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 *g*-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[21].

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.

1. Haimovitz-Friedman, A., Radiation-induced signal transduction response. *Radiat. Res.*, 1998, **150**, 102–108.
2. Jonathan, E. C., Bernhard, E. J. and Mckenna, W. G., How does radiation kill cells? *Curr. Opin. Chem. Biol.*, 1999, **3**, 77–83.
3. Ross, G. M., Induction of cell death by radiotherapy. *Endocr. Related Cancer*, 1999, **6**, 41–44.
4. Lin, X., Ramamurthi, K., Mishima, M., Kondo, A. and Howell, S. B., p53 interacts with the DNA mismatch repair system to module the cytotoxicity and mutagenicity of hydrogen peroxide. *Mol. Pharmacol.*, 2000, **58**, 1222–1229.
5. Dewey, W. C., Miller, H. H. and Leeper, D. B., Chromosomal aberrations and mortality of X-irradiated mammalian cells: Emphasis on repair. *Proc. Natl. Acad. Sci. USA*, 1971, **68**, 667–671.
6. Steel, G. G., Recovery kinetics deduced from continuous low dose-rate experiments. *Radiother. Oncol.*, 1989, **14**, 337–343.
7. Bedford, J. S., Sublethal damage, and chromosomal aberrations in mammalian cells exposed to ionizing radiations. *Int. J. Radiat. Oncol. Biol. Phys.*, 1991, **21**, 1457–1469.
8. Nair, C. K. K., Parida, D. K. and Nomura, T., Radiation protectors in radiotherapy *J. Radiat. Res*., 2001, **42**, 21–37.
9. Tim Lai, Yihuai Gao and Shufeng Zhon, Global marketing of medicinal Ling Zhi mushroom *G. lucidum* products and safety concerns. *Int. J. Med. Mushroom*, 2004, **6**, 189–194.
10. Nishitoba, T., Salo, H., Shirasu, S. and Sakamura, S., Evidence of strain specific terpenoids pattern of *Ganoderma lucidum*. *Agric. Biol. Chem.*, 1986, **50**, 2151–2154.
11. Jones, S. and Janardhanan, K. K., Antioxidant and antitumor activity of *G. lucidum* from South India. *Int. J. Med. Mushroom*, 2000, **2**, 195–200.
12. Gandhi, N. M., Gopalaswamy, U. V. and Nair, C. K. K., Radiation protection by Disulfiram. *J. Radiat. Res.*, 2003, **44**, 265–269.
13. Shety, T. K., Satav, J. G. and Nair, C. K. K., Protection of DNA and microsomal membranes *in vitro* by *Glycyrrhizia glabra* L., against gamma irradiation. *Phytother. Res*., 2002, **16**, 576–578.
14. Bradford, M. M., A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 1976, **72**, 248–254.
15. Beuge, J. A. and Aust, S. D., Microsomal lipid peroxidation. *Methods Enzymol*., 1978, **52**, 302–310.
16. Maurya, D. K., Salvi, V. P. and Nair, C. K. K., Radioprotection of normal tissues in tumour-bearing mice by Troxerutin. *J. Radiat. Res*., 2004, **45**, 221–228.
17. Singh, N. P., Microgel for estimation of DNA strand breaks, DNA protein crosslinks and apoptosis. *Mutat. Res*., 2000, **455**, 111–127.
18. Gandhi, N. M., Maurya, D. K., Salvi, V., Kapoor, S., Mukherjee, T. and Nair, C. K. K., Radioprotection of DNA by glycyrrhizic acid through scavenging free radicals. *J. Radiat. Res.*, 2004, **45**, 461–468.
19. Konca, K. *et al.*, A cross platform public domain PC image analysis program for the comet assay. *Mutat. Res.*, 2003, **534**, 15–20.
20. Sheena, N., Ajith, T. A. and Janardhanan, K. K., Prevention of nephrotoxicity induced by the anticancer drug cisplatin, using *G. lucidum*, a medicinal mushroom occurring in South India. *Curr. Sci*., 2003, **85**, 478–482.
21. Shi, J. H., PSP for the protection of the tumor patients during chemotherapy. In PSP International Symposium (eds Yang, Q. Y. and Kwok, C. Y.), Fudan U, Press, Shanghai, 1993, pp. 271–272.

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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. Plamsid 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 species[1]. 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 economic losses[1]. The genome of CVC and PD isolates of *Xf* has been completely sequenced and annotated[2,3]. However, lack of useful DNA cloning vectors and/or techniques typically used for functional genomic analyses of phytopathogenic 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, conjugation, etc.). Secondly, no standard wide host range shuttle 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 transformation of *Xf*; one with a Tn5 transposase–transposon synaptic complex[4] and two others with narrow host range plasmids that utilize origins of replication derived from *Xf*[5,6]. One plasmid is an integrative vector that carries the CVC chromosomal origin of replication that provides a unstable replication in *Xf*[5]. This vector should be useful for site-directed marker exchange and marker interruption mutations. 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 plasmid[6]. However, this vector proved unstable in the absence of antibiotic selection. We describe here the stable transformation of two *Xf* PD strains using a small, stable, broad host range shuttle vector, pUFR047 through electroporation[7]. 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 *par*A locus[8].

X. fastidiosa PD strains, PD-A[9] and Temecula[4], were grown in PD3 medium[10] supplemented with MOPS (3-4[morphomino] propane sulfonic acid) buffer[7] 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 inoculations[11] of *Catharanthus roseus* (Madagascar periwinkle) and symptoms appeared after three months.

For electroporation, *X. fastidiosa* strains stored in glycerol stocks at –80°C were plated on PD3 agar plates. After ten days of incubation at 28°C, a loop full of culture containing 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 inoculate 30 ml of liquid PD3 medium. After a four-day incubation under the same conditions, the cells of PD-A and Temecula (OD 600 ~ 0.4 to 0.5) were harvested by centrifugation at 3000 *g* at 4°C for 15 min. The pelleted cells were resuspended and washed twice in 30 ml of cold, sterile 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 removed 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[12] of the PD-A transformants; (2) transformation of *E. coli* with these minipreps[12] 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[13] 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 efficiency. The results of an alkaline lysis plasmid miniprep of PD-A transformants and their restriction profile with *Eco*RI and *Bgl*II 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 confirmation 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 selection 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* transformants, 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.

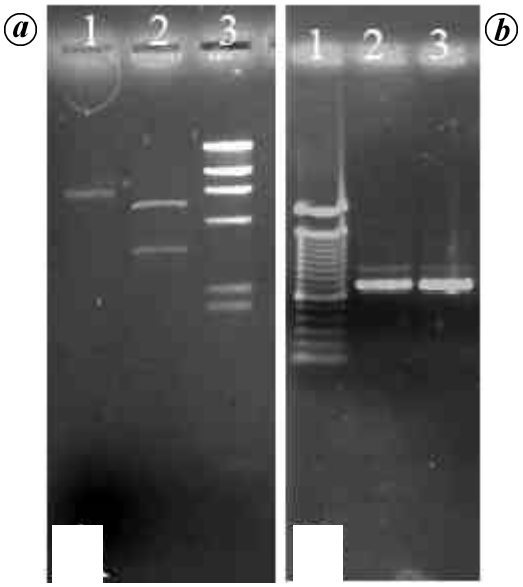

Figure 1. Transformation of *Xylella fastidiosa* PD-A using pUFR047. ***a***, Plasmid DNA extracted from a single colony of PD-A after transformation with pUFR047. Lane 1, Digested with *Eco*RI; lane 2, Digested with *Bgl*II and lane 3, Lambda digested with *Hin*dIII. ***b***, PCR product amplified by *X. fastidiosa*-specific primers RST31 and RST33. Lane 1, 100 bp DNA ladder; lane 2, *X. fastidiosa* PD-A (control) and lane 3, *X. fastidiosa* PD-A/pUFR047.

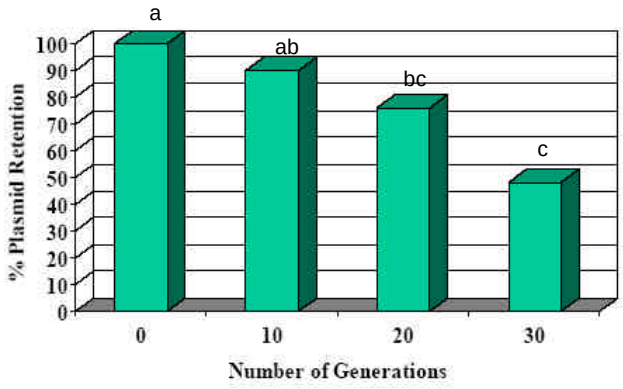

Figure 2. Plasmid maintenance in *X. fastidiosa* strain PD-R in broth culture. Cultures were grown with antibiotic as described to late-exponential-growth phase, and diluted 1/1000 into fresh broth without antibiotic. Growth was continued to late-exponential phase, and the dilution growth procedure repeated for three cycles. Cultures were sampled at the beginning of each cycle, diluted 1 : 10 in a series, and 10 : l 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 $P = 0.05$.

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[8]. 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[2]. The pUFR series of replicative vectors was made stable by the partitioning function of *par*A, derived from the *A. tumefaciens* plasmid pTAR[7]. Plasmid pUFR047 has multiple unique cloning sites, *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-preps than in either *E. coli* or *X. malvacearum* mini-preps, 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.

1. Purcell, A. H. and Hopkins, D. L., Fastidious, xylem limited plant pathogens. *Annu. Rev. Phytopathol*., 1996, **34**, 131–151.
2. Simpson, A. J., Reinach, F. C., Arruda, P., Abreu, F. A. and Acencio, M., The genome sequence of the plant pathogen *Xylella fastidiosa*. *Nature*, 2000, **406**, 151–157.
3. Van Sluys, M. A., Oliveira, M. C., Monteiro-Vitorello, C. B., Miyaki, C. Y. and Furlan, L. R., Comparative analyses of the complete genome sequences of Pierce's Disease and Citrus Variagated Chlorosis strains of *Xylella fastidiosa. J. Bacteriol*., 2002, **185**, 1018–1026.
4. Guilhabert, M. R., Hoffman, L. M., Mills, D. A. and Kirkpatrick, B. C., Transposon mutagenesis of *Xylella fastidiosa* by electroporation of Tn5 synaptic complexes. *Mol. Plant–Microbe Interact*., 2001, **14**, 701–706.
5. Monteiro, P. B., Teixeira, D. C., Palma, R. R., Garnier, M., Bove, J-M. and Renaudin, J., Stable transformation of the *Xylella fastidiosa* Citrus Variegated Chlorosis strain with oriC plasmids. *Appl. Environ. Microbiol*., 2001, **67**, 2263–2269.
6. Qin, X. and Hartung, J. S., Construction of a shuttle vector and transformation of *Xylella fastidiosa* with plasmid DNA. *Curr. Microbiol*., 2001, **43**, 158–162.

7. De Feyter, R., Yang, Y. and Gabriel, D. W., Gene-for-genes interactions between cotton *R* genes and *Xanthomonas campestris* pv. *malvacearum* avr genes. *Mol. Plant–Microbe Interact.*, 1993, **6**, 225–237.
8. De Feyter, R., Kado, C. I. and Gabriel, D. W., Small, stable shuttle vectors for use in *Xanthomonas*. *Gene*, 1990, **88**, 65–72.
9. Hopkins, D. L., Physiological and pathological characteristics of virulent and avirulent strains of the bacterium that causes Pierce's Disease of grapevine. *Phytopathology*, 1985, **75**, 713–717.
10. Davis, M. J., French, W. J. and Schaad, N. W., Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. *Curr. Microbiol*., 1981, **6**, 309–314.
11. Hopkins, D. L. and Adlerz, W. C., Natural hosts of *Xylella fastidiosa* in Florida. *Plant Dis*., 1988, **72**, 429–431.
12. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
13. Minsavage, G. V., Thompson, C. M., Hopkins, D. L., Leite, R. M. V. B. C. and Stall, R. E., Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. *Phytopathology*, 1994. **84**, 456–461.
14. Gabriel, D. W., The *Xanthomonas avr/pth* gene family. In *Plant–Microbe Interactions* (eds Stacey, G. and Keen, N. T.), APS Press, St. Paul, USA, 1999, vol. 4, pp. 39–55.

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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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Keywords: Germplasm, interception, quarantine, *Peronospora manshurica*, soybean.

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[2]. 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 Asia[3,4]. *P. manshurica* has been repeatedly intercepted on soybean seeds imported from several countries[5]; interceptions from Malaysia[6] and Indonesia[7] were of special significance, as it has not been reported from these countries as well. Johnson and Lefebvre[8] were the first to describe oospore-encrusted soybean seeds and concluded that the disease is seed-borne. Seed transmission was established by Jones and Torrie[9] 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