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Research article

The chloroplast localization of protease mediated by the potato rbcS signal peptide and its improvement for construction of photorespiratory bypasses

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Abstract

Location of the proteases would affect on protease stability and photorespiratory bypass pathway, while it is unsolved. Potato rbcS signal peptide was analyzed and constructed into the protease for study of their localization site. The tartronate semialdehyde reductase (EcTSR) proteins could be accurately and efficiently located in chloroplast only when this signal peptide was extended to 80 amino acids. The signal peptide would help malate synthase (CmMS) locate to the surface of chloroplast, to form granules on the outer membrane of chloroplast. The whole spectrum scanning showed that these proteins could enter chloroplast. A signal peptide named PCS1 (Peptide of self-cleavage site 1) carrying a self-cleavage site was designed, and sixteen amino acids from the blue pigment precursor protein of chloroplast positioning signal of *Silene pratensis* were added to the C-terminal of PCS1. Transient expression, Western blot analysis and full-spectrum scanning showed that PCS1 could locate the EcTSR to the chloroplast, after the removal of the signal peptide.

Keywords: chloroplast localization; chloroplast signal peptide; photorespiratory bypass

Introduction

Photorespiration may result in a 25% loss of photosynthates in C3 plants under normal conditions, the value will be even higher if stress conditions, such as drought, heat and high light, are encountered by the plant (Peterhansel and Maurino, 2011). In 2007, bacterial glycolic acid pathways were introduced into chloroplasts to construct the first photorespiration pathway called the Kebeish pathway (Kebeish *et al.*, 2007). Subsequently, glycolate oxidase and malate synthase were located in chloroplast to build the second branch (Maier *et al.*, 2012). By these ways, 75% of glycolic acid produced by photorespiration is transformed into the Calvin cycle.

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The photorespiration bypasses have adverse effects on H_2O_2 signaling (Rojas and Mysore, 2012; Zhang *et al*., 2016), amino acid metabolism and carbon production in plants (Dalal *et al*., 2015; Novitskaya *et al*., 2002). Some studies suggested that the CO2 conductance of chloroplast membrane is a key factor in the effectiveness of photorespiration (Xin *et al*., 2015). Despite these problems, these pathways have attractive prospects for increasing biomass and reducing CO2 deficiency and excess energy damage in stresses such as high temperature and drought. In 2015, Dalal achieved Kebeish branch on *Camelina sativa* to promote its growth and seed yield (Dalal *et al*., 2015). In 2019, the fourth bypass was successfully established in rice chloroplasts using a multi-gene assembly and transformation system (Shen *et al*., 2019).

The first kind of enzymes which were used to construct chloroplast photorespiration metabolic pathway was prokaryotic enzymes, including bacterial GLC (glycolate dehydrogenase), GCL (acetaldehyde polymerase), TSR (hydroxymalonate semi-aldehyde reductase), and CAT (catalase), all of them don't contain subcellular localization signal. The second kind of enzymes are eukaryotic enzymes, including GLO (glycolate oxidase) and MS (malic acid synthase), them are located in peroxidase and glyoxylate circulator respectively. These two types of enzymes, which are required for the construction of photorespiration metabolic pathways, were introduced into chloroplasts via chloroplast localization signal peptides. It is generally believed that chloroplast signal peptide is the strongest signal peptide with the ability of subcellular localization.

Chloroplast localization signal peptides usually contain a large number of hydroxyl amino acid residues (e.g. serine, threonine, proline) and are lack of acidic amino acid residues such as Aspartic acid and Glutamic acid (von Heijne *et al*., 1989). In addition, these signal peptides tend to form hydrophobic alpha-helix structures (Bruce, 2000). The length and primary structure of signal peptides are also diverse (Bruce, 2001). The rbcS chloroplast localization signal peptides of potato were first identified in 1988 (Wolter *et al*., 1988), and have been used for research of Nolke and Houdelet (2014).

Some failure example of location via chloroplast signal peptide would happen, for example, Lubben found that Brom Mosaic virus envelope protein was located on the chloroplast by pea rbcS signal peptide, and its efficiency was only 2.3% (Lubben *et al*., 1989). Byeon found that the rice n-acetyltransferase signal peptide could not locate n-acetyltransferase derived from sheep into the rice chloroplast (Byeon *et al*., 2014).

As an important food crop, the construction of photorespiration metabolic pathway has great potential to improve potato yield. Although it has been reported that enzymes related to photorespiration metabolic pathways e.g., EcGCL, EcTSR, EcCAT, CmMS, and OsGLO3 can be imported into rice or Arabidopsis chloroplast by rbcS signal peptides (Kebeish *et al*., 2007; Maier *et al*., 2012; Shen *et al*., 2019), these enzymes were never tested in potato. In early study, we attempted to introduce enzymes, which were constructed for the photorespiration metabolic pathway, into the potato chloroplast through the chloroplast localization signal peptide. We found that some genes were highly expressed at the mRNA level but the protein level was low. If a key enzyme for metabolic pathway cannot complete its localization in chloroplast or has low efficiency of transportation and cannot obtain its appropriate enzyme activity in the chloroplast, the accumulation of intermediate products would be harmful to the chloroplast.

So, we analyzed the chloroplast localization of these proteases required for the construction of photorespiration pathways. We designed two cutting sites that were consisted of 20 amino acid residues respectively, and these cutting sites was separately added into the C-terminal of the chloroplast localization signal peptides of the potato rbcS gene to construct new signal peptides, and respectively named PCS1 (Peptide of self-cleavage site 1) and PCS2 (Peptide of self-cleavage site 2). Among them, PCS1 contains 16 amino acid residues from the plasmid precursor protein (P07030) of *Silene pratensis*, and its sequence is TVKVAVATPRMSIKAS (Richter and Lamppa, 1998). PCS2 contains 17 amino acid residues from the rbcS protein of *Oryza sativa*. Western blot analysis and full-spectrum scanning showed that PCS1 could locate protease to the chloroplast, after the removal of the signal peptide. PCS1 has potential applications in the construction of photorespiration metabolic pathways and chloroplast metabolic engineering.

Materials and Methods

Reform plasmid pBI121 and constructs the tobacco instantaneous expression vector

Using *Sma*I and *Sac*I to remove GUS gene from original vector pBI121 gi19569229, replace with amplification of eGFP gene gi1721719622 construct carrier pBI-eGFP. Connected to target genes between 35s promoter and eGFP gene, the target gene expression and eGFP genes constitute a box, expression of target genes and eGFP fusion protein.

Building of vectors for protein expression mediated by rbcS chloroplast localization signal peptide (58AA)

Amplified potato rbcS chloroplast localization signal peptide (StTP-58AA gi21562 174bp) sequence and target gene EcGCL, EcTSR, EcCAT (gi49175990), CmMS (hm755991), and OsGLO3 (nc029259). By connecting the pBI-eGFP, pStTP-58AA-EcTSR-eGFP, pStTP-58AA-EcCAT-eGFP, pStTP-58AA-OsGLO3-eGFP, pStTP-58AA-CmMS-eGFP, pStTP-58AA-EcGCL-eGFP were constructed. Details of plasmid structure is presented in Figure 1.

Building of vectors for protein expression mediated by extended rbcS chloroplast localization signal peptide prolongs

The length of 80 amino acids (StTP-80AA gi21562 240bp) and eGFP constituted fusion protein, and its localization in tobacco cells was observed by instantaneous expression. Amplified the encoding gene sequence of StTP-80AA and connected to pBI-eGFP, express fusion protein StTP-80AA-eGFP, this vector called pBIL-eGFP. pBIL-eGFP have one multiple enzyme cutting sites (*Xho*I-*Sal*I-*Kpn*I-*Hpa*I-*Sma*I) which could detect the effect of signal peptides extending to 80 amino acids. It was also advantageous to insert the detection gene. EcTSR, CmMS and eGFP were fused by StTP-80AA, and their localization in tobacco cells was observed by transient expression. The EcTSR gene and StTP-80AA were connected to the pBI-eGFP construction vector pStTP-80AA-EcTSR-eGFP. The CmMS gene and StTP-80AA were connected to the pBI-eGFP construction vector pStTP-80AA-CmMS-eGFP (more details of plasmid structure - Figure 1).

Building of vectors for protein expression mediated by CTPs with self-cutting site

Amplified PCS1 and PCS2 sequences, were added to vector pBI-eGFP, the pStTp-PCS1-eGFP and pStTp-PCS2-eGFP were constructed. The original signal peptide DNA sequence of pStTP-80AA-EcTSR-eGFP was replaced by PCS1 and PCS2 sequence, and pStTP-PCS1-EcTSR-eGFP and pStTP-PCS2-EcTSR-eGFP were constructed (Figure 2 - more details of plasmid structure). See accessory for PCS1 and PCS2 sequence.

Fusion protein instantaneous expression and Western blot analysis

All transient expression vectors that were correctly sequenced were transformed by freezing-thawing method to convert root cancer agrobacterium strain LBA4404. The tobacco aseptic seedling leaves were cut and placed in a Petri dish. The back was dripped into the right amount of bacteria, with a glass syringe suction bacteria liquid penetration blade to wound parent, placed in a Petri dish with a wet filter paper. Covered the Petri dish lid, and avoided light culture for 48h-96h.

Results

80 amino acids of Potato chloroplast localization signal peptides was needed to sufficiently localize EcTSR protein into tobacco chloroplasts

The 58 amino acids of potato rbcS （StTP-58AA） contains information for positioning foreign proteins to the tobacco chloroplast, it could locate EcGCL, OsGLO3, EcCAT in our study (Figure 1a-c).

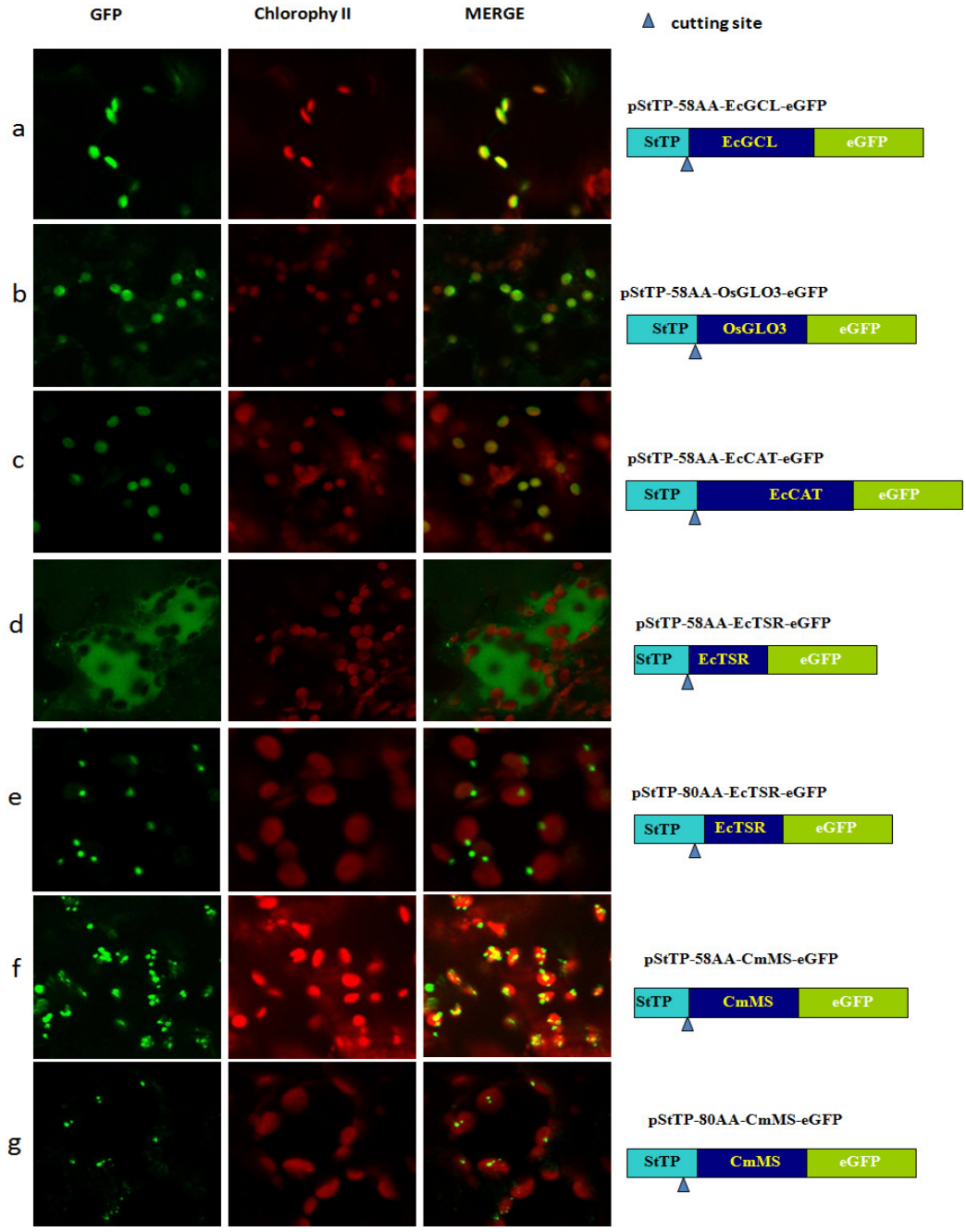

Figure 1. Chloroplast localization of EcGCL, OsGLO3, EcCAT, EcTSR, CmMS mediated by transport peptides of potato rbcS. StTP Chloroplast transport peptides of potato rbcS, OsGLO3 Rice Glycolate oxidase 3, EcGCL *E. coli* Glyoxylate carboligase, EcCAT *E. coli* Catalase, EcTSR *E. coli* hydroxymalonate semi-aldehyde reductase, CmMS cucumber malate synthase

StTP-58AA could not localize EcTSR into the tobacco chloroplasts, and GFP fluorescence was distributed in the whole cell, and its fluorescence could not overlap with chloroplast auto-fluorescence (Figure 1d) (Shen *et al*., 2017). Potato rbcS chloroplast positioning signal peptide was extended to 80 amino acids (StTP-80AA), GFP fluorescence crowded around the chloroplast granuli form and suggesting that the EcTSR chloroplast localization is improved (Figure 1e). CmMS protein was fused with 58 amino acids and 80 amino acid chloroplast localization signal peptides respectively, both of them showed that these proteins were clustered around the chloroplasts (Figure 1f-g).

Signal peptide PCS1 can locate protein and self-cut

PCS1 and PCS2 contain two cutting sites, one cutting site of the potato rbcS signaling peptide was near 58 amino acids, and the other was the additional shearing site. Transient expression indicates that PCS1 and PCS2 was able to well localize EcTSR into tobacco chloroplast, the same as that of the signal peptide of 80 amino acids, all of them located at chloroplast and concentrated in the outer membrane of chloroplast (Figure 2).

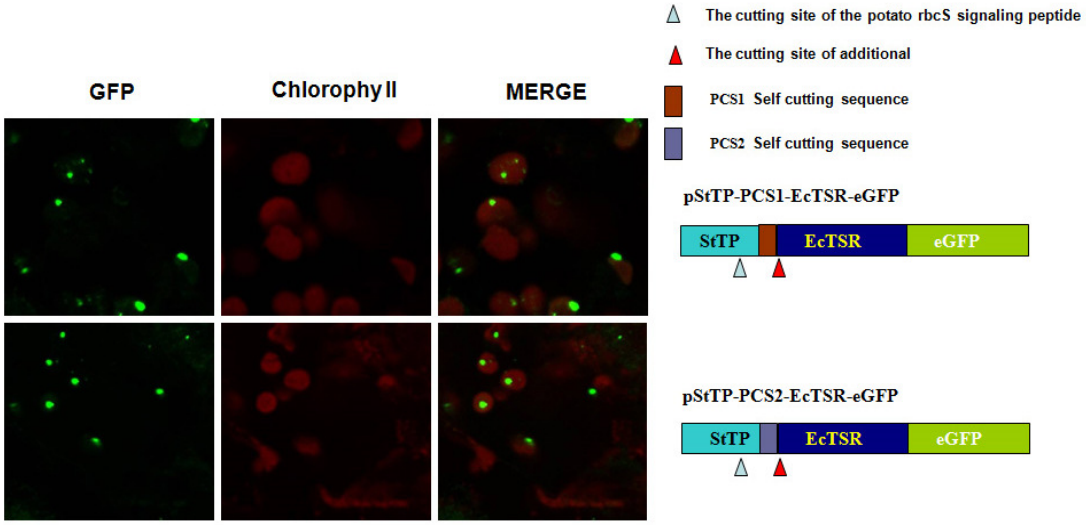

Figure 2. Chloroplast localization of proteins mediated by rbcS transport peptides with Self-cutting sites.StTP chloroplast transport peptides of potato rbcS; EcTSR Tartronate semialdehyde reductase

The protein of transient expression was analyzed, and the pBI-eGFP was used as the reference. The results showed that the 42 amino acids that PCS1 increased compared with StTP-58AA were excised, while the 42 amino acids of PCS2 were not removed (Figure 3a).

The EcTSR protein was mediated by a signal peptide of 58 amino acids. could detect 2 hybridization signals near 60-70kD (Figure 3b). the second detection band was indicated the signal peptide about 6kD was removed. The EcTSR protein was mediated by 80 amino acids, only had 1 protein hybridization signal, and the size was the same as the EcTSR-eGFP protein, and the fusion of signal peptide cutting efficiency was improved.

The EcTSR protein was mediated by PCS1, the signal peptide was removed. The EcTSR protein was mediated by PCS2, the signal peptide was not removed. It can be seen from the above results that the self-cutting site introduced in PCS1 completes the cutting, while the self-cutting site of PCS2 fails to complete the cutting.

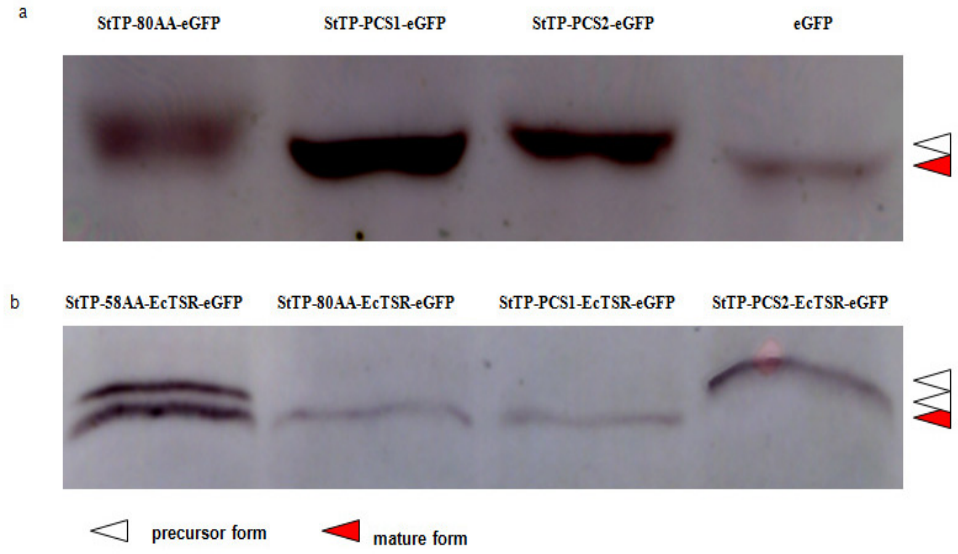

Figure 3. Western blot of the fusion protein with self-cleavage sites. StTP Chloroplast transport peptides of potato rbcS; EcTSR Tartronate semialdehyde reductase, PCS1 Peptide of self-cleavage site 1, PCS2 Peptide of self-cleavage site 2

Full spectrum scanning analysis confirm PCS1-EcTSR-eGFP enter tobacco chloroplast

The subcellular localization of fusion protein StTP-PCS1-EcTSR-eGFP in tobacco leaves was analyzed by using the Zeiss 780/7live laser scanning confocal microscope. As shown in Figure 4, three points in a chloroplast were selected to perform full spectrum scanning, the three points were selected from GFP signals the strongest area 1 (red dots), central area of observation 2 (light green) and edge area 3 (dark blue) and they were analyzed with three areas of 400-700 nm spectral data. The results showed that the peaks of 670-690 nm (ChlorophyII) were basically the same, indicating that all three regions were in the chloroplast. In the vicinity of 509 nm (GFP), area 2 had a high peak value, whose value was weaker than that of region 1, and the observed edge region 3 had no peak at 509 nm. The results showed that fusion proteins entered the chloroplast, and the GFP fluorescence signal was covered by the GFP fluorescence signal of the fusion protein.

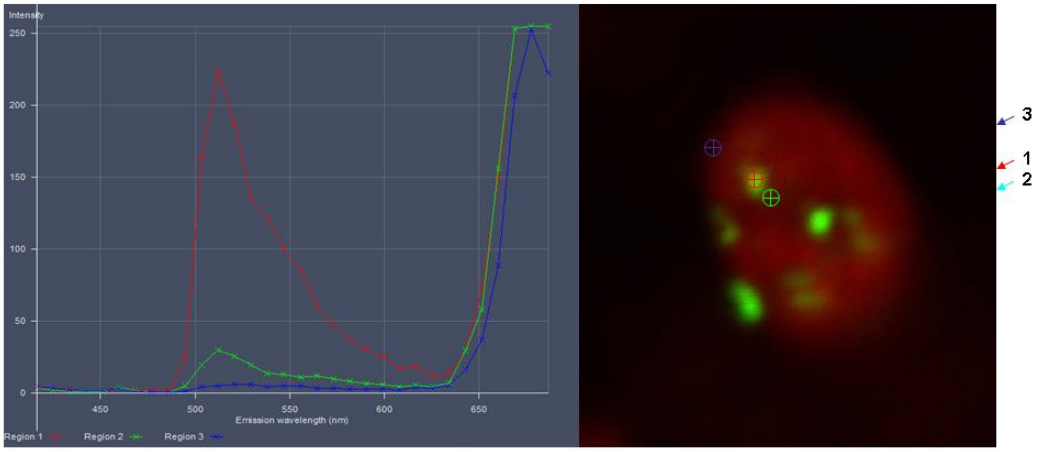

Figure 4. Full spectrum scanning of StTP-PCS1-EcTSR-eGFP fusion protein transient expressed by tobacco leaves

Discussion

It is generally believed that plant plasmids that originate from symbiotic cyanobacteria in cells are semi-autonomous organelles and they always encode 100-300 genes (Bruce, 2000). While, the rest of more than 3000 genes would be encoded and expressed through nuclear genome and all these kinds of protein were transported to chloroplasts by signal peptides to perform their functions (Bruce, 2000). These genes are first translated into precursor proteins in the cytoplasm and transported to the chloroplasts via their N-terminal chloroplast signal peptide (Jarvis, 2008). Chloroplast transport of precursor proteins are divided into three stages: First these proteins were transported to chloroplast outer membrane through cytokines, next the proteins enter into chloroplast stroma by transmembrane transportation, then signal peptides are removed and folded into mature protein (Lee *et al*., 2013).

Potato rbcS signal peptides could not localize EcTSR into chloroplasts or had very low localization efficiency. EPSP from Salmonella typhimurium could only be localized to tobacco chloroplasts when 24 amino acids in the mature region of rbcS were added to pea rbcS signal peptides (Comai *et al*., 1988). Bionda *et al*. (2010) reported that chloroplast signaling peptide with a length of more than 60 amino acids (including the sequence behind the cleavage site) are the most suitable. When the target protein is easy to fold tightly, too short signaling peptides may decrease localization efficiency. The possible reason is that the interaction between signaling peptides and transporters (such as Hsp70) requires a certain space (Bionda *et al*., 2010). According to the literature mentioned above, we extended sequence of the potato rbcS to 80 amino acids in this study. The results proved that the efficiency of localization of EcTSR was significantly improved, which was consistent with the above reports, indicating that the 20 amino acids behind the rbcS cleavage site of potato played a key role in the chloroplast localization of protein EcTSR.

Potato rbcS signal peptide has different localization efficiency for different proteins, and there is no localization, which may be related to the folding speed and strength of the target protein. Similar problems appeared in EcTSR and EcGCL when they located into the chloroplast with the rice rbcS signaling peptide (rCTP). The tertiary structure of EcTSR, rather than the specific amino acid sequence or secondary structure, affects the chloroplast localization function of rCTP, and this effect depends on the degree of proximity between them (Shen *et al*., 2017). This study suggests that whether the length of the N-terminal unfolded region of exogenous protein or the rbcS protein itself would affect chloroplast localization in rCTP mediated chloroplast localization. When the length of the unfolded region is less than 20 amino acids, the efficiency of chloroplast localization decreases.

However, in tobacco research, the tertiary structure of EcTSR may also affect the chloroplast localization function of potato rbcS signal peptide, but the difficulty in locating chloroplasts by EcGCL protein didn't arise. This may be because the localization efficiency depends on both signal peptide and localization protein. This phenomenon deserves further study.

It is noteworthy that after the potato rbcS signal peptide was prolonged, the GFP fluorescence of EcTSR and CmMS which aggregated on the chloroplast outer membrane in granular form could be observed with fluorescence microscopy. In this case, Bionda *et al*. (2010) and other studies have similar reports, and believe that the protein aggregation in the chloroplast outer membrane may be due to its transmembrane efficiency (Lee *et al*., 2006; Bionda *et al*., 2010).

Traditionally, chloroplast proteins remain unfolded with the help of molecular chaperones till they finish transmembrane and folded into functional mature proteins in chloroplasts (Bruce, 2000). Ruprecht *et al*. (2010) pointed out that chloroplast proteins might fold immediately after translation. Folded proteins are firstly transported to the outer membrane surface of chloroplast, then restored to their linear unfolded state by molecular chaperones, and then folded again after transmembrane entry into the chloroplast (Ruprecht *et al*., 2010). According to this view, we believe that the slow recovery of linear state of the CmMS, EcTSR and other proteins may be due to their tight folding when they were transported to the chloroplast outer membrane, thus

affecting the transmembrane efficiency, then resulting in its aggregation in the chloroplast outer membrane. Full spectrum scanning analysis showed that these proteins could enter chloroplasts.

The second problem of protein chloroplast localization is signal peptide cleavage. Chloroplast transport signal peptides are cleaved by chloroplast matrix processing peptidase SPP, while protein transports to thylakoids require thylakoid processing peptidase cleavage (Teixeira and Glaser, 2013). The cleavage and degradation of signal peptides were studied in detail by Richter and Lamppa (2002). They suggested that the cleavage of signal peptides of iron redox protein precursors was determined by the specific interaction of 12 amino acids at the C-terminal of the signal peptide with SPP (Richter and Lamppa, 2002).

After prolonging the potato signal peptide to 80 amino acids, the chloroplast localization of EcTSR protein was improved, but the N-terminal of mature protein was increased by 20 amino acid residues. The addition of this sequence may affect the enzyme activity and physiological function. After connecting eGFP and EcTSR, the signal peptide PCS1 with cleavage site could locate the protein normally and cleave the signal peptide PCS1. PCS1 can increase the localization efficiency of exogenous proteins, and then completely remove signal peptides.

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Accessory

PCS1 DNA sequence

5'-
ATGGCTTCCTCTGTTATTTCCTCTGCAGCTGTTGCTACACGCACCAATGTTACACAAGC
TGGCAGCATGATTGCACCTTTCACTGGTCTCAAATCTGCTGCTACTTTCCCTGTTTCAA
GGAAGCAAAACCTTGACATCACTTCCATTGCTAGCAATGGTGGAAGAGTTAGGTGCATG
CAGGTATGGCCACCAATTAACATGAAGAAGTACGAGACACTCTCATACCTTCCTGATTT
GACTATGAGCTCAACCGTCAAGGTCGCCGTCGCCACCCCCAGGATGTCAATCAAGGCC
TCCATG-3'

PCS2 DNA sequence

5'-
ATGGCTTCCTCTGTTATTTCCTCTGCAGCTGTTGCTACACGCACCAATGTTACACAAGC
TGGCAGCATGATTGCACCTTTCACTGGTCTCAAATCTGCTGCTACTTTCCCTGTTTCAA
GGAAGCAAAACCTTGACATCACTTCCATTGCTAGCAATGGTGGAAGAGTTAGGTGCATG
CAGGTATGGCCACCAATTAACATGAAGAAGTACGAGACACTCTCATACCTTCCTGATTT
GACTATGAGCTCAGGAAATAGCAGCTTCGGAAATGTGTCCAACGGAGGGAGAATACGAT
GTATG-3'

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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