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Yeast two-hybrid and pull-down assays propose an interaction between P50 of apple chlorotic leaf spot virus and PR-10 of *Malus sylvestris* cv. R12740-7A

Y. WANG, N. LI, X. ZHAO, J. HU, Y. HE, T. HU, S. WANG, Y. WANG*, K. CAO

College of Plant Protection, Agricultural University of Hebei, Baoding Hebei, P. R. China

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Summary. – Apple chlorotic leaf spot virus (ACLSV) movement protein (P50) is involved in cell-to-cell transport and influences the long-distance spread of silencing activity. Previously, we obtained 69 P50-interacting proteins from *Malus sylvestris* cv. R12740-7A and using bioinformatics analyzed their biological functions. In this study, we used the GAL4-based two-hybrid yeast system and His pull-down assays to confirm an interaction between PR-10 of *M. sylvestris* cv. R12740-7A and ACLSV P50. Our results provide a theoretical basis for further research on the biological function of PR-10 in ACLSV infection and the interacting mechanism between host and virus.

Keywords: apple chlorotic leaf spot virus; Malus sylvestris cv. R12740-7A; P50; PR-10

Apple chlorotic leaf spot virus (ACLSV) infects a wide range of fruit trees, including apples, pears, plums, quinces, cherries, apricots and peaches (German-Retana, *et al*., 1997; Németh, 1986). It is the most frequently encountered virus in almost all apple orchards in China, often causing latent infections (Desvignes *et al*., 1992; Waterworth, 1993). Although most strains are latent in apple trees, others are responsible for apple russet ring spot, apple top working disease and lethal decline of apple on some rootstock varieties (Desvignes and Boyé, 1989). The virions of ACLSV are flexuous filamentous particles of 720 nm × 12 nm (Brunt *et al*., 1996). The ACLSV genome consists of 7,555 kb long single-stranded RNA molecule, excluding the poly-A tail. Its open reading frames (ORFs) 1, 2 and 3 encode proteins with molecular masses of 216.5, 50.4 and 21.4 kDa, respectively (German *et al*., 1990). The 216.5 kDa ORF encodes a protein possibly involved in viral replication, the 50.4 kDa protein is a putative movement protein, and the 21.4 kDa product is the viral coat protein (German *et al*., 1992).

*Corresponding author. E-mail: wyn3215347@163.com, phone: +863127528157. Y. Wang and N. Li contributed equally to this paper.

Abbreviations: ACLSV = apple chlorotic leaf spot virus; TMV = tobacco mosaic virus; P50 = movement protein; PR-10 = pathogenesis-related protein; Y2H = yeast two-hybrid system

The ACLSV movement protein (P50) is reported to be multifunctional. Observation of transgenic leaves from *Nicotiana occidentalis* expressing the 50 kDa protein fused to enhanced green fluorescent protein (EGFP) revealed that ACLSV P50 targets plasmodesmata and accumulates in the sieve elements of transgenic plant leaves (Yoshikawa, *et al*., 1999, 2006). The protein spreads from cells that produce it into neighboring cells, enabling cell-to-cell trafficking of GFP when P50 and GFP (P50-GFP) are co-expressed in the leaf epidermis. P50-GFP could complement the local spread of the P50-deficient virus when expressed transiently in the leaf epidermis of *Chenopodium quinoa*. Expression of P50-GFP in protoplasts resulted in the production of tubular structures protruding from the protoplast surface (Satoh *et al*., 2000). ACLSV P50 can also interfere with the intra- and inter-cellular targeting and tubule-inducing activity of the 39 kDa putative movement protein of grapevine berry inner necrosis virus (Isogai *et al*., 2003). This protein also has two independently active, single-stranded nucleic acid-binding domains (Isogai and Yoshikawa, 2005). In addition to these functions, a recent study revealed that P50 functions as a silencing suppressor, inhibiting systemic silencing in *N. benthamiana* without interfering with local silencing (Yaegashi *et al*., 2007). Other data suggest that P50 is a unique suppressor, specifically inhibiting the long-distance move-

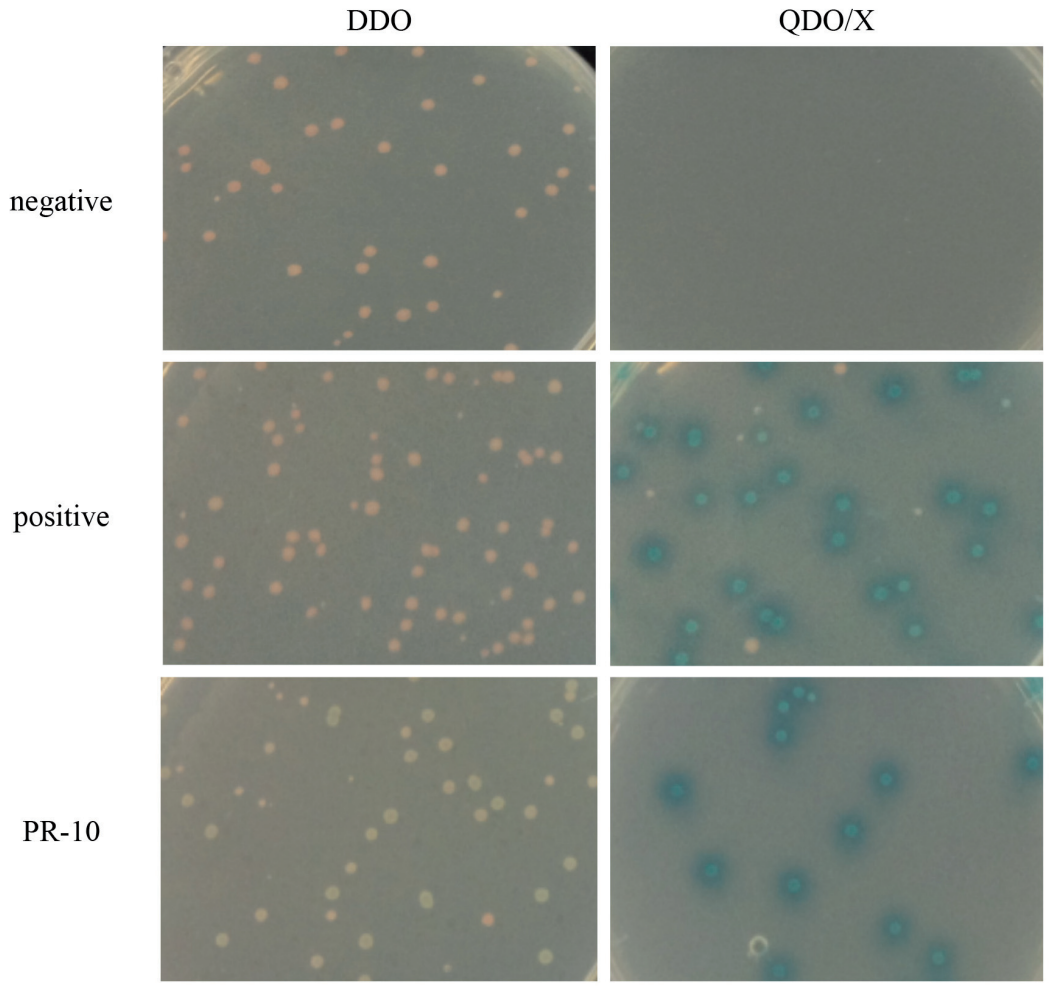

Fig. 1

Interaction between the pathogenesis-related protein (PR-10) from *Malus sylvestris* and the movement protein (P50) from apple chlorotic leaf spot virus in a yeast two-hybrid assay

Negative control: yeast Y2H gold harboring pGBKT7-Lam and pGADT7-T. Positive control: yeast Y2H gold harboring pGBKT7-53 and pGADT7-T. PR-10: yeast Y2H gold harboring pGBKT7-P50 and pGADT7-PR-10. DDO: SD/-Leu/-Trp medium. QDO/X: SD/-Ade/-His/-Leu/-Trp/X-α-Gal medium.

ment of silencing signals through phloem cells (Yaegashi *et al*., 2008). Although many functions of P50 have been reported, little is known about the molecular pathway by which ACLSV P50 interacts with its host. The host proteins essential for interaction with P50 remain largely unknown.

To address this issue, our group used a high-throughput yeast two-hybrid (Y2H) screening by the Matchmaker™ Gold Yeast Two-Hybrid system (Clontech, BD Biosciences, USA) to screen P50-interacting proteins from cDNA library of *M. sylvestris* cv. R12740-7A (Duan *et al*., 2014). To test whether the P50 of ACLSV and the PR-10 from *M. sylvestris* cv. R12740-7A, which was one of P50-interacting proteins obtained by Y2H system, interact physically, we amplified the full-length sequence of PR-10 from the corresponding cDNA clone using PCR, and then cloned it into pGADT7, using the *Eco*RI/*Bam*HI sites, to form pGADT7-PR-10. pGADT7-PR-10 was then used as prey and P50 as bait in the Y2H system. Bait and prey plasmids were mixed and co-transformed into Y2H gold yeast strain. Co-transformation of pGBKT7-53 and pGADT7-T was used as positive controls and pGBKT7-Lam and pGADT7-T as negative controls. Self-activation of P50 and PR-10 has been ruled out in our previous study (data not shown). pGBKT7-P50 and pGADT7-PR-10 were then co-transformed into yeast strain Y2H gold, and the mixture was incubated for 3-5 days at 30°C on SD/-Leu/-Trp (DDO) medium and SD/-Ade/-His/-Leu/-Trp/X-α-Gal medium (QDO/X). Protein interactions were monitored by comparing the growth and color of the yeast with the positive and

negative controls. The results showed that the yeast strain Y2H gold harboring pGBKT7-P50 and pGADT7-PR-10 (AD-PR-10/BD-P50) grew well in the medium lacking tryptophan and leucine, and formed clear blue colonies in QDO/X medium, indicating an interaction between P50 and PR-10 (Fig. 1).

To confirm the interaction between PR-10 and P50, we conducted an *in vitro* pull-down assay. ORF of viral protein P50 in vector pET21a containing His-tag and ORF of *M. sylvestris* cv. R12740-7A PR-10 without His-tag were transformed into *E. coli* Rosetta (DE3) (Novagen). The cells were induced with 0.1 mmol/l IPTG, harvested by centrifugation, and then washed with Tris-buffered saline (TBS, 25 mmol/l Tris, 0.15 mol/l NaCl, pH 7.2) at 4°C. Then 0.1 mol/l phenylmethanesulfonyl fluoride was added to the cells to a final concentration of 1 mmol/l and then sonicated on ice. The lysate was centrifuged at 11,000 × g for 10 min and the precipitate and supernatant were collected for analysis on SDS-PAGE. The virus movement protein (P50) was purified with a 6 × His-tagged protein purification kit (Cwbiotech, China) according to the manufacturer's instructions and 5 µl of the purified protein was separated on SDS-PAGE (Fig. 2). We used western blot analysis with His-HRP to confirm that P50 contained His-tag (Fig. 2, lanes 8–9).

A His pull-down assay was performed using the Pierce™ His Protein interaction pull-down kit (Pierce Biotechnology, USA) according to the instructions of the manufacturer. Briefly, the purified His-tag fusion P50 protein was immobilized on HisPur Cobalt resin for at least 30 min at 4°C with gentle rocking and then each column placed in a collection tube. The tubes were centrifuged at 1,250 × g for 1 min and the supernatant was collected (= bait flow-through). The columns were then washed five times with wash solution. Approximately 800 µl of prepared PR-10 protein sample was then added to the columns and incubated for at least 1 h at 4°C on a gently rotating platform. The columns were inserted into collection tubes and centrifuged at 1,250 × g for 30–60 s (= prey flow-through). The columns were washed several times using 400 µl of wash solution for each washing. Then 250 µl of elution buffer was added to the column and incubated for 5 min with gentle rocking. After centrifugation, bound proteins were removed by the elution buffer (= elution fraction). Each sample was then analyzed by SDS-PAGE (Fig. 3). The elution fraction yielded bands of 50.8 and 17.6 kDa indicating that the P50 of ACLSV had captured the 17.6 kDa PR-10 protein (Fig. 3, lane 6) and the protein PR-10 was confirmed by sequencing (data not shown). To exclude non-specific binding, TBS was used as bait and PR-10 was used as prey protein in the pull-down assay as negative control, excluding PR-10 own affinity to the His resin (Fig 3, lane 7). The protein products expressed by the cells transformed with the empty pET21a vectors were used as prey protein and P50 was used as bait in the pull-down assay as another negative control (data not shown).

Plant viruses spread systemically by cell-to-cell movement through plasmodesmata and are transported on long-distances through the plant's vascular system (Carrington *et al.*, 1996; Lazarowitz, 1999). ACLSV P50 is assigned to the 30K superfamily of virus movement proteins (Mushegian and Koonin, 1993). It has two independently active,

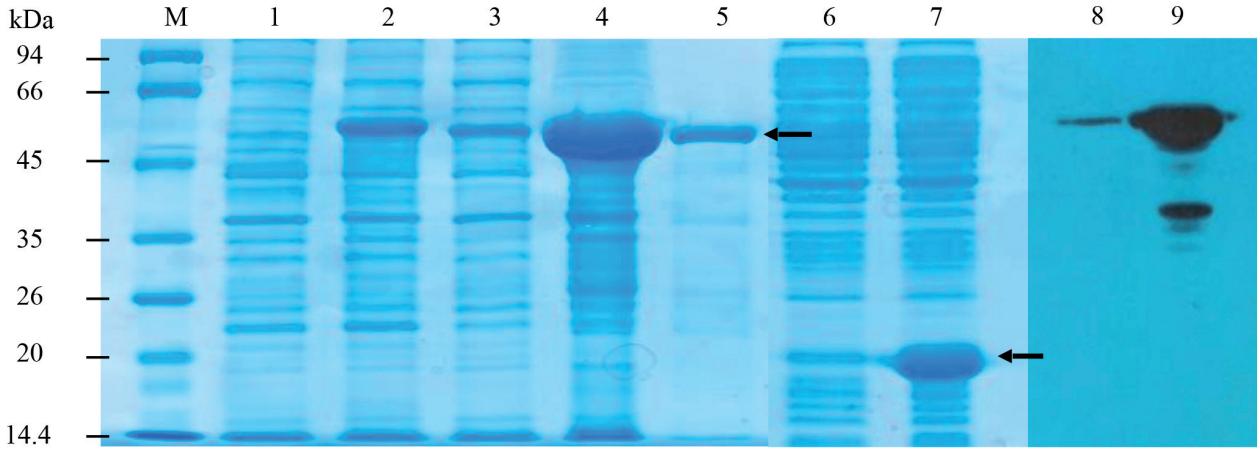

Fig. 2

Expression and purification of the P50 protein of ACLSV and PR-10 from *Malus sylvestris*

12% SDS–PAGE profile of total extracts of *E. coli* Rosetta (DE-3) containing plasmid pET21a-P50, lane 1: uninduced; lane 2: induced by 0.1 mmol/l IPTG; lane 3: supernatant after sonication; lane 4: precipitate after sonication; lane 5: purified lysate. 12% SDS–PAGE profile of total extracts of *E. coli* Rosetta (DE-3) containing plasmid pET21a- PR-10, lane 6: uninduced; lane 7: induced by 0.1 mmol/l IPTG. Western blot analysis of ACLSV P50 protein by His-HRP, lane 8: uninduced; lane 9: after induction. M: Marker.

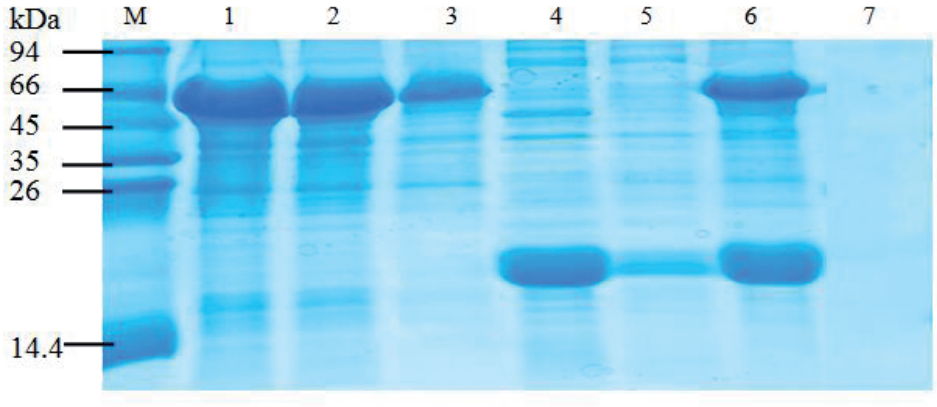

Fig. 3

Pull-down assay of the interaction between the P50 of ACLSV and PR-10 of *Malus sylvestris*

Pull-down assay of, lane 1: bait protein P50; lane 2: bait protein P50 flow-through; lane 3: supernatant of denatured bait protein-resin; lane 4: prey protein PR-10; lane 5: prey protein PR-10 flow-through; lane 6: elution of bait protein P50 and prey protein PR-10; lane 7: negative control (TBS as bait and PR-10 as prey protein). M: Marker.

single-stranded nucleic acid-binding domains (Isogai and Yoshikawa, 2005). Two general mechanisms of action are employed by the 30K superfamily. The first is a tobacco mosaic virus (TMV)-like mechanism that transports viral genomic RNA with a movement protein through plasmodesmata, without extensive structural modifications (Carrington *et al*., 1996; Deom *et al*., 1992; Lazarowitz and Beachy, 1999; Lazarowitz, 1999; Lucas *et al*., 1993), while the second mechanism is tubule based mechanism which moves complete virions along newly formed tubular structures. In addition to the movement protein function, recent work has revealed that P50 also functions as a silencing suppressor and inhibits systemic silencing in *N. benthamiana* (Yaegashi *et al*., 2007).

Until now, most research on ACLSV had focused on the virus itself and little is known about its interaction with host factors. Though many functions of P50 have been reported, there is no experimental evidence indicating a physical interaction between P50 and host factors involved in the plant's immune response. The results of our study, however, suggest that PR-10 from *M. sylvestris* cv. R12740-7A interacts directly with the P50 of ACLSV.

The PR-10 family is one of the most important families among the 17 pathogenesis-related protein families. PR-10 proteins are different from most other PR proteins, as they are typically small, acidic, cytosolic molecules with a conserved three-dimensional structure (Liu and Ekramoddoullah, 2006; Liu *et al*., 2006; Xie *et al*., 2010; Ziadi *et al*., 2001). Two ginseng PR-10 proteins showed RNase activity, and other members of the PR-10 family displayed RNA-degrading activities (Moiseyev *et al*., 1997; Bantignies *et al*., 2000; Kim *et al*., 2008). In addition, CaPR-10 isolated from hot pepper (*Capsicum annuum*) was demonstrated to display RNase activity and is involved in antiviral processes *in vitro* directly (Park *et al*., 2004).

New evidence reveals that PR-10 proteins posses also some other functions (Koistinen *et al*., 2005; Radauer *et al*., 2008; Sikorski *et al*., 1999; Wen *et al*., 1997). For example, Wang *et al*. (1999) reported that PR-10 genes in lilies (*Lilium* spp.) are induced by abscisic acid (ABA) and methyl jasmonate (MeJA), two separate signal transduction pathways found in the plant's anthers and other organs (Wang *et al*., 1999). Several proteins interacting with VpPR-10.1 protein from Chinese grapevine (*Vitis pseudoreticulata*) included CNR8, UFGT6, HSP, DEAD-box, Trx h2, Grx C9 and GLOX. These proteins are closely related to the plant's defensive action against pathogens and also abiotic stress. The grapevine study also suggested that the VpPR-10.1 gene may be involved in hormone signaling, programmed cell death, and defense responses of the plant (Xu *et al*., 2013). Another study showed that the PR-10 protein CaARP functions as an aldo/keto reductase to scavenge cytotoxic aldehydes (Jain *et al*., 2015). Leucine-rich repeat 1 (LRR1) protein interaction with CaPR-10, enhanced HR-like cell death phenotype and activated defense signaling (Choi *et al*., 2012). A positive transcription factor, WRKY b, was shown to bind the PR-10 promoter and activated the defense signaling pathway in pepper (*Capsicum annuum*) (Lim *et al*., 2011).

Several viruses can trigger a PR-10 response in their hosts (Park *et al*., 2004; Pinto and Ricardo, 1995; Puhringer *et al*., 2000; Xu *et al*., 2003). The CaPR-10 protein existed at low levels in the leaf tissue of hot pepper (*C. annuum*), but was dramatically induced when plants were inoculated with TMV-P_0. This increase correlated with an elevated ribonucleolytic activity (Park *et al*., 2004). Cucumber mosaic virus (CMV) and D satRNA (CMV/D satRNA) infected

tomato plants showed specifically activated transcription of several tomato defense-related genes, including PR-10 (Xu *et al*., 2003). The promoter sequence of Ypr10*a from *Malus domestica* has been isolated and characterized. Ypr10*a-GUS transgenic expression in transformed tobacco plants was strongly induced following inoculation with TMV and two potyviruses, tobacco etch virus and tobacco vein mottling virus (Satoh *et al*., 2000).

All researches showed that PR-10 might play a great role in the resistance of plant against virus. Until now, little information is available for the function of PR-10 in *Malus* plants. In this study, we found that PR-10 from *M. sylvestris* cv. R12740-7A interacted directly with the P50 of ACLSV. Our results provide a basis for the biological function of *M. sylvestris* cv. R12740-7A PR-10 in the ACLSV infection process and an interacting mechanism between host and virus. Although the pull-down assay indicated the interaction *in vitro*, it may not occur in living plant cells. Therefore, additional experiments, such as bimolecular fluorescence complementation, co-immunoprecipitation, and the localization of P50 and PR-10 in ACLSV-infected plants by immunoelectron microscopy, should be performed to provide additional lines of evidence for their interaction.

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