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Heterogeneous Expression and Purification of the Wheat VRN1 K-Box Domain Suggest the Formation of a Tetramer of the VRN1 Protein

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Abstract

In cereal species such as wheat (*Ttiticum aestivum*) and barley (*Hordeum vulgare*), many studies have indicated that *VERNALIZATION*1 (*VRN*1) functions as a flowering promoter, which activates florigen gene expression. The wheat florigen gene *Wheat FLOWERING LOCUS T* (*WFT*, which is identical to *VRN*3) is an integrator of the vernalization, photoperiod and autonomous pathways in wheat flowering, and the *WFT* expression is correlated with the *VRN*1 expression. *VRN*1 encodes an APETALA1/FRUITFULL-like MADS-box transcription factor which expression is induced by vernalization, leading to flowering thorough up-regulation of *WFT*. In *Arabidopsis*, it has been reported that protein-protein interactions are keys for MADS-box protein function and MADS-box transcription factors must dimerize to bind to the target gene. In this study, by using gel permeation chromatography (GPC) with purified VRN1 protein, we indicated the possibility that VRN1 protein exists as tetramer-like as flowering homeotic MADS-box proteins in *Arabidopsis*.

Keywords

Flowering, MADS-Box Protein, VRN1, Wheat (*Triticum aestivum*)

1. Introduction

A lot of long-day plant species show an adaptation termed vernalization in which an extended period of low temperature in winter results in the flowering competency during the following spring [1]. Vernalization requirement prevents the plants from transit the reproductive phase before winter when the reproductive

organs would be damaged by cold temperature. Therefore, vernalization requirement is an important adaptive trait for long-day plants which are germinated in autumn. Interestingly, recent studies have revealed that vernalization systems evolved independently in different plant groups [2]. In temperate cereals, such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), three genes have been identified to control the vernalization requirement, namely, *VERNALIZATION* 1 (*VRN*1), *VRN*2 and *VRN*3 [3]. *VRN*1 and *VRN*2 are unrelated to the gene with the same name in *Arabidopsis*.

*VRN*1 encodes an APETALA1/FRUITFULL-like (AP1/FUL-like) MADS-box transcription factor that is up-regulated by vernalization [4] [5] [6] [7]. Historically, as an ortholog of *Arabidopsis* floral MADS-box gene *AP*1 in wheat, *WAP*1 (wheat *AP*1, formerly *TaMADS#*11, DDBJ accession no. AB007504) was cloned by screening a cDNA library from young spikes of common wheat cv. Norin 26 using the degenerate PCR products corresponding to the MADS box region derived from genomic DNA as probes [8] [9]. *WAP*1 has three homoeologous genes located on group 5 homoeologous chromosomes, 5A, 5B and 5D [5]. In barley, the *AP*1 ortholog was firstly reported as *BM*5 [10]. The barley BM5 shares a high degree of predicted amino acid similarity (95%) with WAP1 [6]. Then, it was reported that *VRN*1 located on $5A^m$ chromosome of diploid wheat *T. monococcum* is 98% identical with wheat *WAP*1 [7]. A phylogenetic study of the *AP*1-like MADS box genes indicated that *WAP*1 belongs to one close subclade together with *TaVRT*-1 of wheat [4], *VRN*1 of *T. monococcum* [7], *BM*5 of barley [10] and *LtMADS*1 of *Lolium temulentum* [11], suggesting that they are the same gene and the difference of sequence is due to polymorphism.

The level of *VRN*1 expression is correlated with the level of expression of the wheat florigen gene *Wheat FLOWERING LOCUS T* (*WFT*, which is identical to *VRN*3) [12] [13]. Expression of *VRN*1 gradually increases during the seedling growth stage without vernalization [13], suggesting that the expression of *VRN*1 is also controlled by internal signals such as aging. Furthermore, *VRN*1 is upregulated under a long photoperiod [5] and shows a diurnal expression pattern that is affected by the length of daylight [12] [13]. These observations indicate that *VRN*1 expression is controlled by autonomous and photoperiodic pathways as well as by the vernalization pathway. According to the expression and mutant analysis by our research group, *VRN*1 is thought to up-regulate *WFT* expression [12]. Recently, we demonstrated that VRN1 proteins directly bind to the promoter region of *WFT* gene [14], indicating that VRN1 directly up-regulates *WFT* expression.

In *Arabidopsis*, it has been known that protein-protein interactions are the key for MADS-box protein function, and floral MADS-box proteins function in vivo as part of tetramers or "floral quartets" (dimers of DNA-binding dimers) [15]. The floral quartet model provides a mechanistic explanation for the ABCDE model and proposes that the composition of MADS-box protein tetramers is instructive in determining floral organ identity. Actually, it has been demonstrated that the tetramer formed by SEP3 and the MADS protein, AGAMOUS,

is necessary to activate two target genes, which are required for meristem determinacy [16]. In this study, we revealed the possibility that wheat VRN1 protein exists as tetramer-like as flowering homeotic MADS-box proteins in *Arabidopsis*.

2. Materials and Methods

2.1. Subcloning of VRN1 K-Box

Previously, we cloned a full-length cDNA sequence of *TaMADS#*11 (*VRN-D*1) into the pET30c(+) expression vector plasmid [14]. This plasmid was used as a template to generate K-box (His93 to Glu174) expression vector plasmid. The following PCR reaction conditions were used using KOD-plus-Neo DNA polymerase (Toyobo, Osaka, Japan) and appropriate primers (5'-GGAGATATACATATGCACGAATATAGGAAACTG-3' and 5'-GTGGTGGTGCTCGAGCTCGACGAGTTCCTTCTGG-3'): 2 min at 94˚C, followed by 25 cycles of 10 s at 98˚C, 30 s at 60˚C, and 30 s at 68˚C. The PCR fragment was fused into the multi-cloning site (NdeI-XhoI) of pET21b (Merck Millipore, MA) in-frame with the C-terminal His-Tag coding region using In-Fusion HD Cloning Kit (Takara Bio, Otsu, Japan). This subcloned plasmid was then introduced into *E. coli* BL21-CodonPlus (DE3)-RIL cells.

2.2. Protein Expression and Purification

E. coli cells harboring the plasmid were aerobically precultured at 37˚C in 0.5 L of Luria-Bertani broth. Once turbidity at 660 nm reached 0.5, the culture was supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside, followed by further incubation at 20˚C for 20 h. The cultured cells were collected by centrifugation at 6000 g for 10 min at 4˚C and washed with a buffer containing 20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, and 1 mM phenylmethylsulfonyl fluoride (Buffer A). Cells were then resuspended in the same buffer and ultrasonically disrupted (VCX 750; Sonics & Materials, CT) at 4˚C - 8˚C for 10 min. The cell lysate was centrifuged at 10,000 g for 20 min at 4˚C and the supernatant was collected. The supernatant was applied to a Ni-immobilized metal affinity chromatography column (HisTrap HP column, 5 ml; GE Healthcare, WI) pre-equilibrated with 20 mM imidazole in Buffer A. The column-bound protein was eluted on a linear imidazole gradient (20 to 250 mM) in Buffer A (50 ml). Fractions containing the protein (<15 ml) were concentrated by Amicon Ultra-15 concentrator with a 10,000 molecular weight cutoff membrane (Merck Millipore) to a volume of about 1 - 2 ml. The concentrated fraction was applied to a gel permeation chromatography Superdex 200 10/300 GL column (24 ml; GE Healthcare) pre-equilibrated with 10 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.5, 0.5 M NaCl, 1 mM dithiothreitol (DTT). Fractions containing K-box were collected as the purified protein. Protein concentrations were determined using a Bradford Protein Assay kit (Bio-Rad, CA), with bovine serum albumin as the standard, or by UV spectrophotometry using the molar extinction coefficient ε_{280} =

1490 ($M^{-1}\cdot cm^{-1}$) according to ExPASy ProtParam tool server (http://web.expasy.org/protparam/) [17]. The protein was purified to homogeneity, as determined by 15% sodium lauryl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis followed by Coomassie Brilliant Blue (CBB) R-250 staining.

2.3. Gel Permeation Chromatography

The purified VRN1 K-box was dialyzed at 4˚C overnight against 10 mM 4-(2-hydroxyethyl)piperazine-1-(ethanesulfonic acid) (HEPES), 0.5 M NaCl, 1 mM DTT to remove Tris salt. The protein solution (3.5 mg/ml, 20 µl) was mixed with glutaraldehyde solution (2.5% v/v, 1 µl) or water (1 µl). After incubation at 20˚C for 1 min, the reaction was stopped by adding 2 µl of 1 M Tris, pH 8. The aliquot (10 µl) was mixed with 10 µl of 2 × SDS-PAGE sample loading buffer and heated at 100˚C for 10 min. The samples (10 µl, 15 µg protein) were visualized by SDS-PAGE and CBB R-250 staining. The molecular size of the purified K-box protein was analyzed by gel permeation chromatography (GPC) using the same procedure for the purification method.

3. Results and Discussion

MADS-box proteins are defined by a unique domain structure: in addition to the highly conserved DNA-binding MADS-domain, they have three other domains ("I", "K" and "C"), with the keratin-like K-domain being the most highly conserved and characteristic one [18]. The K-domain mediates protein-protein interaction and is postulated to form several amphipathic a-helices referred to as K1, K2, and K3 [19]. To determine whether wheat VRN1 protein forms multimers, we performed in vitro assay to examine the protein-protein interaction of VRN1 using purified K-box domain. From our previous preliminary experiments, we have found that the VRN1 full-length protein is very unstable for an unknown reason. Therefore, in this study, we used K-box domain which is involved in protein-protein interaction.

At first, we subcloned K-box from a full-length cDNA sequence of *TaMADS#*11 (*VRN-D*1) [9] [14]. Then we constructed *E. coli* protein expression vector (**Figure 1(a)**) which was introduced into *E. coli* cells. Proteins produced by *E. coli* cells were purified and analyzed by sodium lauryl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (**Figure 1(b)**). The SDS-PAGE image indicates purified K-box proteins.

Next, we performed cross-linking assay of the K-box proteins by using glutaraldehyde and SDS-PAGE assay (**Figure 2**). The SDS-PAGE images clearly show monomer, dimer, trimer and tetramer of the K-box proteins. Among these multimers, the dimer was the major product. It is suggested that the K-box forms a dimeric or larger complex. The molecular size of the purified K-box protein was analyzed by gel permeation chromatography (GPC) using the same procedure for the purification method (**Figure 3**, black line). The main peak of the

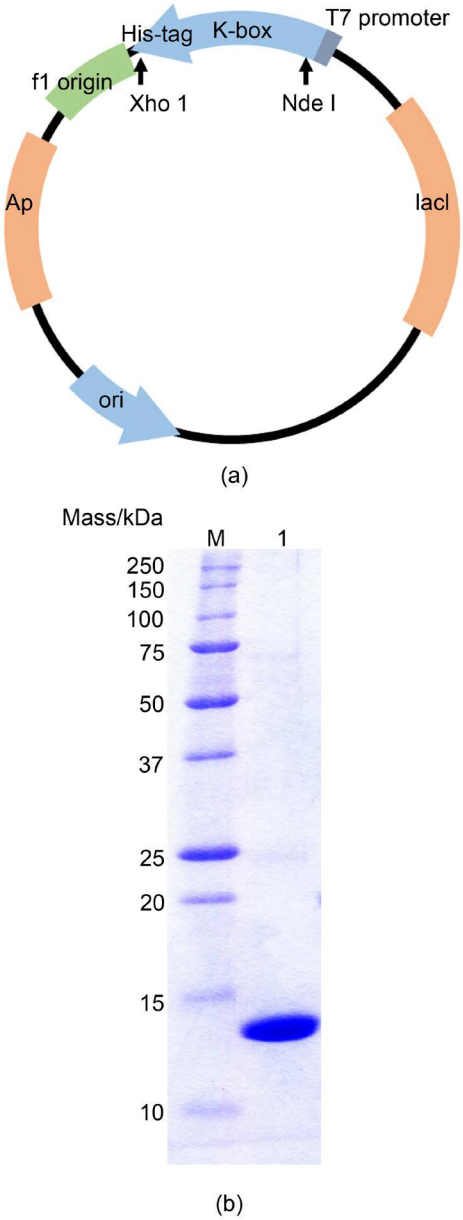

Figure 1. (a) Construction of the expression vector plasmid pET21b with K-box fusion driven by the T7 promoter. A DNA encoding K-box domain of *VRN-D*1 was cloned in-frame into the NdeI-XhoI cloning site. The plasmid has an f1 origin, a pBR322 origin (ori), Ap (ampicillin resistance, *β*-lactamase), and *lacI* gene. (b) SDS-PAGE image in which protein bands were stained with CBB R-250. Lane M, molecular mass standards; lane 1, purified K-box (1 μg). Notice that it shows molecular weight of the VRN1 K-box protein with His-tag.

glutaraldehyde treated (cross-linked) multimeric mixture (**Figure 3**, red line), which was determined to be a dimer by SDS-PAGE, eluted later than the purified K-box protein (before cross-linking). The shape of the dimeric form after cross-linking was suggested to be compact. Assuming that no major conformational change occurred before and after cross-linking, the purified K-box protein is likely to exist in a tetramer formation based on the elution positions of the

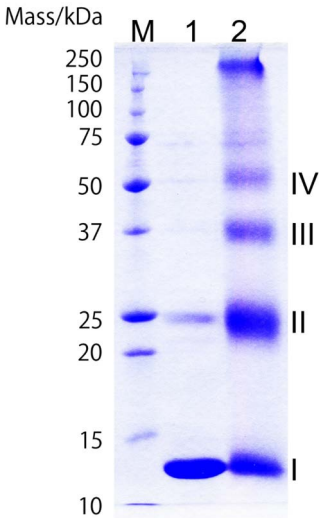

Figure 2. SDS-PAGE images for glutaraldehyde cross-linking experiment. K-box was incubated in the presence of glutaraldehyde (0.12% v/v) to stabilize the possible oligomeric forms of the protein. The cross-linked K-box was analyzed by SDS-PAGE. Lane M, molecular mass standards; lane 1, purified K-box (15 μg, control); lane 2, the cross-linked K-box (15 μg). Roman numerals I-IV correspond to the monomer to tetramer of K-box protein, respectively.

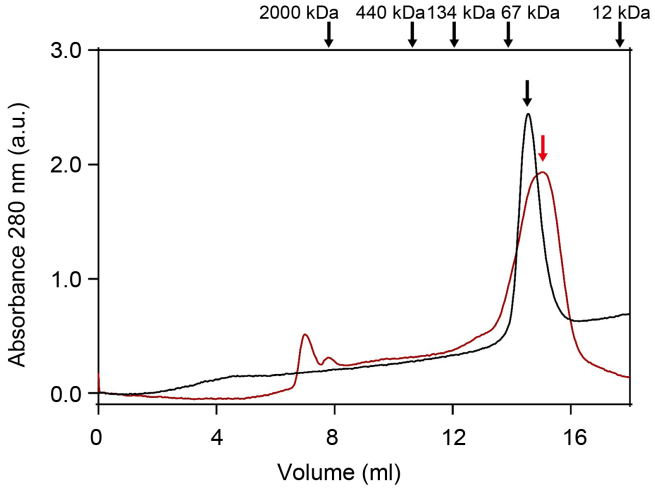

Figure 3. Gel permeation chromatography analysis of purified K-box protein (black line). Red line indicates the glutaraldehyde treated (cross-linked) multimeric mixture. The peaks are shown by black and red arrow, respectively. The elution positions of molecular mass markers are also shown; blue dextran (GE Healthcare), molecular mass = 2000 kDa; horse spleen ferritin, 440 kDa; bovine serum albumin dimer, 134 kDa; monomer, 67 kDa; pigeon cytochrome C, 12 kDa; vitamin B2, 376 Da.

external standards. The elution positions of the external standards (blue dextran (GE Healthcare), molecular mass = 2000 kDa; horse spleen ferritin, 440 kDa; bovine serum albumin dimer, 134 kDa; monomer, 67 kDa; pigeon cytochrome C, 12 kDa; vitamin B2, 376 Da) indicate the purified K-box protein exists as a tetramer formation.

We have been presenting the model in which *VRN*1 directly activates *WFT*

expression since 2009 [12]. In this model, *VRN*1 is upstream of *WFT* and the VRN1 protein directly up-regulates *WFT* expression by binding to the promoter region. This model is consistent with the concept that the level of *VRN*1 expression functions as a threshold for flowering competency in wheat [13]. By using a chromatin immunoprecipitation (ChIP)-seq analysis, VRN1-binding target genes were identified [20]. A total of 289 binding peaks were identified for predicted genes associated with transcribed sequences; one of these was barley *FT*. This result suggests that VRN1 protein binds to the promoter region of the *FT* gene. Finally, we demonstrated that VRN-D1 proteins directly bind competitively and specifically to the CArG-box region in the promoter region of *WFT-D* gene [14]. In this study, we firstly indicated the possibility that the cereal VRN1 MADS-box protein functions as tetramer, especially homo-tetramer, to up-regulate *WFT* gene. In *Arabidopsis*, it is known that the hetero-tetramer network of MADS transcription factors play important roles in a lot of biological processes [21]. If wheat VRN1 protein actually forms homo-tetramers and functions *in vivo*, its mechanism of action is very interesting. The present result will be a clue to elucidate the function of VRN1 protein, that is, how to up-regulate *WFT* expression, in a flowering pathway of wheat.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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