

Short communication

A parasitism gene from a plant-parasitic nematode with function similar to *CLAVATA3/ESR (CLE)* of *Arabidopsis thaliana*XIAOHONG WANG¹, MELISSA G. MITCHUM², BINGLI GAO³, CHUNYING LI⁴, HANANE DIAB⁴, THOMAS J. BAUM⁵, RICHARD S. HUSSEY³ AND ERIC L. DAVIS^{4*}¹Department of Plant Pathology, USDA-ARS, Cornell University, Ithaca, NY 14853, USA²Department of Plant Microbiology and Pathology, University of Missouri-Columbia, Columbia, MO 65211, USA³Department of Plant Pathology, University of Georgia, Athens, GA 30602, USA⁴Department of Plant Pathology, North Carolina State University, Raleigh, NC 27695-7616, USA⁵Department of Plant Pathology, Iowa State University, Ames, IA 50011, USA**SUMMARY**

The *Hg-SYV46* parasitism gene is expressed exclusively in the dorsal oesophageal gland cell of parasitic stages of the soybean cyst nematode, *Heterodera glycines*, and it encodes a secretory protein that contains a C-terminal motif of the *CLAVATA3/ESR*-related (CLE) family in *Arabidopsis thaliana*. In shoot and floral meristems of *Arabidopsis*, the stem cells secrete *CLV3*, a founding member of the CLE protein family, that activates the *CLV1/CLV2* receptor complex and negatively regulates *WUSCHEL* expression to restrict the size of the stem cell population. Mis-expression of *Hg-SYV46* in *Arabidopsis* (ecotype Columbia-0) under control of the *CaMV35S* promoter resulted in a *wus*-like phenotype including premature termination of the shoot apical meristem and the development of flowers lacking the central gynoecium. The *wus*-like phenotype observed was similar to reports of over-expression of *CLV3* and *CLE40* in *Arabidopsis*, as was down-regulation of *WUS* expression in the shoot apices of *35S::Hg-SYV46/Col-0* plants. Expression of *35S::Hg-SYV46* in a *clv3-1* mutant of *Arabidopsis* was able partially or fully to rescue the mutant phenotype, probably dependent upon localization and level of transgene expression. A short root phenotype, as reported for over-expression of *CLV3*, *CLE40* and *CLE19* in roots, was also produced in primary *35S::Hg-SYV46/Col-0* transgenic plants. The results suggest a functional similarity of *HG-SYV46* to plant-secreted CLE ligands that may play a role in the differentiation or division of feeding cells induced in plant roots by parasitic nematodes.

INTRODUCTION

Cyst nematodes (*Heterodera* and *Globodera* species) are microscopic worms that penetrate host plant roots as motile juveniles

and select plant cells with meristematic potential to induce elaborate syncytial feeding sites that serve the essential nutritional needs of the subsequent sedentary nematode life stages (Hussey and Grundle, 1998). Secretory proteins encoded by 'parasitism genes' expressed within the nematode's oesophageal gland cells are released through its stylet (oral spear) to induce re-differentiation of recipient plant cells into syncytia (Davis *et al.*, 2000). The elaborate morphological changes observed in syncytial cells are accompanied by profound changes in plant cell gene expression within the affected cells, often relatively early during the establishment of the feeding site (Gheysen and Fenoll, 2002). Evidence is now emerging that not only is feeding cell phenotype a product of augmented plant cell gene expression, but that feeding cell phenotype may be under considerable direct control from signal molecules secreted by the nematode during the parasitic relationship (Davis *et al.*, 2004).

The *Hg-SYV46* parasitism gene (GenBank accession no. AF273728) of the soybean cyst nematode, *Heterodera glycines*, was cloned through yeast secretion signal peptide selection of an *H. glycines* oesophageal gland cell-specific cDNA library and it is highly expressed exclusively within the dorsal oesophageal gland cell of *H. glycines* during syncytium formation in plants (Wang *et al.*, 2001). *Hg-SYV46* encodes a polypeptide of 139 amino acids with a functional N-terminal signal peptide for secretion. The complete *HG-SYV46* polypeptide is unique to *H. glycines* (Wang *et al.*, 2001); however, it does contain the conserved C-terminal motif shared by the *CLAVATA3/ESR*-related (CLE) protein family (Olsen and Skriver, 2003) in which *CLV3* is a central member. Nine amino acid identities in the C-terminal 14 amino acid motif region exist between *CLV3* and *HG-SYV46* (Olsen and Skriver, 2003) that include a highly conserved glycine residue that is important for *CLV3* function (Fletcher *et al.*, 1999). All of the CLE genes encode small proteins that contain an N-terminal signal peptide for secretion or membrane anchor and a conserved 14 amino acid motif located at or near the carboxyl terminus (Cock and

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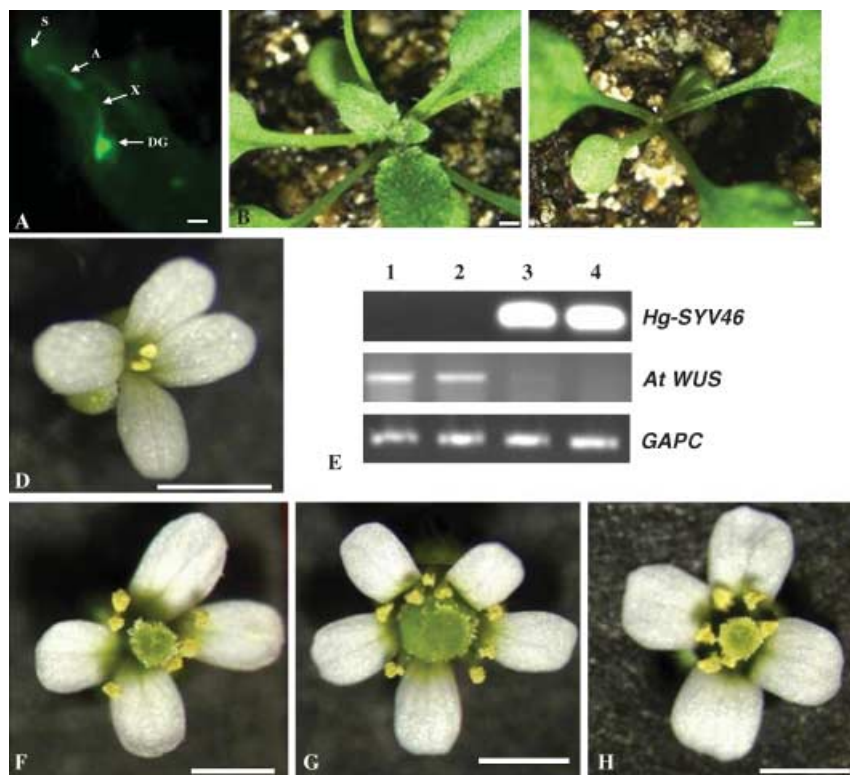


Fig. 1 Localization of HG-SYV46 in *Heterodera glycines* and effects of *Hg-SYV46* expression in transgenic *Arabidopsis thaliana*. (A) Polyclonal antibodies localized HG-SYV46 within the dorsal oesophageal gland cell, its extension and the gland cell ampulla at the base of the stylet (feeding spear) of a parasitic stage of *H. glycines* dissected from a host plant root. (B) The developing shoot of a wild-type seedling (ecotype Columbia-0) at 20 days post-germination. (C) A *35S::Hg-SYV46* transgenic Arabidopsis plant at 20 days post-germination showing an arrested shoot apical meristem (arrow). (D) Flower of a *35S::Hg-SYV46* transgenic Arabidopsis plant showing a decreased number of stamens and missing carpels as compared with wild-type, a phenotype similar to a *wus* flower (Laux *et al.*, 1996). (E) RT-PCR analysis of *Hg-SYV46* and *WUS* expression in the shoot apices of *Hg-SYV46* non-expressed (*promoterless::Hg-SYV46*, lanes 1 and 2) and expressed (*35S::Hg-SYV46*, lanes 3 and 4) individual transgenic Arabidopsis lines from 10-day-old seedlings. Expression of the *Arabidopsis gapC* gene was used as an internal control. (F) A wild-type Arabidopsis flower containing four petals, six stamens and two fused carpels. (G) Flower of an *Arabidopsis clv3-1* mutant (Clark *et al.*, 1995) carrying more floral organs in all whorls than wild-type. (H) A fully restored flower of a *clv3-1* mutant with wild-type floral organ size and number obtained from a *35S::Hg-SYV46/clv3-1* transgenic plant. Abbreviations: DG = dorsal gland, X = extension, A = ampulla. Scale bars: 0.1 mm in A, 1.0 mm in B–D and F–H.

McCormick, 2001; Sharma *et al.*, 2003). Unlike all other putative *CLE* genes reported to date, only *CLV3* and *CLE40* contain introns (Fletcher *et al.*, 1999; Hobe *et al.*, 2003; Sharma *et al.*, 2003). *CLE40*, which is expressed at low levels throughout plant tissues, including roots (Sharma *et al.*, 2003), encodes a potentially secreted peptide with close structural similarities to *CLV3* (Hobe *et al.*, 2003). *CLE40* is a functional equivalent to *CLV3* when expressed under the control of *CLV3* promoter (Hobe *et al.*, 2003). *Hg-SYV46* also contains introns in its genomic sequence (X. Wang *et al.* unpublished observations), and its encoded polypeptide is 50% identical to *CLE40* in the 14 amino acid conserved *CLE* motif.

Two synthetic peptides were produced (Eurogentec Inc., Belgium) to antigenic regions of the predicted HG-SYV46 protein (GenBank accession no. AF273728) including the peptide STGD-KKTANDGSGNN at the N-terminus of the mature protein and the

peptide PVNESKRLSPSGDPH near the C-terminus. The two synthetic peptides were co-injected into rabbits (Eurogentec, Inc.) to generate anti-HG-SYV46 polyclonal sera, and specific binding of the anti-HG-SYV46 sera to each individual peptide was confirmed by enzyme-linked immunosorbent assay. The anti-HG-SYV46 sera bound only to secretory granules within the dorsal oesophageal gland secretory cell (Fig. 1A) of parasitic stages of *H. glycines* dissected from soybean roots. The polyclonal sera bound to dorsal gland secretory granules that had migrated anteriorly through the cellular extension to the dorsal gland ampulla (collecting reservoir) at the base of the nematode stylet (Fig. 1A). The specificity of the polyclonal sera to the HG-SYV46 parasitism protein was by design because neither of the HG-SYV46 peptide sequences chosen for synthesis exists in any other parasitism protein identified within the oesophageal gland cells of *H. glycines* (Gao *et al.* 2001, 2003; Wang *et al.*, 2001). The presence of a functional

secretion signal peptide (Wang *et al.*, 2001) and movement of HG-SYV46 from the gland cell to the nematode stylet during parasitism have suggested an important role of secreted HG-SYV46 in plant parasitism.

CLV3, which is specifically expressed in the stem cells of shoot and floral meristems of Arabidopsis, encodes a secreted peptide ligand that functions in controlling the balance between meristem cell proliferation and differentiation through its interactions with the CLV1/CLV2 receptor complex and the WUS transcriptional factor (Brand *et al.*, 2000; Fletcher *et al.*, 1999; Lenhard and Laux, 2003; Rojo *et al.*, 2002; Schoof *et al.*, 2000). Expression and potential *in planta* secretion of HG-SYV46 by parasitic *H. glycines*, and HG-SYV46 similarity to the CLE protein family at the C-terminal conserved region (Olsen and Skriver, 2003), has prompted investigations to determine if this nematode parasitism gene has similar functions to plant CLE genes.

Full-length *Hg-SYV46* cDNA was cloned behind the *CaMV35S* promoter, and the resulting transgene *35S::Hg-SYV46* was introduced into wild-type (ecotype Columbia-0) *Arabidopsis thaliana* plants via *Agrobacterium*-mediated floral dip transformation (Clough and Bent, 1998). In 60% of the 240 *35S::Hg-SYV46/Col-0* primary transgenic plants studied, unlike the wild-type plant (Fig. 1B), a *wus* phenotype (Laux *et al.*, 1996) was observed where the shoot meristem ceased initiating organs after generation of the first leaves (Fig. 1C). Later in development, these plants produced flowers that either did not open or lacked the central gynoecium (Fig. 1D). The root systems of *35S::Hg-SYV46/Col-0* transformants demonstrating a strong *wus* phenotype were stunted (data not shown), similar to the short root phenotype resulting from misexpressions of *CLE19* and *CLE40* in roots of Arabidopsis (Casamitjana-Martinez *et al.*, 2003; Fiers *et al.*, 2004; Hobe *et al.*, 2003). Transgene expression in the primary *35S::Hg-SYV46/Col-0* plants showing *wus* phenotype was confirmed by RT-PCR using total RNA from leaf tissues of these plants. The remaining 40% of the primary transgenic plants either showed a much weaker *wus* phenotype that produced occasional *wus* flowers (Laux *et al.*, 1996) or developed like wild-type plants due, presumably, to insufficient transgene activity to induce an observable phenotype. The anti-HG-SYV46 sera did not detect the expected HG-SYV46 protein on Western blots of total proteins extracted from Arabidopsis transformants that demonstrated high or low levels of *Hg-SYV46* expression. Definitive immunodetection of any CLE protein, however, has been lacking (Fiers *et al.*, 2004; Lenhard and Laux, 2003; Nishihama *et al.*, 2003), potentially due to *in planta* processing of CLE polypeptides to produce a functional peptide ligand (Casamitjana-Martinez *et al.*, 2003).

Many phenotypic features observed in the *35S::Hg-SYV46* transgenic plants, such as cessation of organ formation and reduction in organ number in the inner floral whorls, may be caused by a failure to maintain an adequate number of stem cells in shoot and floral meristems. In the shoot meristem of

Arabidopsis, the WUSCHEL (WUS) homeodomain transcription factor (Mayer *et al.*, 1998) promotes stem cell formation and maintenance that acts antagonistically to the CLV pathway (Brand *et al.*, 2000; Lenhard and Laux, 2003; Schoof *et al.*, 2000). Over-expression of the CLV3 signal severely down-regulates WUS expression (Brand *et al.*, 2000). Similar to *CLV3*, over-expression of *CLE40* results in a down-regulation of WUS during development (Hobe *et al.*, 2003). The reduction of WUS transcripts in the arrested shoot apices of *35S::Hg-SYV46/Col-0* transgenic plants was also confirmed by RT-PCR analysis (Fig. 1E). This result suggests that HG-SYV46 could activate the CLV signalling pathway to repress WUS expression. Recent studies have shown that many CLE proteins, including CLE40, can interact with the CLV1/CLV2 receptor complex (R. Simon, personal communication) in the shoot of Arabidopsis. Expression of *Hg-SYV46* in *clv1* and *clv2* mutant Arabidopsis (Clark *et al.*, 1993; Kayes and Clark, 1998) will indicate a more direct interaction of this nematode ligand with the CLV1/CLV2 complex.

The *35S::Hg-SYV46* transgene was also transformed in *clv3-1* mutant Arabidopsis (Clark *et al.*, 1995) to investigate further a potential CLV3 function. As compared with the wild-type Arabidopsis flower (Fig. 1F), *clv3-1* mutant plants produce enlarged shoot and floral meristems (Fig. 1G) with extra floral organs in each whorl (Clark *et al.*, 1995). Among the 125 primary *35S::Hg-SYV46/clv3-1* transgenic plants analysed, about one-third partially or fully rescued the mutant shoot and floral phenotype (Fig. 1H), and about 20% of the primary transformants produced the *wus*-like phenotype (Laux *et al.*, 1996). The data suggested that HG-SYV46 could complement activity in the CLV3 signal transduction pathway when in proper quantitative balance and localization within the *clv3-1* background. A complementation study expressing *Hg-SYV46* under the control of a native *CLV3* promoter (Brand *et al.*, 2002) in a *clv3* null mutant (Clark *et al.*, 1995) is underway to determine more definitively the potential functional equivalency of HG-SYV46 and CLV3.

So what is the biological relevance of a potential secreted plant signal peptide mimic from a root-parasitic nematode that has CLV3-like activity in plant shoots? A CLV-like signalling pathway in control of Arabidopsis root meristem maintenance has been suggested from investigations of over-expression of *CLV3*, *CLE40* and *CLE19* in roots (Casamitjana-Martinez *et al.*, 2003; Fiers *et al.*, 2004; Hobe *et al.*, 2003). Both *CLE40* and *CLE19* express at low level in wild-type Arabidopsis root tissues, and over-expression of either gene produces a short root phenotype in which the primary root meristem is gradually consumed and becomes fully differentiated during root development (Casamitjana-Martinez *et al.*, 2003; Fiers *et al.*, 2004; Hobe *et al.*, 2003). A short root phenotype was also observed in primary *35S::Hg-SYV46/Col-0* transgenic plants, and a more detailed phenotypic study of those roots is under way. It has been hypothesized that the CLE19 polypeptide might function as a differentiation signal

to be perceived by a CLV1-related receptor kinase complex in roots to induce cell differentiation (Casamitjana-Martinez *et al.*, 2003; Fiers *et al.*, 2004). In this respect, a similar secreted CLE mimic from a nematode may function as one component of a pathway to redirect and maintain the differentiation of root vascular cells into elaborate feeding sites, potentially via a CLV1-like receptor.

A recent microarray study indicates that vascular cell-specific CLE and CLV receptor genes are expressed in the hypocotyl of *Arabidopsis* (E. Beers, personal communication). Thus, the potential exists that secreted HG-SYV46 might alternatively function by competitively inhibiting the ability of a similar endogenous plant CLE ligand to interact with a common host CLV-like receptor in roots, subsequently to augment normal vascular cell differentiation. In this model, a mutation in a putative native CLV1-like receptor might similarly promote differentiation towards other cell types (e.g. feeding cells) as opposed to wild-type vascular cells. Interestingly, hyperinfection of a *har-1* mutant of *Lotus japonicus* by root-knot nematodes has recently been reported (Lohar and Bird, 2003). *HAR1*, a CLV1-like gene, has recently been shown to function in roots of *L. japonicus* to balance cell proliferation and differentiation in the root apical meristem (Krusell *et al.*, 2002; Nishimura *et al.*, 2002).

The possibility that some CLE polypeptides such as CLE19 in roots may function to inhibit (meristem) cell division as opposed to stimulating cell differentiation has not been excluded (Fiers *et al.*, 2004), and this observation also relates to potential secreted CLE and the nematode feeding cell phenotype. Considerable alterations in plant gene expression, including a number of cell cycle genes, that may restrict cell division to maintain the integrity of nematode feeding cells have been observed (Gheysen and Fenoll, 2002). As further plant root CLE and CLV1-like mutants and genes are identified, their potential effects on parasitism by nematodes may clarify a role of secreted CLE mimics and CLV1-like receptors in nematode feeding cell development.

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