in the DNA. The damages by ionizing radiation to DNA can cause loss of viability of the cells exposed to radiation. The alkaline comet assay is an elegant and effective technique to monitor the extent of DNA damage and its protection. When human leukocytes are exposed to γ-radiation ex vivo, the cellular DNA undergoes damage, as reflected in the increase of the comet parameters (% DNA, tail length, tail moment and olive tail moment). The presence of aqueous extract of *G. lucidum* during irradiation of cells decreases the comet parameters, indicating its significant role in protection. One of the deleterious consequences of DNA damage from exposure to ionizing radiation is the induction of cancer. Protecting cellular DNA from radiation damage might result in the prevention of the cancers induced by radiation. Fungal polysaccharides of comparable structure and function as those found in *Ganoderma*, have undergone rigorous clinical trials. Based on such indirect experimental evidence, it is hypothesized that this medicinal mushroom polysaccharide might render significant relief from the side effects of both chemotherapy and radiotherapy.

The result of the present investigation reveals the potential of *G. lucidum* in radiation protection not only in radiotherapy, but also in accidental radiation exposure. The findings also suggest the possibility of using this medicinal mushroom extract as adjunct therapy in cancer radiotherapy and chemotherapy.


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### Stable transformation of *Xylella fastidiosa* with small repW shuttle vector pUFR047

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*Xylella fastidiosa* (Xf) is a fastidious, xylem-inhabiting, Gram-negative bacterium that causes serious plant diseases in a wide range of plant species. The most serious diseases are Pierce’s Disease (PD) of grape and Citrus Variegated Chlorosis (CVC). Functional genomic analyses of Xf have been severely limited by lack of a stable replicative shuttle vector. Plasmid pUFR047, small, stable, wide host range, conjugative and repW shuttle vector have been successfully transferred into *Xf* strains by electroporation. The vector replicated in a stable manner for over thirty generations of growth in the absence of antibiotic selection in *Xf* strains.

**Keywords:** Citrus variegated chlorosis, electroporation, Pierce’s disease, shuttle vector.

*XYLELLA FASTIDIOSA* (Xf) is a xylem-inhabiting, Gram-negative bacterium that causes serious diseases in a wide range of plant species¹. Two of the most serious of these

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are Pierce’s Disease (PD) of grape and Citrus Variegated
Chlorosis (CVC); both are currently inflicting major eco-
nomic losses. The genome of CVC and PD isolates of Xf
has been completely sequenced and annotated. However,
lack of useful DNA cloning vectors and/or techniques
typically used for functional genomic analyses of phyto-
pathogenic bacteria have impeded progress of Xf strains
in functional genomics. There are two practical difficulties
in performing functional genomic analyses on any Xf
strain. First, Xf strains are nearly fastidious; it takes roughly
five to six days for a bacterial streak to appear on an agar
plate using the best medium, and it takes about ten days
for a liquid culture to grow to sufficient density to use the
cells for most routine purposes (DNA extraction, conju-
gation, etc.). Secondly, no standard wide host range shut-
tle vectors (with repW, repP or repQ replicons) have been
reported to be stably moved into and maintained in any Xf
strain. There have been only three reports of transforma-
tion of Xf; one with a Tn5 transposase–transposon synaptic
complex and two others with narrow host range plasmids
that utilize origins of replication derived from Xf. One
plasmid is an integrative vector that carries the CVC
chromosomal origin of replication that provides an unstable
replication in Xf. This vector should be useful for site-
directed marker exchange and marker interruption muta-
tions. The second is a replicative shuttle vector that carries
the pUC origin for replication in Escherichia coli and a
rolling circle replicon derived from a cryptic CVC plas-
mid. However, this vector proved unstable in the absence
of antibiotic selection. We describe here the stable trans-
formation of two Xf PD strains using a small, stable, broad
host range shuttle vector, pUFR047 through electropora-
tion. This vector is one of a series of well-characterized
conjugational shuttle vectors based on repW, and is widely
used to shuttle DNA fragments from E. coli to various
species and strains of Xanthomonas, where the vector is
stabilized in the absence of antibiotic selection by the para
locus.

X. fastidiosa PD strains, PD-A and Temecula, were
grown in PD3 medium supplemented with MOPS (3-
4[morpholino] propane sulfonic acid) buffer at 28°C. Agar
was added to solidify media at 15 g/l; plates streaked
with PD strains were wrapped with parafilm to prevent
desiccation during incubation. Liquid cultures were grown
with low-speed rotatory agitation (100 rpm). Both strains
were confirmed to be pathogenic by xylem puncture in-
oculations of Catharanthus roseus (Madagascar per-
wickie) and symptoms appeared after three months.

For electroporation, X. fastidiosa strains stored in gly-
cerol stocks at −80°C were plated on PD3 agar plates. After
ten days of incubation at 28°C, a loop full of culture con-
taining 3–4 colonies were inoculated into 5 ml of liquid
PD3 medium and incubated for six days with low-speed
agitation. Two millilitres of this culture was used to inoc-
ulate 30 ml of liquid PD3 medium. After a four-day incu-
bation under the same conditions, the cells of PD-A and
Temecula (OD 600 ~ 0.4 to 0.5) were harvested by centri-
fugation at 3000 g at 4°C for 15 min. The pelleted cells
were resuspended and washed twice in 30 ml of cold,
stere 10% glycerol. The final cell pellet was resuspended
in 0.3 ml of 10% glycerol and held on ice. A 70-µl aliquot
of cell suspension was mixed with 0.5–1 µg of pUFR047
plasmid DNA dissolved in 1 mM Tris-HCl, pH 8. The
mixture of cells and DNA was transferred into a cold
0.1 cm electroporation cuvette and kept on ice for 1 min.
The cells were electroporated at 1.8 kV to generate a pulse
of 5.8 to 6.0 ms duration with the Electroporator 2510 system
(Eppendorf Scientific). Cells were then immediately re-
moved from the cuvette, one ml of liquid PD3 was added,
and the cells were incubated at 28°C overnight with constant
rotary shaking at 100 rpm to allow expression of antibiotic
resistance. Transformants were selected by plating 200 µl
of cell suspension on PD3 agar supplemented with 2 µg
gentamycin per ml for 20 days at 28°C. The colonies on
selection medium were picked individually, grown in PD3
medium containing gentamycin. The presence of pUFR047
in the transformants was confirmed by three different
methods: (1) agarose gel electrophoresis of alkaline lysis
minipreps of the PD-A transformants; (2) transformation
of E. coli with these minipreps on LB agar medium
supplemented with ampicillin (50 µg per ml) followed by
miniprep agarose gel detection of pUFR047 in the E. coli
and (3) PCR of the transformants with the X. fastidiosa
specific primers RST31 and RST33.

By electroporation plasmid pUFR047 reproducibly
transferred into both the PD-A and Temecula strains of X.
fastidiosa at a frequency of ca. 50 transformants/µg DNA.
The presence of restriction/modification enzyme systems
in the Xf genome may explain this low-to-moderate effi-
ciency. The results of an alkaline lysis plasmid miniprep
of PD-A transformants and their restriction profile with
EcoRI and BglII gave the fragments of expected size for
pUFR047 (Figure 1 a). Based on band intensity, the copy
number of the plasmid mini-preps of pUFR047 extracted
from PD-A or Temecula appeared to be ca. 50% higher
than that of this plasmid extracted from X. campestris pv.
malvacearum or E. coli (data not shown). The confirma-
tion of Xf PD-A transformants by PCR indicated that they
were indeed Xf strains (Figure 1 b). Plasmid transfer into
Xf strains by conjugation was inefficient and difficult to
reproduce because slow growth of Xf required a long se-
lection period on agar plates which resulted always in the
appearance and overgrowth of E. coli donor and/or helper
colonies that became spontaneously resistant to antibiotics.

Replicative stability of pUFR047 was measured in the
absence of antibiotic selection for 30 generations. PD3
broth (10 ml) containing 2 µg gentamycin (Gm) per ml
was inoculated with single colony isolates of Xf trans-
formants, shaken at 28°C to late-exponential-growth phase
(8 to 9 days), and diluted 1/1000 into fresh broth without
antibiotic. Growth was continued to late-exponential phase,
and the dilution-growth procedure repeated for three cycles.
Cultures sampled at the beginning of each cycle were plated on both selective and nonselective medium by serial ten-fold dilutions. Serial ten-fold dilutions were performed in MOPS buffer, pH 6.2, containing 0.001% Silwet L-77 (polyalkyleneoxide modified heptamethyltrisiloxane; OSI Specialties, Inc, Friendly, WV) to disperse cell clumps.

Use of Silwet L-77 greatly facilitated reproducibility in the cell counts by dramatically dispersing clumped bacteria and did not appear to be toxic to either PD strain at concentrations used (data not shown). The number of colonies on both selective and nonselective media was counted and per cent plasmid retention calculated. The data were analysed for analysis of variance using SPSS statistical package to find the significance of observed differences ($P = 0.05$). After 30 generations of growth in three experiments, 48% of the cells retained the plasmid in the absence of antibiotic selection (Figure 2). This level of stability is within the range of that observed with other IncW plasmid vectors in the pUFR series in Xanthomonas, where retention ranged from 74 to over 99%, depending on the strain\(^6\). Monteiro et al.\(^5\) used plasmid vectors p16KdAori and p16Kori, carrying the 9a5c chromosomal origin of replication (oriC) to transform CVC strain, but did not stably replicate in 9a5c CVC isolate. These authors suggest that a limited level of DnaA expression in X. fastidiosa would not allow extrachromosomal replication of oriC plasmids. Plasmid pUFR047 is one of a series of repW vectors that has proven useful in functional genomic analyses of various xanthomonads\(^14\). Perhaps significantly, Xylella is taxonomically closer to Xanthomonas than any other bacterial genus\(^5\). The pUFR series of replicative vectors was made stable by the partitioning function of parA, derived from the A. tumefaciens plasmid pTAR\(^7\).

Plasmid pUFR047 has multiple unique cloning sites, the lacZ for blue/white detection of cloned inserts in E. coli, and can stably harbour inserts of 30–40 kb to make cosmid derivatives\(^14\). Interestingly, the copy number appeared to be significantly higher in X. fastidiosa mini-plasmids than in either E. coli or X. malvacearum mini-plasmids, and the DNA extracted from X. fastidiosa was of surprisingly high quality. This small, stable shuttle vector should facilitate functional genomics analyses in this important plant pathogenic species.

**Figure 1.** Transformation of Xylella fastidiosa PD-A using pUFR047. a, Plasmid DNA extracted from a single colony of PD-A after transformation with pUFR047. Lane 1, Digested with BglII and lane 3, Lambda digested with HindIII. b, PCR product amplified by X. fastidiosa-specific primers RST31 and RST33. Lane 1, Digested with HindIII. Lane 2, Lambda digested with HinEII and lane 3, Lambda digested with HinEII.

**Figure 2.** Plasmid maintenance in X. fastidiosa strain PD-R in broth culture. Cultures were grown with antibiotic as described to late-exponential-growth phase, and diluted 1/1000 into fresh broth without antibiotic. Growth was continued to late-exponential phase, and the dilution growth procedure repeated for three cycles. Cultures were sampled at the beginning of each cycle, diluted 1:10 in a series, and 10:1 droplets were plated onto two plates each of nonselective and selective media. Per cent retention of plasmid is shown as a function of the number of generations of growth after release of antibiotic selection. Values are means of three replications. Data represented by bars with same letter are not significantly different according to Fisher’s least significant difference test at $P = 0.05$.

Interception of *Peronospora manshurica* in soybean germplasm imported during 1976–2005

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Quarantine processing of 20,108 soybean germplasm samples received during 1976–2005 resulted in the interception of *Peronospora manshurica*, the downy mildew fungus in 1994 samples (9.91%) from 16 countries, including Malaysia and Indonesia, from where it has not been reported. The interception has great quarantine significance in view of the fact that this fungus has not yet been reported from India, is destructive in nature, its oospores can survive for several years in seed and soil, a large number of physiological races exist and zero tolerance limit is prescribed for quarantine purposes. Country-wise details on the introduction of germplasm and interception of downy mildew are presented.

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GLOBALLY there are several instances of plant disease epidemics due to movement of infected seed/other planting materials. Soybean, *Glycine max* (L.) Merrill having both protein and oil, is an important source of low-cost food. The importance of soybean in India has become overwhelming in view of the shortage of edible oil in the country. At present, there is a large area under soybean cultivation in India, particularly in Bihar, Gujarat, Himachal Pradesh, Madhya Pradesh, Maharashtra, Karnataka, Rajasthan and Uttar Pradesh. During 2004–05, the total area under cultivation in India was 7.2 m ha and total production was 6.5 million metric tons with an average yield of 0.76 mt/ha (ref. 1). India occupies 7.14% of total world area and only 2.37% of total world production; the average yield is about one-third of world average.

A large number of soybean germplasm is being introduced in the country for crop-improvement programmes. A number of pathogenic fungi were intercepted during quarantine processing of imported soybean germplasm*. Among the interceptions, the most important one is downy mildew of soybean caused by *Peronospora manshurica* (Naum.) Syd., as this mildew is widely distributed the world over, including several countries in Asia but has not yet been reported from India as well as South Asia7,8. *P. manshurica* has been repeatedly intercepted on soybean seeds imported from several countries9; interceptions from Malaysia9 and Indonesia7 were of special significance, as it has not been reported from these countries as well. Johnson and Lefebvre9 were the first to describe oospore-encrusted soybean seeds and concluded that the disease is seed-borne. Seed transmission was established by Jones and Torrie9 in 1946.

During the last 30 years (1976–2005), a total of 20,108 seed samples of soybean germplasm were received for quarantine clearance. Country-wise introduction of samples and detection of *P. manshurica* are presented in Table 1. Seed samples were first examined under a stereoscopic binocular microscope for the presence of crust of oospores of downy mildew. Seed samples found treated with *Rhizobium* culture or found free by initial stereo binocular observations, were subjected to washing test. Seeds were stirred in water in a test tube and the suspension was observed in petri plates under stereo binocular microscope. Seed suspensions free from oospores were subjected to centrifugation at 5000 rpm for 10 min; pellets were obtained and examined under compound microscope for the presence of oospores. Seed samples found free by visual inspection/washing test, were randomly tested by staining the teased seed coat with 2,3,5-triphenyltetrazolium chloride (TTC).

Some of the seed samples under stereoscopic microscope showed dull, milky white to light brown crusts on the seed surface. Crusts at higher magnification (50X) showed a