaZDS-eGFP and the empty pC2300-35S-eGFP were transformed into Chinese chrysanthemum protoplasts. The specific steps are as follows:
The isolated protoplasts were placed on ice for 30 min. After they settled, most of the supernatant was discarded, and an appropriate volume of MMG solution was added to a specific concentration (106 cells/ml); 100 μl of protoplasts was taken to 2 ml centrifuge tube. Add 10 μg of the transformed plasmid to the bottom of centrifuge tube and mix gently; add an equal volume of pEG-4000 transfection solution, mix gently and let stand for 10 min at room temperature; add 700 μl W5 solution,
upside down mix well, centrifuge at 100 ×g for 2 min at room temperature; aspirate most of the supernatant, leave 50-100 μl of supernatant, and suspend the protoplasts; add new WI solution to 1 ml, and let it react at room temperature and dark for 24 h.

2.7. Subcellular localization
Protoplasts expressing the GFP fusion protein were observed under a fluorescence microscope, and images were captured using an Olympus BX51 fluorescence microscope equipped with a DP70 camera (Olympus, Japan), photographed and recorded.

3. Results

3.1. BoaZDS subcellular localization prediction in Chinese kale
The subcellular localization software WoLF PSORT predicts that BoaZDS may be localized on the chloroplast.

3.2. Construction of BoaZDS subcellular localization vector
The successfully constructed Chinese kale BoaZDS subcellular localization vector (Fig. 1) was subjected to plasmid extraction, and the obtained plasmid was subjected to electrophoresis (Fig. 2), which was consistent with the expected sequence length and was used for subsequent experiments.

![Figure 1. Schematic diagram of the vector construction of the pC2300-35S-BoaZDS-eGFP](image1)

![Figure 2. Electrophoresis detection of the BoaZDS subcellular localization vector](image2)

3.3. Separation and purification of Chinese kale protoplasts
The protoplasts after overnight enzymatic hydrolysis were isolated and purified to obtain a protoplast density of (10⁶ cells/ml), which can be used for subsequent protoplast transformation experiments.
Figure 3. The isolated and purified leaf mesophyll protoplasts of Chinese kale

3.4. BoaZDS subcellular localization in Chinese kale
The subcellular mapping software WoLF PSORT was used to predict the mapping of Chinese kale chinensis to chloroplasts. To further investigate the subcellular localization of Chinese kale, the constructed pC2300-35S-BoaZDS-eGFP recombinant plasmid and pC2300-35S-eGFP were transfected into the protoplasts of Chinese kale. The transient expression of BoaZDS in protoplasts was observed by fluorescence microscopy. Figure 4 shows that BoaZDS only detected clear GFP fluorescence signals on chloroplasts, but no GFP signal was found in other parts of protoplasts (Fig. 4). This result is consistent with the software predictions, demonstrating that the BoaZDS is specifically localized on the chloroplast.

Figure 4. Subcellular localization of BoaZDS
A: Transient expression of BoaZDS-GFP fusion protein in Chinese kale protoplasts;
B: Transient expression of GFP in Chinese kale protoplasts; Bar= 30μm

4. Discussion
At present, methods for protein subcellular localization include: fusion reporter gene localization (commonly used reporter genes including GFP, RFP, YFP, BFP, GUS, etc.), immunohistochemical localization, proteomic localization technology, etc. [11]. In this experiment, we first predicted that BoaZDS was located on the chloroplast of Chinese kale; then, the target gene BoaZDS was fused with the reporter gene eGFP to construct a fusion gene expression vector, which was expressed in the Chinese kale protoplast. It is further proved that BoaZDS is located on the chloroplast of Chinese kale.
This result speculates that the *BoaZDS* gene is involved in plant photosynthesis and may have protective effects on chloroplasts.

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