Fungal Zoospore-Mediated Delivery of a Foreign Gene to Wheat Roots

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ABSTRACT

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The primitive fungus *Olpidium brassicae* is an obligate plant parasite that acts as a natural virus vector transmitting tobacco necrosis virus and certain other viruses to roots of many monocotyledonous and dicotyledonous plants. Plasmid pAM981, carrying the chloramphenicol acetyltransferase (CAT) gene, was packaged by dissociated capsid protein of tobacco necrosis virus. The resulting nucleoprotein complexes were acquired by *O. brassicae* zoospores and transmitted to wheat roots. Transient expression of CAT in wheat roots was detected, indicating that transformation can be achieved if plants can be regenerated from root tissue.

Additional keywords: genetic engineering, plant transformation, vector.

Interest in transformation of crop and horticultural plants with enhanced agronomic traits and resistance to disease and drought is very high. Transformation of many dicotyledonous plants with the aid of *Agrobacterium* has become fairly routine. However, transformation of some of the most important crop plants, such as the cereals and legumes, has proven difficult with *Agrobacterium*. *Agrobacterium* can attach to wheat callus cells, but no stable transformation has been obtained (3). Foreign DNA has been introduced into plants by means other than *Agrobacterium*. Recent reports have described wheat and rice transformation and regeneration with electroporation (13,14,20). DNA-coated microprojectiles used to bombard intact maize, rice, and immature wheat embryo cells; direct DNA injection of immature rice inflorescences; and imbibition of DNA by dry embryos have also been used as delivery systems (4,9-11,23).

Olpidium is an obligate fungal parasite of plant roots. It has a very wide host range and worldwide distribution. Monocotyledonous as well as dicotyledonous plants are infected. *Olpidium* is a primitive, zoosporic, nonmycelial fungus that infects plant cells in the cortex of the root tip in the meristematic and cell elongation regions. *Olpidium* was the first fungus identified as a vector of a plant virus, tobacco necrosis virus (TNV) (16). The virus and its satellite virus (STNV), also *Olpidium* transmitted, have a worldwide distribution (7,8). Both TNV and STNV are isometric, nonenveloped plant viruses with monopartite, single-stranded RNA genomes (1,7). TNV virions are about 28 nm in diameter and have a single capsid protein (M_r 30,000) (21). The smaller STNV has virions that are 17 nm in diameter and have a single coat protein (M_r 22,000) (1).

Attachment of both TNV and STNV to the zoospore flagellum and outer membrane has been clearly demonstrated (19). Stobbs et al (15) have also shown that cucumber necrosis virus, similarly vectored by *O. radicale*, both binds to and is internalized within zoospores. *Olpidium* zoospores exposed to ^{125}I-labeled TNV and roots and then processed for autoradiography showed TNV only external to zoospores that were attached to the root surface (W. G. Langenberg, *unpublished*). Transmission of TNV to roots of plants by *Olpidium* zoospores is efficient. Virus can be acquired and transmitted from a virus solution so dilute that mechanical inoculation fails to establish infection (5).

We describe here experiments in which the bacterial chloramphenicol acetyltransferase (CAT) reporter gene was transferred to wheat roots by zoospores of *O. brassicae* (Woronin) P. A. Dang and expressed in those roots.

MATERIALS AND METHODS

Isolation and purification of TNV and STNV. A mixture of TNV and STNV was isolated as described previously (25).

Preparation of capsid protein. The virus-containing band was removed from a gradient and dialyzed free of CsCl against several changes of glass-distilled water (12,000-14,000 MW cut-off; Spectrapor, Los Angeles, CA). The virus concentration was estimated by absorbance at 260 nm with an extinction coefficient of 5.5 mg/cm^2 (21). The mixture of TNV and STNV (0.3-0.7 mg/ml) was disrupted by the addition of EDTA, pH 8.0, to 15 mM and incubation at 5 C for 1 h. KCl was then added to a final concentration of 0.4 M. The solution was incubated for another hour at 5 C, and then protease-free ribonuclease (bovine pancreatic ribonuclease, type X11-A; Sigma, St. Louis, MO) was added (1 μg/ml,) and the solution was incubated at room temperature for 1 h. This RNase-containing capsid protein solution was used to encapsidate DNA.

Source and construction of plasmid pAM981. The plasmid pAM981 was constructed by ligating a fragment containing cauliflower mosaic virus 35S promoter-CAT fusion and T-DNA (transferred DNA) nopaline synthase gene terminator from pCaMVCN (Pharmacia, Uppsala, Sweden) into the *Hin*cII site of pUC19 (24) (Fig. 1).

Encapsidation of plasmid pAM981 in TNV structural protein. Capsid protein and CsCl-purified plasmid DNA (also dialyzed

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against glass-distilled water) were mixed at a 2:1 ratio of protein to DNA (w/w). The mixture was dialyzed against glass-distilled water for 2 h at 5 C and then against glass-distilled water containing 10 mM NaCl, 10 mM $MgCl_2$, and 30 mM $CaCl_2$ overnight (18 h) at 5 C.

Acquisition of pseudovirions by *Olpidium* zoospores. *Olpidium* can be freed of naturally occurring TNV and STNV by air drying roots containing *Olpidium* resting spores (2,17). Virus-free *Olpidium* was isolated from 23-yr-old dried lettuce roots and cultured on mung bean (*Vigna radiata* (L.) R. Wilcz.), wheat, and lettuce roots.

Transmissibility of the virus by *Olpidium* was maintained by regularly (once every 2–3 mo) reisolating virus transmitted to mung bean roots by zoospores. Local lesions on mung bean roots served as a source of inoculum to increase the virus in primary leaves of cowpea seedlings by manual inoculation. Unless this precaution was taken, mutants that were not fungus transmissible came to predominate, and *Olpidium* zoospores failed to acquire nucleoprotein particles and transmit them to wheat roots.

Water was withheld for 12–24 h from wheat plants that had been root inoculated with a zoospore suspension 3–6 wk earlier and grown in sand in small plastic pots. Plants were removed from the pots, and adhering sand was rapidly rinsed off the roots with running tap water. Roots were then placed in a minimal amount of distilled water. Zoospores were released from zoosporangia within minutes. Fifteen minutes later, the water was decanted into 50-ml centrifuge tubes, and zoospores were concentrated by centrifugation for 5 min at 4,000 *g* in a swinging bucket rotor (HB-4) in a refrigerated RC-2B centrifuge (Sorvall, Norwalk, CT). Refrigeration was necessary because *Olpidium* zoospores are sensitive to temperature (18). At the conclusion of the centrifugation, water was aspirated to approximately the 2-ml level, the centrifuge tube was gently swirled, and pseudovirions were added. In a typical experiment, the pseudovirions were produced from 200 μg of capsid protein and 100 μg of plasmid DNA and added to 5×10^6 zoospores. The solution of zoospores and pseudovirions was incubated at 20 C for 30 min. No attempt was made to remove unencapsidated DNA or excess TNV capsid protein.

Wheat seedlings (allowed to germinate for about 36 h) with primary roots 0.5–1.5 cm in length were placed in the zoospore-pseudovirion solution in the dark for 4 h at 20 C. Controls consisted of wheat primary roots exposed to zoospores and unencapsidated plasmids, to unencapsidated plasmids only, or to zoospores only. Other controls consisted of manual inoculation of Celite-dusted cowpea primary leaves, wheat leaves, or roots with pseudovirions encapsidating plasmid pAM981.

Fig. 1. Features of plasmid pAM981 (24).

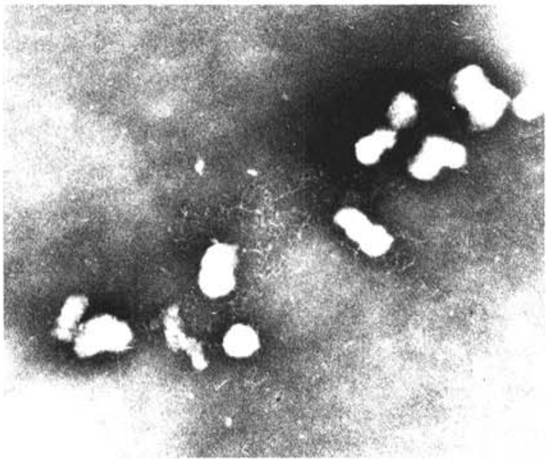

Fig. 2. Nucleoprotein particles of tobacco necrosis virus structural proteins encapsidating a 4.7-kb plasmid or plasmids.

Heat treatment of roots and growth of plants. After the incubation period, seedlings were placed in distilled water maintained at 40 ± 0.5 C for 10 min to kill zoospores (18), removed with forceps, and blotted on paper towels. Seedlings were planted in 6-cm-diameter plastic pots in coarse autoclaved sand and grown for 2 days in continuous light at 20 C. Pots were watered with a 1:5 diluted Hoagland balanced salt solution without micronutrients. In the control experiments, no zoospores or very few survived the heat treatment.

CAT assays. Root tips were ground in 100 μl of plant extraction buffer (12) in a microfuge tube with a pestle (Kontes Scientific Glassware, Vineland, NJ) and assayed for CAT activity with ^{14}C-chloramphenicol according to Gorman et al (6).

RESULTS AND DISCUSSION

Since *Olpidium* zoospores naturally mediate the transfer of genetic material (viral RNA) into plants, we investigated whether other nucleic acids could be similarly introduced into plants with the fungal-viral system. Therefore, studies were carried out to determine 1) whether TNV or STNV virions could be disassembled and the released capsid proteins then assembled into particles with exogenous nucleic acids, including plasmid DNA; 2) whether such particles would bind to *Olpidium* zoospores; and 3) whether encapsidated DNA would be transferred into root cells and subsequently released and transcribed.

Analysis by electron microscopy of negatively stained preparations revealed that particles of approximately 90 nm were formed after completion of dialysis only when both DNA and protein were present (Fig. 2). Although the nucleoprotein complexes are considerably larger than the TNV icosahedron of 30 nm, the pAM981 plasmid was efficiently and stably encapsidated in TNV structural protein. Evidence that the nucleoprotein particles encapsidated the plasmids and that these did not adhere to the outside of the particles is presented in Figure 3. Plasmid DNA was not external to nucleoprotein particles, because plasmid DNA survived digestion of particles with DNase (Fig. 3, lane 5).

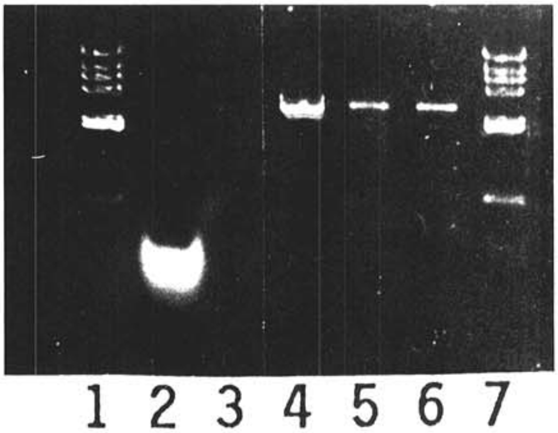

Fig. 3. Evidence for particle encapsidation of plasmid DNA. All DNA samples were digested with *Hin*dIII before they were loaded onto a 1% agarose gel containing 0.1 μg of ethidium bromide per milliliter as a stain. Lanes 1 and 7, DNA size markers of lambda DNA digest with *Hin*dIII ranging in size from 23,130 to 561 bp (Bio-Rad Laboratories, Hercules, CA). Lane 2, unencapsidated plasmid pAM981 DNA after DNase treatment. Lane 3, no DNA was present in the last supernatant of a pseudovirion preparation after three high-speed centrifugations of pseudovirions through a 20% sucrose cushion at 246,000 g_{max} in an SW 55 rotor (Beckman Instruments, Fullerton, CA) for 1.5 h. Lane 4, untreated unencapsidated plasmid DNA. Lane 5, DNA phenol extracted from pseudovirions that had been treated with DNase. Plasmid DNA remained intact. Lane 6, plasmid DNA phenol extracted from pseudovirions.

To test CAT expression in wheat root cells, roots (now 3–6 cm long) were washed free of sand, excised, and processed for CAT activity. As shown in lane 1 of Figure 4, CAT activity was present to a significant degree only in the treatment in which wheat seedlings received zoospores and nucleoprotein particles. The experiment described here was repeated twice with the same result. Each experiment was performed with newly isolated pAM981 plasmids that were encapsidated in freshly prepared, dissociated capsid proteins of TNV.

In control experiments, Celite-dusted leaves of cowpea and wheat seedlings and wheat roots were manually inoculated with particles carrying the pAM981 plasmid suspended in distilled water. Leaf and root tissues were assayed for CAT activity 60–70 h later. No CAT activity was found in any of the manually inoculated tissues (Fig. 5). It was thus not possible to manually inoculate plants with nucleoprotein particles. *Olpidium* zoospores with attached pseudovirions were harvested for CAT assay, but no CAT activity was detected (Fig. 6).

Some TNV isolates contain STNV. STNV cannot replicate independently. Both TNV and STNV have a wide host range and are probably common viruses in roots of plants worldwide. Both viruses are transmitted by *Olpidium* zoospores, although each has a distinct structural protein and no serological relationship exists between them (7,8). Our TNV purification and capsid protein isolation removed most of the contaminating STNV. Samples of different batches of purified virus or pseudovirions were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and silver stained for protein. Only one band of 30-kDa protein was found corresponding to the size of TNV structural protein (Fig. 6). Some degradation of TNV capsid protein from EDTA- and KCl-treated virions was apparent in some batches but not in others (Fig. 6). The arrow on the right side of Figure 6 indicates where a band of STNV capsid protein (22K) would have been had it been present in detectable amounts.

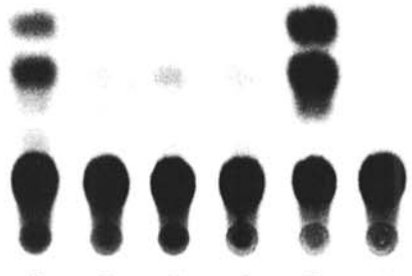

Fig. 4. Assay for chloramphenicol acetyltransferase (CAT) activity in root 60 h after penetration by *Olpidium* zoospores carrying nucleoprotein particles with pAM981 (lane 1) and in control experiments. Roots showing transient CAT activity; lane 2, roots exposed to zoospores and unencapsidated plasmids; lane 3, roots with unencapsidated plasmids only; lane 4, roots and zoospores only; lane 5, CAT enzyme positive control; and lane 6, negative control, no CAT enzyme.

Artificial mixtures of purified STNV and TNV were separated with relative ease by several cycles of density gradient centrifugation (22). Uyemoto et al (22) showed that it was practically impossible to free an existing TNV isolate of STNV. Repeated single-lesion isolation combined with a series of density gradient centrifugations of purified TNV failed to remove STNV. This can now be attributed to heteroencapsidation. For this reason and because the amount of STNV capsid protein in TNV capsid protein preparations is negligible (Fig. 6, lanes 1–5), we have not attempted to free our Nebraska isolate of STNV.

In successful experiments, zoospores were derived from wheat cultures of *Olpidium* and used to transmit pseudovirions to wheat. The influence of other *Olpidium* hosts and recipient plant species on the efficacy of nucleoprotein particle transfer by zoospores remains to be determined.

Although introduction of foreign DNA into plant cells and its transient expression do not guarantee a stable transformation, introduction of foreign DNA into plant cells by electroporation, particle bombardment, and polyethylene glycol treatment has resulted in stable transformation in several crop plants.

The plasmid used for transformation can be modified to accommodate short stretches of ribosomal DNA sequences or T-DNA border sequences that might enhance stable integration into plant genomes.

The system described here has the potential to provide a new transient expression system for intact plants. In particular, studies of organ-specific expression of plant gene promoters require the use of transgenic plants and thus are time consuming. The method outlined with virus capsid protein and *Olpidium* may provide a rapid means for carrying out such studies and will also likely shed light on the nature of viral-fungal-plant interactions. The system may be useful in investigating the viability of double-stranded RNAs. It also may provide an alternative to "agroinfection" and particle gun systems, which are currently the most common methods available for the analysis of the infectivity of cloned material from viruses for which infection with naked nucleic acid is not possible.

Local isolates of *Olpidium* and TNV should do as well as the Nebraska isolate did in encasement of other plasmids.

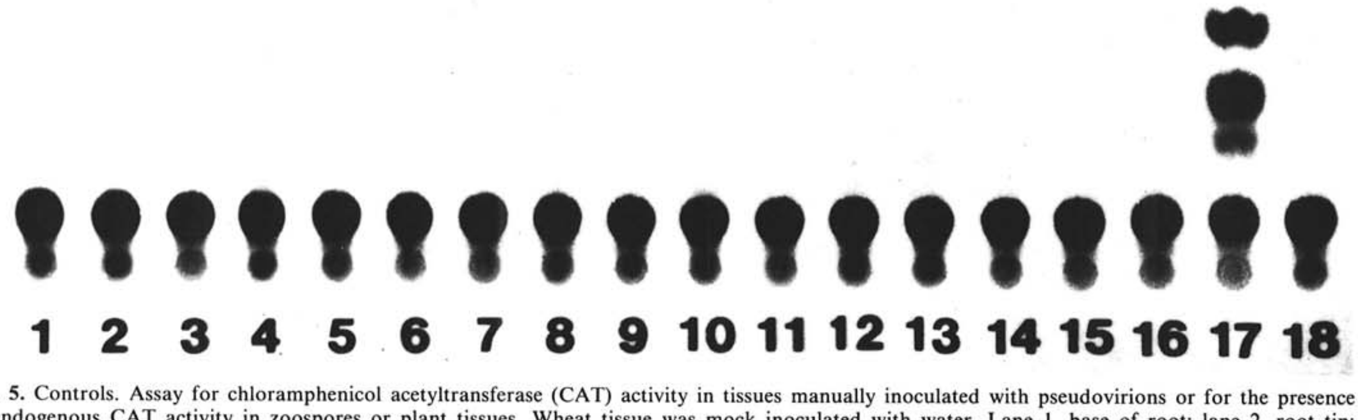

Fig. 5. Controls. Assay for chloramphenicol acetyltransferase (CAT) activity in tissues manually inoculated with pseudovirions or for the presence of endogenous CAT activity in zoospores or plant tissues. Wheat tissue was mock inoculated with water. Lane 1, base of root; lane 2, root tip; lane 3, wheat leaf (wheat tissues inoculated with pseudovirions); lane 4, root tip; lane 5, root base; lane 6, leaf tip; lane 7, leaf base; lane 8, other combined root tips (wheat tissue immersed in pseudovirions for 2 h); lane 9, root base; lane 10, root tip; lane 11, leaf; lane 12, cowpea cotyledons inoculated with water; lane 13, cowpea cotyledons inoculated with pseudovirions; lane 14, newly emerged trifoliate cowpea plant manually inoculated with pseudovirions; lane 15, *Olpidium* zoospores; lane 16, zoospores with pseudovirions; lane 17, CAT enzyme positive control; and lane 18, water-only control. None of the tissues or zoospores showed CAT activity.

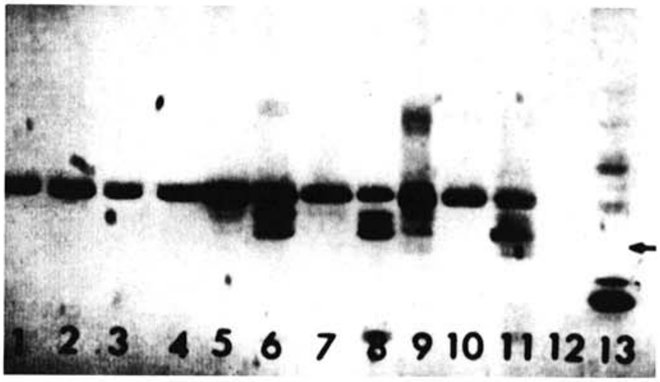

Fig. 6. Analysis of tobacco necrosis virus (TNV) capsid protein for purity by gel electrophoresis and silver staining. Lanes 1–5, purified TNV preparations as a source of capsid protein for encapsidation. Only one band of 30K is visible. Lanes 6–8, capsid proteins after EDTA-KCl disruption of TNV virions. Some degradation may have taken place during storage. Lanes 9–11, same as lanes 6–8, but preparations were not boiled in sodium dodecyl sulfate. Lane 12, blank. Lane 13, protein standards: 66.3K bovine serum albumin, 55.4K glutamic dehydrogenase, 43K ovalbumin, 39K aldolase, 28.8K carbonic anhydrase, 17.5K TMV capsid protein, and 14.3K lysozyme. Arrow indicates where a protein of 22K (satellite virus of TNV) would have been had it been present.

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