Variable domain I of nematode CLEs directs post-translational targeting of CLE peptides to the extracellular space

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Effector proteins expressed in the esophageal gland cells of cyst nematodes are delivered into plant cells through a hollow, protrusible stylet. Although evidence indicates that effector proteins function in the cytoplasm of the syncytium,[1-3] technical constraints have made it difficult to directly determine where nematode effector proteins are initially delivered: cytoplasm, extracellular space, or both. Recently, we demonstrated that soybean cyst nematode CLE (HgCLE) propeptides are delivered to the cytoplasm of syncytial cells. Genetic and biochemical analyses indicate that the variable domain (VD) sequence is then required for targeting cytoplasmically delivered nematode CLEs to the apoplast where they function as ligand mimics of endogenous plant CLE peptides.[4] The fact that nematode CLEs are targeted through the gland cell secretory pathway and delivered as mature propeptides into plant cells makes it impossible for these proteins to be subsequently delivered to the extracellular space via co-translational translocation through the endoplasmic reticulum (ER) secretory pathway of the host cell. However, when expressed in transgenic plants, if the mature nematode CLE propeptide harbored a functional cryptic signal peptide, it could possibly traffic to the apoplast through the ER secretory pathway by co-translational translocation. Here, we present evidence that VDI, the N-terminal sequence of the VD of HgCLE2,[4] is sufficient for trafficking CLE peptides to the apoplast and that trafficking is indeed through an alternative pathway other than co-translational translocation.

VDI is Sufficient to Target CLE Peptides to the Extracellular Space

All plant CLE proteins contain N-terminal signal peptides that can target plant CLEs through the ER secretory pathway to function in the extracellular space.[5,6] Overexpression of Arabidopsis *CLAVATA3* (*AtCLV3*),[7] the best studied plant *CLE* gene, suppresses the expression of the homeobox gene *WUSCHEL* (*WUS*),[8] causing a *wus* mutant phenotype. Overexpression of *AtCLV3*$^{\Delta SP}$ abolishes its function because AtCLV3 is not properly targeted to the extracellular space, where it has been shown to function[4,6] (**Fig. 1**). The same has recently been shown for several other Arabidopsis CLEs.[9] In a previous study of AtCLV3,[10] deletion of the VD (AtCLV3$^{\Delta 23-66}$) or of the extension sequence downstream of the AtCLV3 CLE motif (AtCLV3$^{\Delta EXT}$) did not affect function. However, four amino acids of the VD immediately N-terminal of the 12-amino acid CLE motif were included in the VD deletion construct. In this study, deletion of both the VD and the extension sequence (AtCLV3$^{\Delta 23-66\Delta EXT}$) did not affect AtCLV3 function (**Fig. 1**), which indicates that 16 amino acids (amino acids 67–82) spanning the CLE motif are sufficient for AtCLV3 function when targeted to the apoplast by its signal peptide. Recently, we demonstrated that the nematode HgCLE2 VD can target the CLE motif of HgCLE2 and AtCLV3 to the

Figure 1. Overexpression of HgCLE2 variable domain I (VDI) targets the AtCLV3 CLE domain to the apoplast for function in Arabidopsis. Design of overexpression constructs. *AtCLV3,* full length *AtCLV3*; *AtCLV3ΔSP*, deletion of signal peptide from *AtCLV3*; *AtCLV3Δ23-66*, deletion of variable domain (VD) from *AtCLV3*; *AtCLV3Δ23-66ΔEXT*, deletion of both the VD and extension sequence after the CLE motif from *AtCLV3*; *HgCLE2VDI-AtCLV3Δ1-66ΔEXT*, VDI of HgCLE2 fused to the 16-amino acid CLE motif region sequence of *AtCLV3*.

Figure 2. Sequence alignment of the variable domain I (VDI) region of different cyst nematode CLE proteins reveals a high level of sequence conservation. Hg, *Heterodera glycines*; Hs, *Heterodera schachtii*; Gr, *Globodera rostochiensis*.

apoplast for function.[4] In this study, we tested whether the N-terminal sequence of the VD (VDI) is sufficient for the trafficking function. VDI was fused with the 16-aa AtCLV3 CLE motif region sequence and overexpressed in Arabidopsis. All 135 T1 seedlings displayed clear *wus* phenotypes (**Fig. 1**), which suggests that VDI of HgCLE2 is sufficient to target CLE peptides to the extracellular space for function. Since these results are based on overexpression studies in Arabidopsis, we speculated that VDI might harbor a cryptic signal peptide sequence involved in targeting CLE peptides to the ER secretory pathway to function in the extracellular space.

Identification of a Predicted Cryptic Signal Peptide Sequence in VDI

The presence of a putative cryptic signal peptide in the VDI sequence of HgCLE2

was suggested by a bioinformatics analysis. Two independent bioinformatics protocols were designed that employed the SignalP version 3.0 software.[11] Both protocols predicted a cryptic signal peptide in VDI. In the first protocol, a signal peptide was detected using the entire sequence of HgCLE2. In the second protocol, an N-terminal region of HgCLE2 (residues 1–41) was removed, and signal peptide detection was repeated to confirm that the region does not affect the prediction score. This established the left boundary of the predicted peptide. To determine the right boundary, 5, 10 and 15 residues were incrementally removed from the remaining sequence and reanalyzed with SignalP using the same parameters. Together, the bioinformatics analysis predicted a cryptic signal peptide in VDI between residues I42 and A64 of HgCLE2 (**Fig. 2**).

Besides the two CLE proteins from soybean cyst nematode,[4] we recently

reported on the identification of two *CLE* gene sequences, *HsCLE1* and *HsCLE2*, from the beet cyst nematode (*Heterodera schachtii*), a close relative of soybean cyst nematode.[12] Several CLE sequences from potato cyst nematode (*Globodera rostochiensis*) have also been reported.[13] So far, all of the cyst nematode CLEs identified to date can mimic plant CLEs when overexpressed in plants without their signal peptide sequences.[4,12,13] These data indicate that the function of the VD in retargeting CLE peptides to the apoplast is conserved across cyst nematodes. Not surprisingly, a T-Coffee sequence alignment among nematode CLEs revealed a high level of sequence conservation in VDI spanning the region that contains the predicted cryptic signal peptide sequence in HgCLE2 (**Fig. 2**). This suggests that cyst nematodes may utilize the same mechanism to traffic CLE proteins to the extracellular space of plant cells.

VDI Directs Post-Translational Targeting of CLE Peptides to the Extracellular Space

To test the function of the putative cryptic signal sequence, a C-terminal eGFP translational fusion to VDI was constructed to determine subcellular localization in onion epidermal cells. AtCLV3SP-eGFP and 35S-eGFP were included as positive and negative controls, respectively. These constructs were transiently expressed in onion epidermal cells by gold particle bombardment, and the GFP signal in the extracellular space was examined after plasmolysis.[14] AtCLV3SP-eGFP effectively targeted eGFP to the extracellular space (**Fig. 3A**); whereas, cells expressing 35S-eGFP accumulated GFP in the cytoplasm and nucleus, not in the extracellular space (**Fig. 3B**). Cells expressing HgCLE2VDI-eGFP accumulated GFP in the cytoplasm and nucleus similar to 35S-eGFP (**Fig. 3C**). These data indicate that the identified cryptic signal peptide is not functioning as a canonical ER-targeting sequence; therefore, VDI targeting of nematode CLEs to the extracellular space by co-translational translocation into the ER secretory pathway does not explain the results of overexpression studies. This is consistent with the fact that the mature CLE propeptide is delivered into syncytial cells by the nematode. Thus, VDI is targeting nematode CLEs to the extracellular space post-translationally through an alternative trafficking mechanism, which may be precluded by the GFP tag in this assay because of its molecular weight and/or structural differences from CLE peptides.

A BLAST search[15] to identify proteins that contain a subsequence similar to the cryptic signal peptide identified in HgCLE2 VDI was performed. The BLAST search detected proteins that share a surprisingly long stretch of 9 identical residues or 10–11 similar residues when considering positive substitutions with the cryptic signal peptide. An alignment using the full-length HgCLE2 sequence
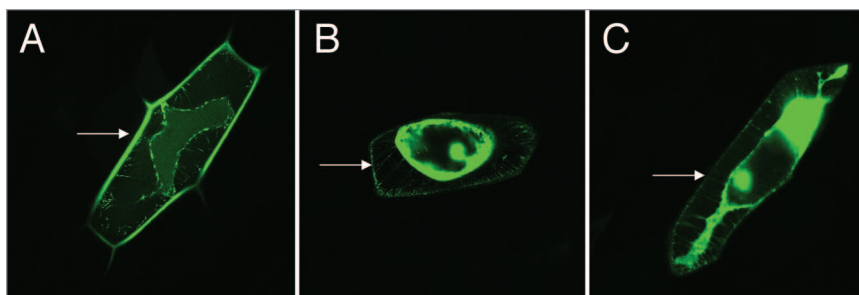
Figure 3. Localization of eGFP in onion epidermal cells after plasmolysis. (A) AtCLV3SP-eGFP; (B) 35S-eGFP; (C) HgCLE2VDI-eGFP. Arrows point to eGFP accumulation in the extracellular space in (A), but not in (B and C).

with these proteins revealed only ~3–7% sequence identity, suggesting that these proteins are not related. Interestingly, however, a large number of these proteins are transporters from a number of different species, including plants (e.g., *Oryza sativa*) and bacteria (e.g., *Burkholderia vietnamiensis*). Further studies will be necessary to determine exactly how the VDI sequence facilitates transport of nematode CLEs to the extracellular space.

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