Genome sequence variation in the constricta strain dramatically alters the protein interaction and localization map of Potato yellow dwarf virus

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Abstract

The genome sequence of the constricta strain of Potato yellow dwarf virus (CYDV) was determined to be 12 792 nt long and organized into seven ORFs with the gene order 3′-N-X-P-Y-M-G-L-5′, which encodes the nucleocapsid, phospho, movement, matrix, glyco, and RNA-dependent RNA polymerase proteins, respectively, except for X, which is of unknown function. Cloned ORFs for each gene, except L, were used to construct a protein interaction and localization map (PILM) for this virus, which shares greater than 80 % amino acid similarity in all ORFs except X and P with the sanguinolenta strain of this species (SYDV). Protein localization patterns and interactions unique to each viral strain were identified, resulting in strain-specific PILMs. Localization of CYDV and SYDV proteins in virus-infected cells mapped subcellular loci likely to be sites of replication, morphogenesis and movement.

INTRODUCTION

Although the coding capacity of viral genomes is low, it is common for each encoded protein to interact with multiple target factors, often located in different subcellular loci [1–3]. Additionally, sequence divergence among viral strains can have profound effects on virulence, symptom development or adaptation to new hosts and vectors [4–8]. As such, determination of the subcellular localization of viral proteins and mapping their interacting partners is fundamental to understanding virus–host interactions [2, 9–12]. Of particular interest in this regard are viruses that are able to replicate in diverse cell types, as proteins encoded by these viruses must contain domains that mediate interaction with factors in evolutionarily divergent hosts.

Rhabdoviruses infect a broad range of hosts, and members of this group includes viruses that infect humans, terrestrial animals/vertebrates, fish, arthropods and plants [9, 13]. Currently, the plant-adapted rhabdoviruses are assigned to two genera, and two more recently described genera, Dichorhavirus and Varicosavirus, contain members with segmented genomes that also infect plants [9, 14, 15]. The genus Cytorhabdovirus, for which the type species is Lettuce necrotic yellows virus, contains those plant rhabdoviruses that replicate and undergo morphogenesis in the cytoplasm of infected cells [16]. Potato yellow dwarf virus (PYDV) is the type species of the genus, Nucleorhabdovirus, while Orchid fleck virus is the type species of the genus Dichorhavirus. Both of these genera are typified by the nucleotrophic character of member viruses [9, 14, 17, 18].

PYDV was first reported as a highly destructive pathogen of potato (Solanum tuberosum), and early research of this virus contributed significantly in the arena of virus–insect interactions [19, 20]. At least seven strains of PYDV have been described at the level of vector-specificity and biological variation in symptom severity [20, 21]. Of these, two strains distinguished by their differential transmission by leafhopper vectors, Aceratagallia sanguinolenta and Agallia constricta, referred to hereafter as sanguinolenta yellow dwarf virus (SYDV; also called PYDV-New York) and constricta yellow dwarf virus (CYDV; also called PYDV-New Jersey), respectively, became the predominant research strains that served as early models for defining the ultrastructure and cytopathology of plant-adapted rhabdoviruses [22–24] and development of sucrose-gradient centrifugation as an method for preparing virus samples for electron microscopy.

New sequence: the complete 12 792 nt genome of CYDV has been deposited into Genbank as accession KY549567.
analytical method [25]. Symptom severity of SYDV is
greater than CYDV in Nicotiana benthamiana, and, in our
hands, is easier to purify given its higher titre in that host
[18, 26, 27].

The genome of SYDV was characterized previously [18] and,
since then, those of several segmented and non-segmented
plant rhabdoviruses have been described [2, 9–12]. Collectively,
the pattern that has emerged is that the protein interaction
and localization maps (PILMs) for each virus are unique
[2, 9–12]. Much of this variation is attributable to highly
divergent genomic sequences among the viral species. In light
of this, we sought to determine if lesser variation in genome
sequence could profoundly affect PILMs at the level of viral
strains, instead of between viruses. As such, we developed a
PILM for the CYDV strain of PYDV and compared it to that
of SYDV [18]. It is clear from our studies that even modest
changes in sequence variation can affect the topology of
PILMs. These studies provide a link between the molecular
features of rhabdovirus strains and their differential interac-
tions with host and vector species.

RESULTS

Genome sequence of CYDV

The complete 12 792 nt genome of CYDV, deposited in
GenBank as accession KY549567, was determined. The anti-
genomic sequence has the coding capacity for ORFs, encod-
ing proteins greater than 100 aa each (Fig. 1a). The
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Terminal sequences and gene junctions in the
CYDV genome

Regarding SYDV reported previously, the leader and trailer
terminal sequences of CYDV have a complete base comple-
mentarity over only a very short region, namely the terminal
nine bases of the genome (Fig. 1c) [18].

A conserved gene junction with the consensus 3’-
AAUUAUUUUU GGG UUG-5’ (Fig. 2a) was located
between each of the ORFs in the CYDV genome, as well as
the leader (ldr)/N gene junction. This junction differs from
that for SYDV only with respect to the position of the ade-
nine in the poly-U track (Fig. 2b). Overall, the CYDV junc-
tions share a similar tripartite organization with that of
other plant-adapted rhabdoviruses, namely: region 1, con-
sisting of a poly-U track that serves as template for poly-
adenylation of nascent mRNA transcripts; region 2, a triplet
of guanosyl residues; and region 3, the transcriptional start
site, consisting of UUG. As is typical for rhabdoviruses,
each individual gene junction differs slightly from the con-
sensus sequence. Most notably for CYDV, the intergenic
spacer in the N/X and G/L junctions contained an addi-
tional guanosine residue (Fig. 2a).

Predicted features of PYDV proteins

Generally, the predicted sizes of CYDV-encoded proteins
are the same as, or slightly smaller, than their SYDV cog-
nates. The N, X and G proteins are approximately 1, 1 and 3 kDa smaller than their SYDV cognates, respec-
tively, whereas the P, Y, M and L proteins are of equivalent
sizes for both viruses.

Various protein localization prediction algorithms were
used to identify potentially biologically relevant motifs in
the CYDV-encoded proteins. A subset of this information
is provided in Table 1. Regarding its SYDV cognate, protein
localization prediction algorithms failed to identify a
nuclear localization signal (NLS) in the CYDV nucleocapsid
protein. Furthermore, the primary structure of CYDV-N
does not contain the mapped QKRANEEAPPAAQKR
bipartite NLS found in SYDV-N [29]. Algorithm-predicted
NLSs were identified in the phosphoprotein, matrix protein
and polymerase protein.

Both CYDV and SYDV N proteins have a predicted isoelec-
tric point (pI) of 7.62. The X protein of CYDV has a pre-
dicted pI of 3.87, slightly more acidic than the pI 4.5 of its
SYDV cognate. Similar to CYDV-X, the P protein at pI 6.23
is slightly more acidic than the 7.72 of its SYDV cognate.
The putative movement protein, CYDV-Y, has a pI of 6.6
while that of SYDV is 7.0, both matrix proteins sharing a pI
close to 9.0. Both CYDV and SYDV glycoproteins have a pI
around 4.6. However, consistent with other proteins, the CYDV-L at pI 6.75 is greater than one log more acidic than its SYDV cognate (pI 7.99).

Although the CYDV-G ORF predicts a smaller protein than its cognate, the relative molecular weight based on the electrophoretic mobility of CYDV-G was reported to be greater than that for SYDV-G (92 kDa versus 85 kDa) [27]. The CYDV-G and SYDV-G proteins are predicted to have seven N-linked glycosylation sites each, and six and nine, respectively, O-linked glycosylation sites. The actual degree of glycosylation has not been mapped physically, and therefore the reason for the difference in electrophoretic mobility of these proteins remains equivocal.

**Localization of CYDV protein fusions in plant cells**

In order to test whether the sequence variation between SYDV and CYDV influenced protein localization, we determined the subcellular localization patterns for six CYDV proteins in planta and compared these data to published results for SYDV [18]. Each of the N, X, P, Y, M and G proteins was expressed as a GFP fusion in transgenic *N. benthamiana*, which expressed RFP fused to histone 2B (Fig. 3). In contrast to GFP-SYDV-N, whose localization was distributed evenly...
across the nucleoplasm, GFP:CYDV-N localized in subnuclear loci with a cross-sectional area of about 2 µm. GFP:CYDV-X distributed throughout the cell, with accumulation in the nucleus. GFP:CYDV-P accumulated in puncta distributed throughout the nucleoplasm, but was excluded from the nucleolus. GFP:CYDV-Y partitioned between the cell periphery and the nuclear envelope, suggesting a membrane association for this protein. Regarding the SYDV matrix protein, the cognate CYDV protein was exclusively nuclear when expressed as a GFP fusion. GFP:CYDV-G associated with endomembranes, with the most easily detectable signal localized on the nuclear envelope.

**Interaction matrix for CYDV proteins**

In addition to protein localization studies, we investigated whether the determined sequence divergence between the two viral strains impacted the interaction of CYDV proteins, relative to the interactions observed for SYDV [18]. In order to make direct comparisons, the same type of bimolecular fluorescence complementation (BiFC) assays was used to define the interaction and localization patterns of CYDV proteins (Fig. 4). While all pairwise interactions were tested, in the four protein fusion orientations possible with BiFC, only a subset of the data is reported here. The N, X, P, Y, M and G proteins were tested in all pairwise interactions and against glutathione-S-transferase (GST), which served as a non-binding control (Fig. 4). The L protein was not included in these experiments as we were unable to detect GFP fusions of this protein in planta (data not shown). None of the CYDV proteins showed interaction with GST. Positive BiFC interactions were detected for the pairs N/N, N/X, N/P, N/Y, N/M, N/G, P/P, X/P, X/Y, X/M, and M/M. No other interactions were detected. The X protein did not interact with the G protein.

The resulting BiFC and localization data were integrated into a CYDV PILM, which differs significantly from that of SYDV (Fig. 5). The N/Y, Y/Y and G/G interactions were unique to SYDV, while the M/P and X/M interactions were unique to CYDV.

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**Table 1. Features of PYDV proteins determined by predictive algorithms**

| ORF | MW (kD) | TM | pI | Predicted NLS | Putative function | Highest scoring virus/E-value (BLAST) |
|-----|---------|----|----|---------------|------------------|---------------------------------------|
| 1   | 51      | None | 7.62 | KRTAEDATTQQTQK* | Nucleocapsid (N) | PYDV-N/0.0 |
| 2   | 9       | None | 3.87 | -               | Unknown (X)     | PYDV-X/4e-04 |
| 3   | 31      | None | 6.23 | PAKSRRK       | Phosphoprotein (P) | PYDV-P/2e-103 |
| 4   | 32      | None | 6.60 | -               | Movement (Y)    | PYDV-Y/3e-161 |
| 5   | 29      | None | 8.85 | KRTVADPPFLKLRRKSE | Matrix protein (M) | PYDV-M/2e-131 |
| 6   | 67      | aa 575–597 | 4.56 | -       | Glycoprotein (G) | PYDV-G/0.0 |
| 7   | 220     | aa 510–529 | 6.73 | KKLVPTNHPDNLKKR | Polymerase (L)  | PYDV-L/0.0 |

*This putative NLS was not predicted computationally and is instead the region of the CYDV-N protein corresponding to the mapped NLS in SYDV [29].
Localization of PYDV proteins in virus-infected plant cells

It has been reported previously that localization patterns of plant-adapted rhabdovirus proteins can differ markedly in the context of infected cells compared to single protein expression in virus-free cells [30]. Given this precedent, we expressed GFP fusions of proteins from both CYDV and SYDV in transgenic *N. benthamiana* plants expressing RFP targeted to the endomembrane system, which provided a facile means to track changes in plant nuclear proteins as well (Figs 6, 7).

GFP:CYDV-N was unevenly distributed throughout the nucleoplasm, while the GFP:CYDV-X and GFP:CYDV-P proteins exhibited a more even distribution throughout the nucleoplasm. In the case of the P protein, the punctate nuclear localization pattern observed when localized in virus-free cells was absent in virus-infected cells. The Y protein showed accumulation on the cell periphery, as well as the nuclear envelope. The GFP:CYDV-M protein co-localized with membranes that accumulated in intranuclear spherules. Regarding its localization pattern in virus-free cells, the GFP:CYDV-G protein accumulated primarily on perinuclear membranes and the nuclear envelope.

In contrast to its cognate protein, GFP:SYDV-N was distributed evenly across the nucleoplasm in virus-infected cells. GFP:SYDV-X protein showed greater partitioning between the nucleus and cytoplasm than GFP:CYDV-X, which was primarily nuclear in the context of infected cells. GFP:SYDV-P was observed on large sub-nuclear foci in virus-infected cells, in a pattern clearly distinguishable from that produced by GFP:CYDV-P. GFP:SYDV-M localized to intranuclear membranes in virus-infected cells, while GFP:SYDV-G was found on endomembranes in the presence or absence of virus.

**DISCUSSION**

We have produced a PILM for the CYDV strain of PYDV. The SYDV and CYDV strains represent the closest related plant-adapted rhabdoviruses for which PILMs have been produced. Despite their close sequence relatedness, there are significant contrasts in the protein interaction and localization patterns, which provides insights at the molecular and cellular levels for the contrasting biology of these viruses.

Of particular interest is the difference in CYDV-N of the region spanning the NLS that was mapped in SYDV-N protein (QKRANEEAPPAAQKR) [29]. While the 10 aa spacer is maintained between the paired KR residues essential for nuclear localization, the sequence of the spacer is not conserved, nor are the KR residues flanked by glutamines. Functional mapping will have to verify the KRTAE-DATTQQTKR sequence in CYDV-N as being a bona fide NLS. If this is indeed the case, then the charge and sequence variation may explain the marked difference in localization...
patterns of the PYDV-N proteins, particularly as variation in NLS sequence greatly impacts affinity and isoform selectivity for nuclear import receptors including importin-α, which is the presumed karyopherin for SYDV-N [29, 31]. If this is not the NLS region, then CYDV-N must utilize an entirely different signal to facilitate targeting of this protein to the nucleus. However, given the 71% sequence identity (83% similarity) of the CYDV- and SYDV-N proteins, there is no readily identifiable region that might encode an alternative NLS in CYDV-N.

In addition to differences in nuclear localization patterns per se, an intriguing result is the observation that CYDV-Y is targeted to the nuclear envelope, while its SYDV cognate is not. The primary structure of these proteins is 74% identical and 88% similar, with the dissimilar residues dispersed over the entire length of the proteins. As such, there is no obvious region in CYDV-Y that readily accounts for targeting of this protein to the nuclear envelope. However, differential interaction with a nuclear transport receptor may account for the differential localization pattern [32]. Therefore, the nuclear envelope-targeting region will have to be mapped physically [29]. The difference in partitioning of the Y proteins is maintained in the context of infection, with the SYDV-Y accumulating in the nucleus whereas the CYDV-Y does not. Assuming that the Y protein mediates cell-to-cell transport of PYDV strains, then a differential in the efficiency of transport may, in part, account for the differential symptom severity of these viruses in plants.

| BiFC | CFP | Overlay |
|------|-----|---------|
| N/N |     |         |
| N/X |     |         |
| N/P |     |         |
| N/Y |     |         |
| N/M |     |         |
| N/G |     |         |
| P/P |     |         |
| N/GST | | |

*Fig. 4.* All pairwise combinations for CYDV-encoded proteins, except L, were assayed in bimolecular fluorescence complementation experiments. Specific combinations are listed on the left-hand side of each column of single-plane confocal micrographs that show the location of YFP fluorescence (BiFC) relative to that of the CFP-marked nucleus (CFP). Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing CFP fused to the nuclear marker protein Histone 2B. The merger of the BiFC and CFP channels is also shown (Overlay). Protein fusions to each half of YFP were tested in all pairwise interactions, of which only a subset is shown here. Glutathione-S-transferase (GST) was used as a non-binding control. The majority of BiFC-negative results are not shown, save those necessary to confirm specificity of binding in the positive assays. Scale bar, 10 µm.
While the effect of any single difference in the localization and interactions of PYDV proteins on the general interaction of this virus with its plant host cannot be determined from the present study, collectively it stands to reason that a ‘summation’ of these differences has resulted in vector and plant selectivity. More broadly, it is interesting to note that every plant-adapted rhabdovirus has a unique PILM [2, 10, 11, 18]. All of these PILMs were constructed using BiFC assay in leaf epidermal cells of *N. benthamiana*. Given the uniformity of assay conditions, the differential interactions should be a reflection of the intrinsic properties of each viral protein. BiFC is known to report only very stable interactions, and thus a lack of detecting any particular interaction, e.g. P/P for SYDV or N/N for CoRSV, does not mean that these interactions do not occur but only that they are not stable enough to yield BiFC-positive results. Each protein–protein interaction in the BiFC, and every interaction in general, is governed by a particular dissociation constant ($K_D$) [33]. Thus, variation in PILMs, in part, likely represents variation in $K_D$ for each viral protein. Extrapolating from the PILMs, it is not uncommon for viral proteins to interact with at least one, and often many more, host cell proteins [1, 2, 34]. Therefore, virus evolution, in particular adaptation to hosts and vectors, must be restricted or permitted according to the efficiency of binding of interaction domains in viral and host proteins [35–37]. Moreover, infection by viruses induces global changes in alternative splicing of host mRNAs [38]. This alternate splicing may alter protein-interaction domains in host factors [39, 40]. Furthermore, interaction with viral proteins can cause dramatic changes in localization of host factors, which may alter their ability to interact with their normal interactors [2]. Coupled with this is the extensive alteration of host cells, particularly nuclear structure, that is evident in CYDV- and SYDV-infected cells. In short, the protein interactome encountered by individual viral proteins is likely to be something quite different from that which exists in the absence of infection.
Taken together, we posit that virus–host cell compatibility is governed, in part, by summation of the efficiency by which viral proteins interact with each other and with host factors. These interactions, in turn, are governed by the $K_D$ for each interaction. By corollary, adaptation to new hosts or vectors will be governed by the existence of requisite interaction domains in host factors in new hosts, or sufficiently rapid selection of virus variants from the quasi-species cloud upon entry to a new host or vector. This hypothesis is supported by phylogenetic evidence, which demonstrates that plant-adapted species group according to their insect vector, thus it is likely that insects are the key driver of speciation for this group of viruses [18]. It is intriguing that the X protein of CYDV is more closely related to its cognate protein in EMDV than to SYDV. While there is no firm evidence for recombination between or among these viruses, the solanaceous hosts common to both may have provided such an opportunity [41]. Thus, variation in PILMs is likely expected given the diverse host range that can be collectively infected by the plant-adapted rhabdoviruses for which PILMs have been generated. Furthermore, within a single-host species, e.g. *N. benthamiana*, plant-adapted rhabdoviruses exhibit a wide range of pathogenicity, with some viruses expressing a recovery phenotype (SYNV) [26], taking exceptionally long (weeks) to establish infections (PYDV) [26], or requiring plants to be maintained at elevated temperatures in order to establish systemic infections (CoRSV) [10].

Mechanistic investigation of the hypotheses above will require expansion of the availability of recombinant viral systems [42, 43] and detailed biochemical characterization of rhabdoviral protein complexes, with particular attention paid to the determination of $K_D$s for interactions contributing to PILMs, as well as a broader characterization of host factors that interact with plant rhabdoviral proteins [2]. However, the availability of a significant number of PILMs raises intriguing questions about their underlying molecular basis, which have implications for understanding the evolutionary trajectories of these viruses.

**METHODS**

**Virus maintenance and purification**

All plants, including transgenic *N. benthamiana* lines expressing autofluorescent proteins fused to histone 2B, a
nuclear marker, or RFP-HDEL (endomembrane marker), were maintained in the greenhouse under conditions reported previously [30]. Potato yellow dwarf virus strain CYDV (American Type Culture Collection accession PV-233) was maintained by serial passage in N. benthamiana and N. rustica plants housed in insect-proof cages. As reported for SYDV, CYDV was purified on sucrose density gradients, as described previously [26]. Field isolates of CYDV were collected from infected tomato (Solanum lycopersicum) in 2010, and black nightshade (Solanum nigrum) and pepper plants (Capsicum annum) in the fall of 2016 in Beltsville, MD (Hammond, unpublished data). This isolate will hereafter be referred to as CYDV\textsuperscript{MD}.

**Isolation of total RNA, RT-PCR**

Total RNA was extracted from plant tissues using the Qiagen RNAeasy Plant minikit (Qiagen). Except where noted, first-strand cDNA synthesis and PCRs were carried out using Superscript reverse transcriptase IV (Thermo Fisher Scientific) and Phusion high-fidelity DNA polymerase (Finnzymes), respectively.

**ION Torrent sequencing**

The genomic sequence of CYDV was determined using the same ION Torrent sequencing pipeline utilized for determination of the CoRSV genome [10]. All library construction and sequencing steps were performed by staff of the Advanced Genetic Technology Center (University of Kentucky). Poly(A)\textsuperscript{−}-RNA was purified from total RNA isolated from CYDV-infected leaves of N. rustica at 30 days post inoculation using a Dynabeads mRNA Purification Kit according to the manufacturer’s instructions. Template cDNA was prepared using an IonPGM Template OT (OneTouch) 200 Kit. Sequencing was performed with an Ion PGM Sequencing 200 Kit and the Ion 316 chip. Contigs were assembled from the high-quality read data using the Trinity assembler package [44].

**RACE**

3'- and 5'-RACE were performed with the BD-SMART RACE cDNA Amplification kit according to the manufacturer’s instructions (Thermo-Scientific). For these analyses, cDNA was synthesized by MMLV reverse transcriptase, and PCRs were conducted with Advantage-II DNA polymerase (Clontech).

**DNA sequence analysis**

Homology searches were used to compare CYDV sequences to the genomes of other rhabdoviruses using various BLAST tools provided on the National Center for Biotechnology Information (NCBI) server. ORFs were identified using the ORF finder search tool [45]. The primary structures of proteins encoded by CYDV were analysed using a variety of algorithms provided by the Expasy proteomics server [46], including Compute pI/MW [47], PSORT for prediction of protein localization [48], SignalP for prediction of signal peptide cleavage sites [49] and NetNGlyc for prediction of N-glycosylation sites [50].

**Phylogenetic analysis**

All L-protein sequences used in the sequence alignment study were obtained from data deposited in the NCBI database. In addition to that for CYDV, L-gene sequences utilised in phylogenetic analyses include the following: Coffee ringspot virus – Lavras strain (CoRSV; KF812526), Eggplant motiled dwarf virus (EMDV; NC_025389), Lettuce necrotic yellows virus (LNYV; AJ867584), Maize mosaic virus (MMV; AY618418.1), Sonchus yellow net virus (SYNV; L32603.1), Maize fine streak virus (MFSV; AY618417.1), Potato yellow dwarf virus (PYDV; NC_016136.1), Maize Iranian mosaic virus (MIMV; DQ186554), Northern cereal mosaic virus (NCMV; NC_002251.1), Orchid flock virus (OFV; NC_009609), Rice yellow stunt virus (RYSV; NC_003746.1), Taro vein chlorosis virus (TaVCV; NC_006942.1) and Vesicular stomatitis Indiana virus (VSIV; NC_001560.1).

Sequence alignment and phylogenetic trees, generated using the neighbour-joining method with a bootstrap test with 1000 replicates, were conducted using the Phylogeny.fr suite of online tools, as described previously [51, 52]. A partial sequence of the L gene from CYDV\textsuperscript{MD} (isolated from tomato) was recovered by PCR using generic plant rhabdovirus primers [53]. This sequence had a 99 % nucleotide sequence identity to CYDV, and was deposited in GenBank as accession (no. JQ405264).

**Protein expression in plant cells**

Sequence-validated clones in vector pDONR221 (Invitrogen) of all CYDV ORFs, except L, were recombined into the appropriate binary vectors for localization of fluorescent protein fusions in plant cells [30, 54]. Tests for protein interactions were conducted using BiFC assays as described previously [10–12, 55]. Importantly, the conversion of the pSAT-based vectors to allow Gateway recombination-based cloning entirely eliminated the high background when ‘empty’ vectors expressing the two halves of YFP were co-expressed. As such, we have determined that false positives are less likely to occur when using the pSITE-BiFC vectors [2, 55]. Therefore, the vectors employed in this study were pSITE-2CA (GFP fusions) and localization experiments, and the pSITE-BiFC-nEYFP and pSITE-BiFC-cEYFP vectors for BiFC assays. Recombinant vectors were transformed into Agrobacterium tumefaciens strain LBA4404. Agroinfiltration for expression of protein fusions in plant cells was conducted essentially as described previously [30].

**Laser scanning confocal microscopy**

Microscopy for this study was conducted using an Olympus FV1000 laser-scanning confocal microscope as described previously [30].

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This research was conducted to the highest ethical standards.

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