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研究課題名（和文）Production of recombinant full-length membrane-spanning vacuolar sorting receptor of soybean and complex formation with cargo protein

研究課題名（英文）Production of recombinant full-length membrane-spanning vacuolar sorting receptor of soybean and complex formation with cargo protein

研究代表者

Ｃａｂａｎｏｓ　Ｃｅｒｒｏｎｅ・Ｓａｌａｍａｔ（Cabanos, Cerrone Salamat）

京都大学・農学研究科・准教授

研究者番号：５０８７５４７４

交付決定額（研究期間全体）：（直接経費）　　2,200,000円

研究成果の概要（和文）：酵母を利用して、大豆液胞選別受容体(GmVSR)の組み換えタンパク質の調製を試みた。全長のGmVSR について構造解析に必要な量を精製することが困難であった。次に、リガンド結合に必要なドメインのみ(GmVSRlum)をピキア酵母で発現させた。高い発現レベルを示したが、リガンド結合について検出できる条件を見出すことは困難であった。そこで、ホモロジーモデリングによる3Dモデルを予測し、これに基づいて、GmVSRにおけるR102、F106、D136、E139がリガンドとの相互作用に寄与することを推定した。GmVSRの発現条件のさらなる最適化を行い、これらの残基の重要性について検証する必要がある。

研究成果の学術的意義や社会的意義
The results of this study will serve as a stepping-stone towards elucidating the structure and binding mechanism of VSR to its cargo. The findings of the study will also help expand and transform protein sorting biology and could bring insight into the development of protein-rich crops.

研究成果の概要（英文）：Using codon optimized constructs, I transformed and optimized expression of the full-length soybean vacuolar sorting receptor in yeasts, S. cerevisiae and Pichia pastoris. Although GmVSR in Pichia had higher expression level than Saccharomyces, the yield was very low for use in structural elucidation. I then designed a minimal construct with luminal region only (GmVSRlum) containing essential binding domains. GmVSRlum showed promising yield, however, upon testing for cargo binding, it did not show any binding at all. Instead of 3D determination by X-ray crystallography, I carried out homology modeling via RaptorX and AlphaFold2 to obtain its predicted 3D model and, based on this, propose that residues R102, F106, D136, E139 contribute to soybean VSR-cargo interaction. Here, I was not able to establish the yeast expression system suitable for soybean VSR. In the future, I surmise that mutagenesis in the protein combined with molecular dynamics simulations might help solve this problem.

研究分野：applied agricultural biochemistry

キーワード：sorting receptor　vacuole　seed storage protein　soybean

１．研究開始当初の背景

The seeds of higher plants accumulate large quantities of seed proteins including storage proteins and lectins. These proteins are localized in two different compartments. One is protein bodies (PB) for zeins and prolamins that are directly derived from endoplasmic reticulum (ER) in maize and rice endosperm. Another is protein storage vacuole for globulins and albumins which is a special type of vacuole [Cabanos et al, 2021]. During seed maturation, the precursors of globulins and albumins are synthesized on rough ER and are sorted to protein storage vacuoles (PSV) where they are converted into mature forms and are accumulated. These protein are transported to these vacuoles through targeting signals known as vacuolar sorting determinants (VSDs). Many VSDs for seed proteins have been found in plants. So far, there are 3 types of VSDs that have been discovered: sequence-specific (ssVSDs), C-terminal (ctVSDs), and physical structure/conformation VSDs (psVSDs). The proteins containing the VSDs are in turn recognized and transported to PSV by important proteins known as vacuolar sorting receptors (VSRs). Most studies on VSRs were from *Arabidopsis* and only limited data available on soybean. Soybean VSR is unique in that it has broader specificity for ligands - all type of VSDs exist in its cargo proteins unlike that of *Arabidopsis* VSR (Maruyama et al, 2018). Since soybean VSR bind to all three types of VSDs available, this makes it a good model for plant VSRs. VSRs are type I integral membrane proteins containing a large luminal domain (~550 residues) followed by a single transmembrane domain and a short cytoplasmic tail (~40 residues). The transmembrane domain and cytosolic tail are required for the homomeric interaction of VSR, which is crucial for vacuolar protein sorting. VSRs have 3 main regions: luminal, transmembrane (TMD) and C-terminal domains (CT). The luminal region further contains three domains: a protease-associated (PA) domain at the N terminus, Central domain (CD) in the middle, and three epidermal growth factor repeats (EGF) at the C-terminus (Cabanos et al, 2021). So far, only the PA domain (only ~20% of whole protein) of *Arabidopsis* has a 3D structure and bound to a cargo-peptide (Luo et al, 2014 and Tsao et al, 2022). Although PA domain is responsible for cargo specificity, it is believed that CD also interacts with cargo. The role of the EGF is also not fully understood, but it is hypothesized to induce conformational changes in both PA and CD for them to recognize the cargo and regulate ligand protein-receptor dissociation (Luo et al, 2014, Maruyama et al, 2015). The TMD and CT domains are for targeting the VSR to the vacuoles by interacting with Adapter-protein complex. They key question is this - what are the exact roles of the abovementioned domains in cargo binding?

２．研究の目的

This study seeks to develop a recombinant production system for full-length soybean, *Glycine max*, VSR (GmVSR), perform functional characterization and complex formation with 11S globulin cargo protein. The results of this study will serve as a stepping-stone towards elucidating the structure, either through x-ray crystallography or single particle cryo-electron microscopy. The structures in turn will shed light into the whole binding mechanism at the molecular level, i.e., specific domains involved, of VSRs in soybean.

３．研究の方法

(1) Design of constructs and development of a yeast expression system, using either budding yeast, *Saccharomyces cerevisiae* or methylotrophic yeast, *Pichia pastoris,* for GmVSR.

Full-length soybean VSR was synthetically codon optimized for yeast expression. GmVSR full-length was cloned into pRS426 vector for *Saccharomyces cerevisiae* as well as into pPICZαA and pPICZB for *Pichia pastoris* host. GmVSR tagged at the end part (C-terminus) with flag or flag-polyhistidine tandem tag was inserted into linearized vectors by HiFi Assembly (NEB). Subsequently, truncated GmVSR, containing essential luminal region, was also constructed by cloning into pPICZαA. Transformation into *Saccharomyces* and *Pichia* was done via electroporation. Expression test was performed in synthetic complete media without Uracil followed 24 hr expression induction in the presence of galactose for *Saccharomyces*. For *Pichia pastoris* induction, cell culture was grown in YPD with 0.2mg/mL zeocin, overnight. Cells were then harvested, re-suspended in buffered minimal-glycerol media until it reaches logarithmic phase and induction was done by exchanging media with buffered minimal-methanol. Induction was maintained by addition 0.5% methanol every 24 hours. Expression was continued for ~48-60 hours..

(2) Development of a protocol for GmVSR purification to homogeneity with yield suitable for functional characterization and future structural elucidation.

For intracellularly expressed GmVSR, yeast cell lysis from 10-20g pellet was performed using a Bead-beater (Biospec) in 50mM HEPES Buffer, 0.15M NaCl, 10% glycerol, protease inhibitors. Total protein was extracted with 4% CHAPS from total lysate by mixing for 2 hours at 4ºC. This was then clarified by centrifugation at 10,000xg. Supernatant was diluted with same buffer to a final concentration of 0.1% CHAPS and was mixed with 200µl anti-Flag or nickel Sepharose resin. For secreted GmVSRlum, culture media was harvested and clarified by centrifugation. Protein was captured by binding for 2 hours in rotary mixer at 4ºC. The resin was then harvested and transferred to 5ml tube and washed serially with a total of 15ml of 50mM HEPES Buffer, 0.4M NaCl, 10% glycerol, 0.2% DDM. Finally, GmVSR was eluted with 50mM HEPES Buffer, 0.15M NaCl, 10% glycerol, 0.2% DDM, 0.2mg/ml Flag peptide for anti-flag affinity or 50mM HEPES Buffer, 0.15M NaCl, 10% glycerol, 0.2% DDM, 0.4M imidazole for nickel affinity purification. Size-exclusion chromatography was done using Superose 6 increase 3.2/300 (GE Life Sciences) using the same elution buffer without imidazole.

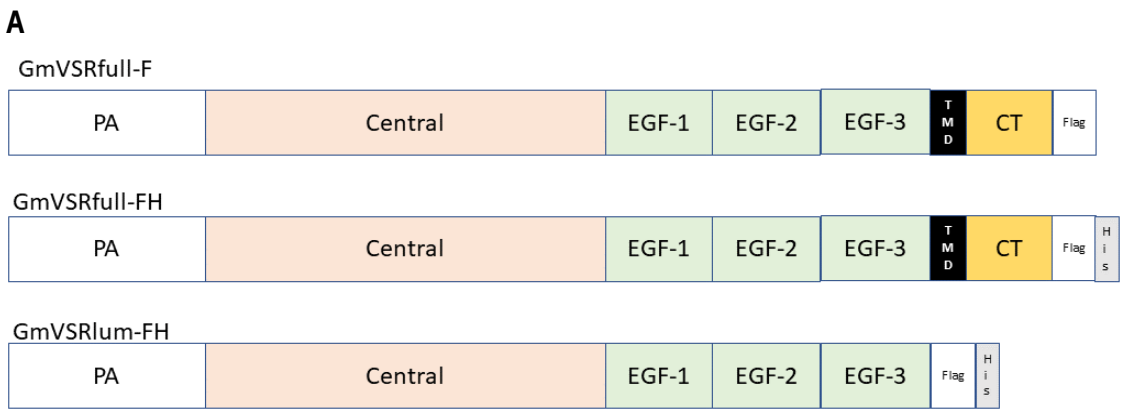
(3) Optimize protocol for complex formation between the purified GmVSR and its known cargo peptide – (recombinant GST-ctVSD peptide).

To facilitate in vitro binding assay, a synthetic recombinant cargo was first constructed by fusing Glutathione S-transferase protein (GST) with a known ctVSD (PLSSILRAFY) from soybean 7S globulin. This was expressed in *E. coli* and purified with GST-affinity purification following manufacturer's protocol. Binding test was done by mixing 20µg of GmVSRlum with ~30-50µg of GTS-ctVSD cargo and incubating for at least 30min on ice. Presence of complex was determined by capturing with 20µl of nickel Sepharose resin for 2 hours in a rotary mixer. Resin was then washed extensively, spun down, and eluted with same buffer as above. Presence of both GmVSRlum and cargo protein bands upon SDS-PAGE analysis means positive binding.

４．研究成果

(1) GmVSR expression in yeasts

Figure 1A shows the comparison of the domains present in full-length and truncated GmVSR that was used in this study. GmVSRfull contains the PA, CD, EGF repeats and CT domains, while GmVSRlum contains only luminal region, comprising the PA, CD and EGF domains. Upon expression test, GmVSRfull was observed to be expressed in *Saccharomyces cerevisiae* but in very low level. No intracellular expression of GmVSRfull and GMVSRlum was observed in *Pichia pastoris* pPICZB constructs. However, a positive and higher expression was observed in *Pichia pastoris* pPICZαA construct. This not surprising since secretion vectors containing α-mating factor signal have been used for some membrane proteins in the past to improve expression and they remain intracellularly due to their membrane localization (Byrne, 2015). I performed medium scale Pichia expression and purification for pPICZαA-GmVSR full-length but yield was very low (20-25ug/0.8 L). I then tested for expression of pPICZαA-GmVSRlum in culture media (no intracellular expression was observed) and was shown to have the high expression (~1-2mg/L culture) (Fig. 1B).



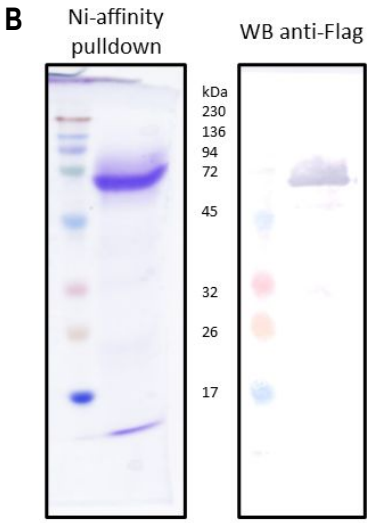


Fig. 1. (A) The GmVSR constructs and their domains with either Flag or Flag-polyhistidine tandem tags. (B) Nickel-affinity pulldown of GmVSRlum expressed in *Pichia pastoris* X-33 showing the Coomassie blue stained protein band and its western blot detection with anti-Flag antibody.

(2) Purification of GmVSR to homogeneity

Briefly, 0.6 L of media from GmVSRlum culture was clarified by centrifugation at 6000 rpm. Media was then manually applied to Nickel Sepharose HisTrap FF (Cytiva) 5ml using syringe. The column was washed 10 times column volume and eluted using the same buffer with 0.4M imidazole. Size-exclusion chromatography was done using Superose 6 increase 3.2/300 (GE Life Sciences) using th same buffer without imidazole. Figure 2 shows the size-exclusion chromatography profile of the protein. GmVSRlum came out as monodispersed peak and appears to be stable even after leaving it for 24hrs at 4ºC based on peak profile (Fig. 2A). The final yield was ~1mg/L culture which is suitable for structural elucidation either by x-ray crystallography or cryo-EM (Fig. 2B).

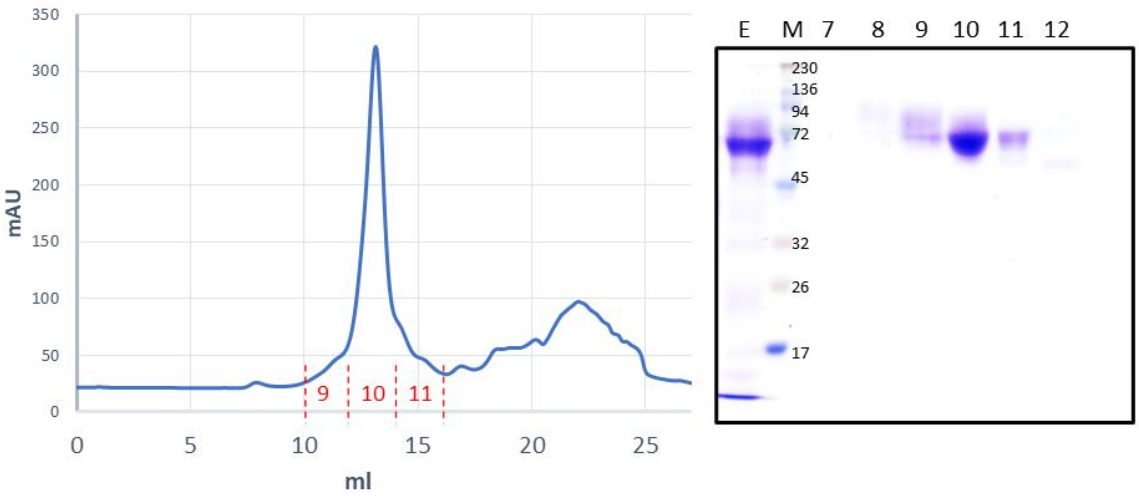


Fig. 2. (A) Size-exclusion chromatography profile of GmVSRlum. (B) SDS-PAGE analysis of the fractions obtained. E, denotes eluate after nickel affinity purification; M, Dynamarker Protein Multicolor III ladder; 7-12, gel filtration fractions.

(3) Complex formation between the purified GmVSR and cargo-peptide (GST-ctVSD)

Complex formation was tested by incubating GmVSRlum with 3-5 fold molar excess of cargo, made from fusing GST protein and known ctVSD peptide, for at least 30min with rotation at 4ºC. However, after several trials, I was not able to show that GmVSRlum binds to cargo (Fig. 3). Due to absence of complex formation and to aid in predicting possible interaction sites between GmVSR and cargo, I carried out structure homology modeling via RaptorX and AlphaFold using default settings to obtain the predicted 3D model for the full-length protein. GmVSR shares a protein sequence identity of approximately 85% with pea vacuolar sorting receptor BP-80 and 69 % with Arabidopsis VSR1. Similar to VSRs from other plants, soybean VSR has the three main regions and has the same sub-domains in the luminal region (Fig. 4A). The structural alignment of the homology model of GmVSR-PA domain with *Arabidopsis* VSR-PA shows the typical seven-stranded barrel domain surrounded by six peripheral helices (Fig. 4B). Based on the key residues for recognition of ctVSD in *Arabidopsis*

VSR (Tsao et al, 2022), I propose that residues R102, F106, D136, E139 of the cargo binding loop of soybean VSR contribute to cargo or ligand interaction (Fig. 4C).

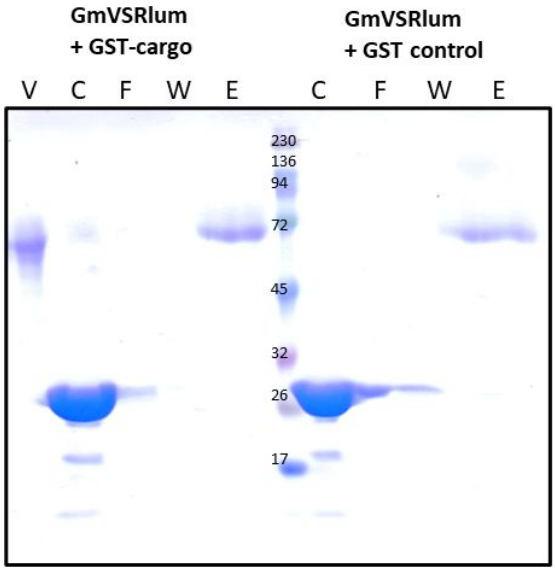


Fig. 3. (A) Absence of binding of GmVSRlum to GST-cargo/stVSD and (B) to GST alone as negative control. V, denotes GmVSRlum input; C, cargo or ligand; F, flow-through; W, wash; and E, as elution fractions.

In this study, I was not able to establish the yeast expression system suitable for a functional soybean VSR. I surmise that in future studies random mutagenesis in combination with molecular dynamics simulations might help solve this problem and increase the affinity of the ligand or cargo protein to the binding pocket.

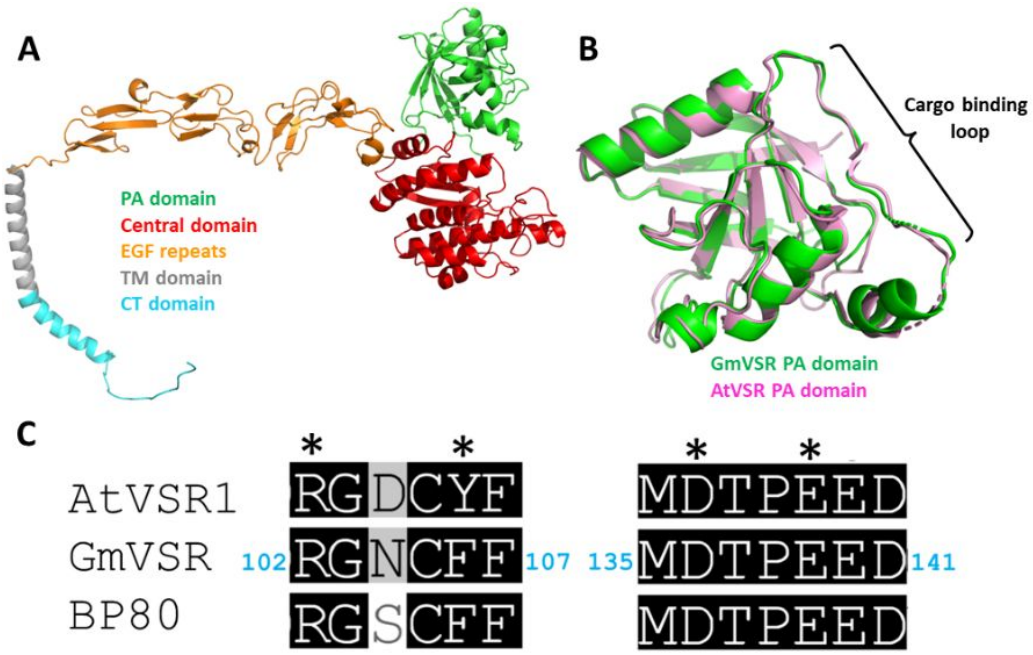


Fig. 4. (A) Homology model of GmVSR. (B) Structural alignment of VSR-PA domains from soybean and Arabidopsis. (C) Putative key residues (asterisks) within cargo-binding loop important for GmVSR-cargo interaction.

＜引用文献＞

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５．主な発表論文等

〔雑誌論文〕　計1件（うち査読付論文　1件／うち国際共著　1件／うちオープンアクセス　0件）

１．著者名 Cabanos Cerrone、Matsuoka Yuki、Maruyama Nobuyuki	４．巻 143
２．論文標題 Soybean proteins/peptides: A review on their importance, biosynthesis, vacuolar sorting, and accumulation in seeds	５．発行年 2021年
３．雑誌名 Peptides	６．最初と最後の頁 170598～170598
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.peptides.2021.170598	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関