



Characterization of Vacuolar Iron Transporter 1 (VIT1) from *Tulipa gesneriana*

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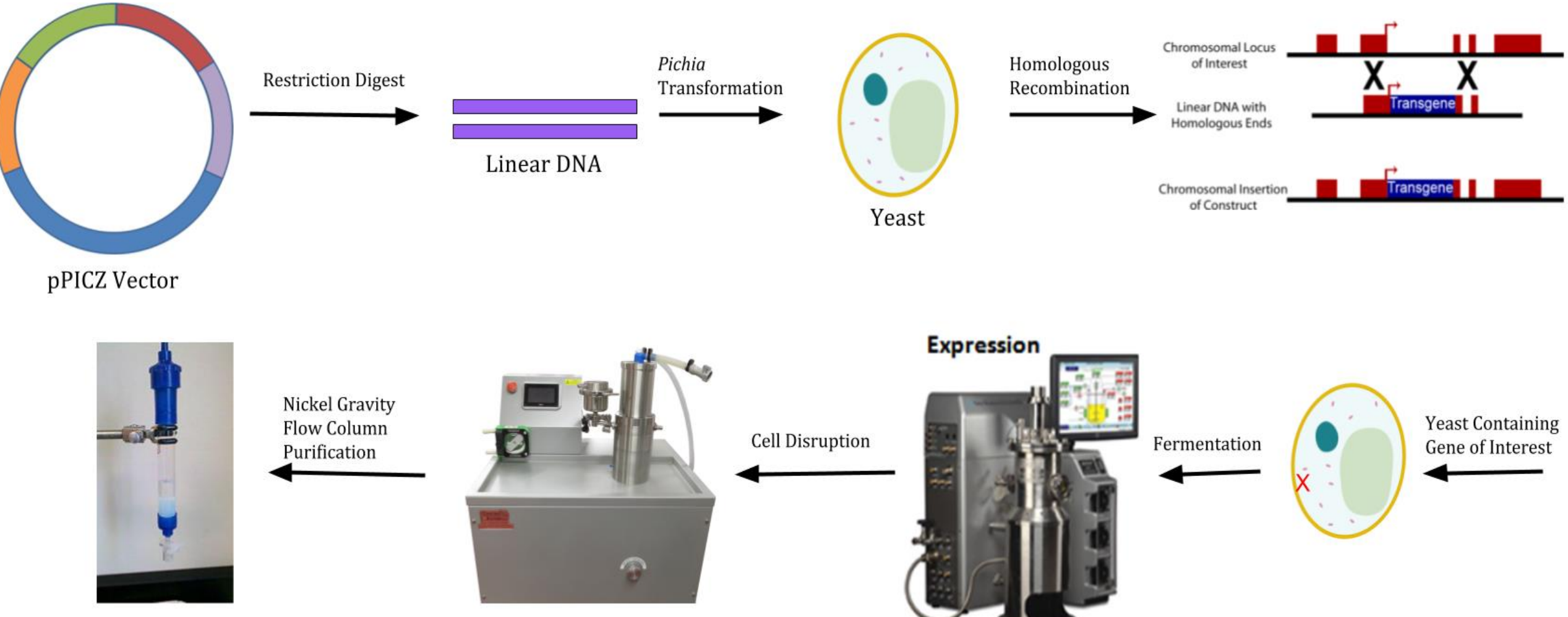
Introduction

The protein TgVit1 is a member of the CCC1 family. Members of the CCC1 family are transporters that move iron and/or manganese from the cytosol to the vacuole. TgVit1 is exclusively a vacuolar iron transporter that resides in the inner bottom epidermal layer of tulip petals. TgVit1 protein involvement in the localization and regulation of iron, specifically with iron sulfate and ferrous, enables iron homeostasis within membranous cells. TgVit1 proteins transport iron to anthocyanin cells. Anthocyanins thrive on a regulated amount of iron. When iron is transported in excess or in deficiency, the amount of anthocyanin cells decreases. Anthocyanins are crucial cells in plants, protecting them from senescence, abiotic stressors, and oxidation. TgVit1's transportation of iron into anthocyanin provides petal pigmentation. When TgVit1 concentration is at its strongest, anthocyanin pigments turn blue. However, the areas where TgVit1 is less abundant are often purple; TgVit1 is 25 times more bountiful in blue areas than in purple areas [1]. Understanding the structure and binding abilities of TgVit1 would provide the ability for artificial stabilization of the protein, therefore enabling anthocyanin function and production to be more efficient.

Methods

Materials and Preparation

After receiving the gene of interest, TgVIT1, it was cloned as a synthetic codon-optimized gene into a linearized pPICZ expression vector. This was then propagated in TOP10 *E. Coli* cells. A restriction digest was performed, followed by a *Pichia* transformation through electroporation. Through homologous recombination, the gene of interest was integrated into the yeast expression system. *P. pastoris* was grown in a ten liter BioFlow 415 Bioreactor and induced slowly with methanol. After 24 hours, the yeast was harvested. The harvested cells were then disrupted at 40,000 psi using a Constant Cell Disruptor. Membranes were isolated from the lysate and purified using a His-60 gravity nickel column. The protein was further purified through gel filtration using FPLC. The collected protein was concentrated and subjected to crystallization trials and was also used as an antigen in nanobody cell surface display methodologies.



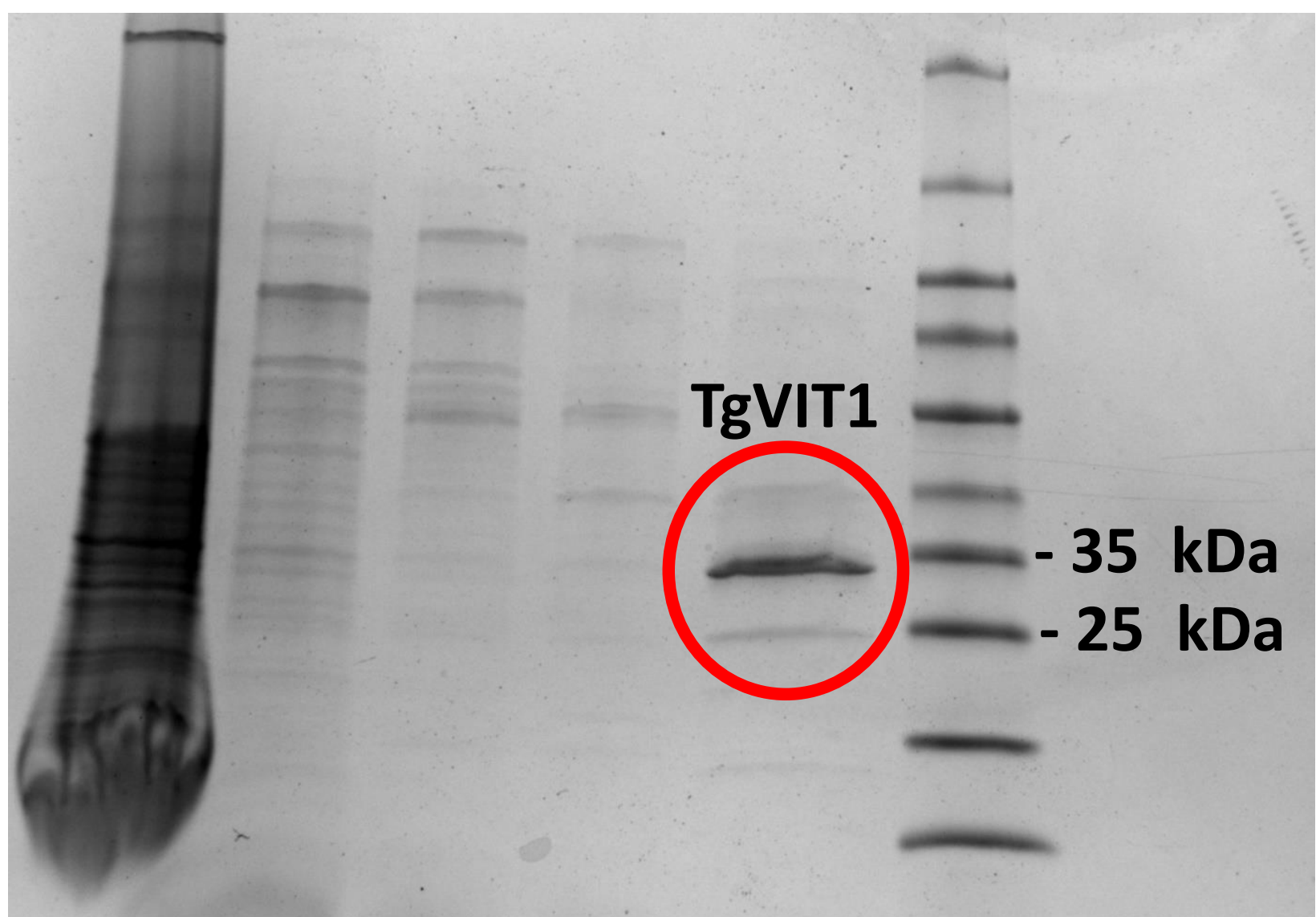
Cell Sorting

A solution of TgVIT1, His-Tag 647, Saline Buffered Phosphate (PBS) and a bacterial library were combined to a 4 milliliter volume and put into the cell sorter. The probe binding to the His-Tag in the antigen had an emission of 667 nanometers that fluoresced when the antigen was bound by a sequence in the bacterial library. The cells were sorted based on fluorescence. After the first sorting, the collected sample was resorted in order to select out the binders with a stronger affinity. The sample was the strongest binders was then plated onto an LB plate containing carbenicillin and kanamycin for overnight growth and future sequence determination.

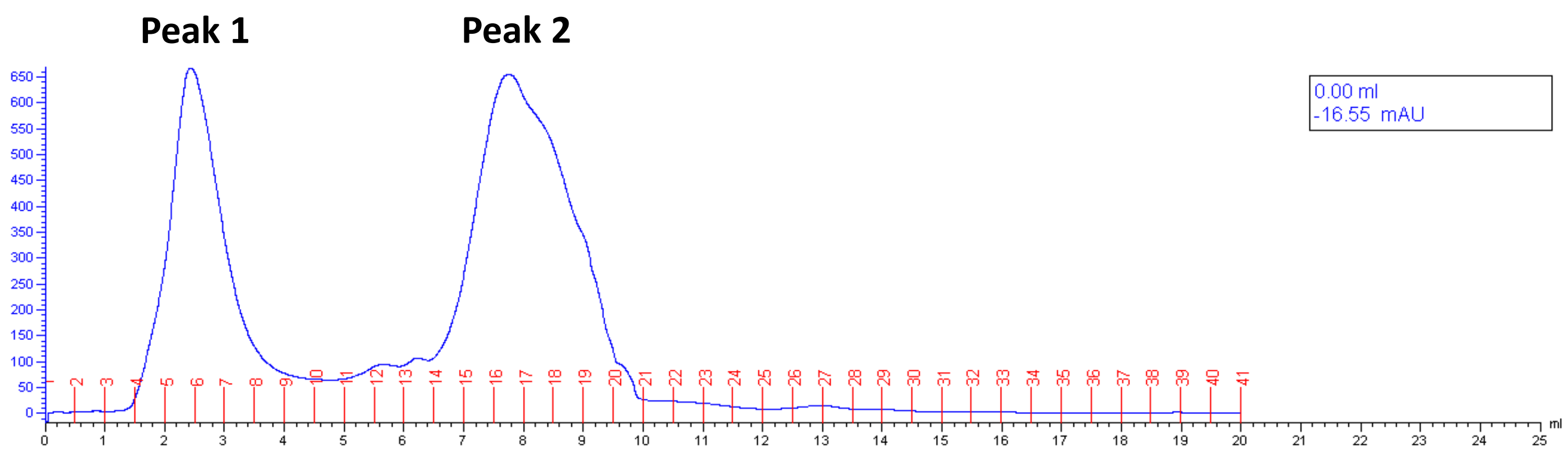
Protein Crystallization

Purified TgVIT1 was concentrated to ~8 mg/mL and used in vapor diffusion protein crystallization experiments with various concentrations of precipitants and salts over varying pH ranges.

Results

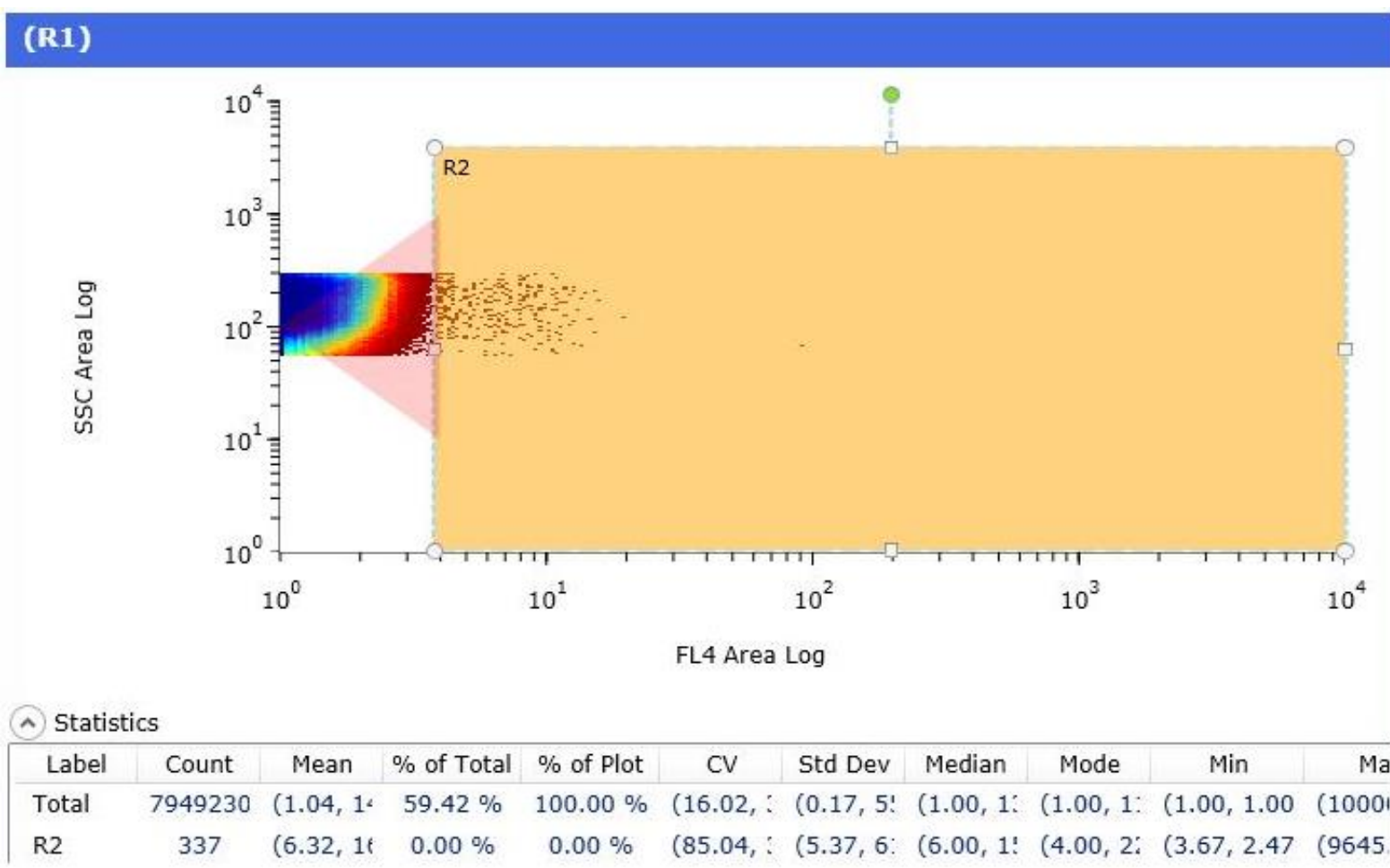


The first lane on the SDS gel represents the flow through and the adjacent lanes represent three washes followed by an elution using 300 mM imidazole. After His-60 purification, the protein of interest is seen at approximately the expected molecular weight of 29 kDa.

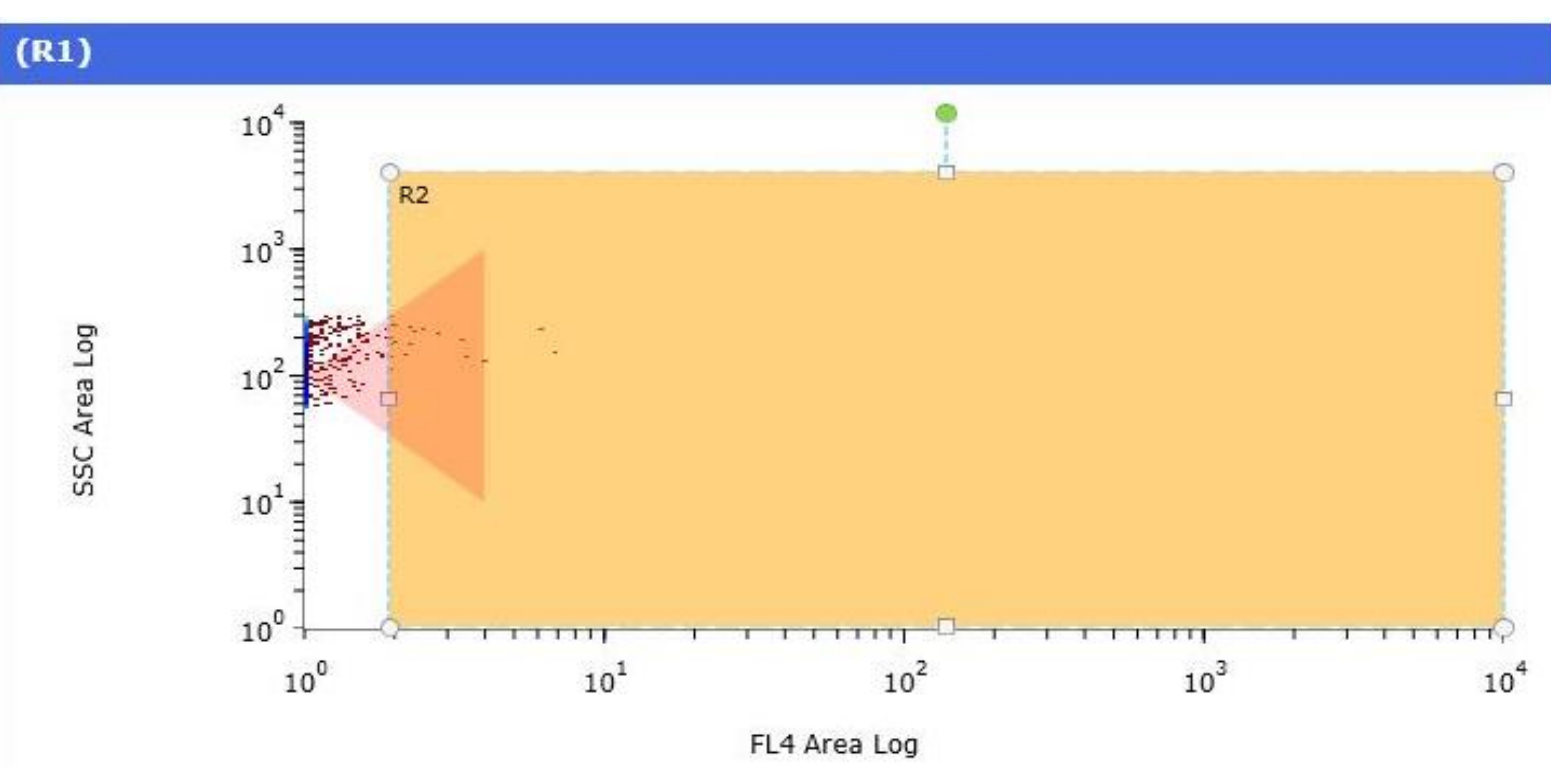


The SEC trace shows the protein coming off (peak 1) followed by a separation from a large contaminant (peak 2). Only the peak with our protein of interest was collected for cell sorting and crystallization trials.

The initial sort demonstrates the potential binders to VIT1 gated at R2.



Label	Count	Mean	% of Total	% of Plot	CV	Std Dev	Median	Mode	Min	Ma
Total	7949230	(1.04, 1)	59.42 %	100.00 %	(16.02, 1)	(0.17, 5)	(1.00, 1)	(1.00, 1)	(1.00, 1.00)	(1000)
R2	337	(6.32, 1)	0.00 %	0.00 %	(85.04, 1)	(5.37, 6)	(5.00, 1)	(4.00, 2)	(3.67, 2.47)	(9645)



Label	Count	Mean	% of Total	% of Plot	CV	Std Dev	Median	Mode	Min	Ma
Total	5018	(1.01, 1)	64.86 %	100.00 %	(20.78, 1)	(0.21, 5)	(1.00, 1)	(1.00, 1)	(1.00, 1.00)	(1000)
R2	21	(2.57, 1)	0.27 %	0.42 %	(90.06, 1)	(2.32, 4)	(2.00, 1)	(2.00, 1)	(1.92, 2.47)	(9645)

The re-sort gated the binders at R2 with the stronger affinity to the antigen, eliminating the non-specific interactions from the initial sort.

Conclusion

Residing in the epidermal layer of tulip petals, the iron transporter protein TgVIT1 is involved in the localization and regulation of iron. TgVIT1 is involved in the localization and regulation of iron while enabling iron homeostasis within membranous cells. The TgVIT1 protein plays a fundamental role transporting iron to anthocyanins cells that thrive on a regulated amount of iron. If the iron levels are too high or too low the amount of anthocyanin cells decreases. Anthocyanin is crucial as it protects the plant from external dangers such as senescence, abiotic stressors, and oxidation. Furthermore, anthocyanin provides the tulip petals with pigmentation. When TgVIT1 levels are high, the anthocyanin pigment turns blue and while TgVIT1 levels are low, the anthocyanin pigment turns purple. Our results are a basis for further trials that could provide insight for the function of VIT1 as an iron transporter in the cell but in order to learn more about the transporter, researchers must replicate our work in order to determine it's validity. After our work is labeled as valid, researchers could explore TgVIT1's structure by utilizing an x-ray diffractor to analyze the 3-dimensional structure of the protein. This would give a better understanding of the protein and how it functions. Furthermore, once the protein is stabilized and enough information is known about it characteristics, researchers could exploit TgVIT1 in order to genetically modify plants that can sustain themselves in high or low iron environments.

References

- [1] Kim, Sun A., et al. Localization of Iron in Arabidopsis Seed Requires the Vacuolar Membrane Transporter VIT1. *Science*, 24 Nov. 2006.
- [2] Momonoi, Kazumi, et al. A Vacuolar Iron Transporter in Tulip, TgVit1, Is Responsible for Blue Coloration in Petal Cells through Iron Accumulation. *Plant Journal*, 24 Apr. 2009.
- [3] Shi, Pengbao, et al. *Iron Supply Affects Anthocyanin Content and Related Gene Expression in Berries of Vitis Vinifera Cv. Cavernet Sauvignon*. *Molecules*, 14 February 2017.

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