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THE PRESERVATION OF CHIHUAHUAN PLANT GENOMES THROUGH IN VITRO BIOTECHNOLOGY: DNA BANK-NET, A GENETIC INSURANCE POLICY

ROBERT P. ADAMS

DNA Bank-Net, Baylor University, B.U. Box 7372, Waco, Texas 76798

ABSTRACT

The first plant to plant gene transfer occurred in 1983. Since then, genes have been transferred to plants from viruses, bacteria, fungi, and even mice. These genetic transfers are done to attain insect, bacteria, viral, and fungal resistance, balanced protein content, more efficient photosynthesis, nitrogen fixation and salt and heavy metal tolerance. These kinds of gene transfers from one unrelated organism to another indicate that we must now view the world's genetic resources from a horizontal perspective in which gene transfers will cut across species, genera, family, and even kingdom boundaries. For example, no longer are we constrained to breed wheat to its nearest relatives, but may now transfer a gene for insect resistance directly from cowpeas to wheat.

Areas of the world such as the Chihuahuan Desert and tropical rain forests are rich sources of unusual plants that have potential medicinal, natural insecticide, and herbicide uses. Yet, these resources are rapidly being lost, particularly in the tropics. In vitro technology is presented for preserving DNA for use in genetic engineering. This paper explores some of the constraints faced in the extraction and preservation of DNA and the formation of an international network of DNA banks for utilization in genetic engineering in both this and the next century. DNA Bank-Net is the only organization that is extracting and storing genes (DNA) from a wide spectrum of plants for use as genetic insurance.

RESUMEN

La primer planta que transpuso los genes a otra planta ocurrió én 1983. Desde entonces los genes han sido transladado a otras plantas de parte de viruses, bacterias, fungus, y tambien ratones. Estos translados genéticos son echos para obtener insectos, bacteria, viral y resistencia fungi, contenido de proteina balanciada, mas efficiente photosinthesis, fijación de nitrogina, y sal y metales de tolerancia pesada. Estas clases de genes transladadas de un organismo no relacionado a otro indica que nosotros debemos ahora ver los recursos genéticos mundiales de una perspectiva horizonal en el cual los genes transladados contara con un fin de especies, genera, familia y tambéin kingdom. Por ejemplo, ya no estamos detenidos a procrear al trigo a sus parientes mas cercanos, pero ahora podemos trasladar un gen para resistencia de insecto directamente de chicharos al trigo.

Areas del mundo como el desierto de Chihuahua y selvas de lluvias tropicales son cursos ricos de plantas inusuales que tienen usos de medecina potencial, insecticida natural y herbicida. Todavia, estos recursos se estan haciendo rapidamente perdidos, particularmente en los trópicos. La technologia in vitro es presentada para preservar la DNA para el uso en la ingenieria genética. Este papel explora algunos constreñimientos que estan haciendo la extración y preservación del DNA y la formación de una malla internacional de los bancos del DNA para la utilización de la ingenieria genetica, en ambos este y el siguente siglo. El banco de DNA es la unica organización que esta sacando y guardando genes de (DNA) de una espectra muy amplia de plantas para uso como aseguranza genética.

The first direct transfer and expression of a plant gene into another plant was reported in 1983 when Murai et al. (1983) detailed the production of the bean protein, phaseolin, in sunflowers. New recombinant (transgenic) plants are now being reported with increasing frequency. For example, a strain of cowpea, *Vigna unquiculata*, discovered in the Ilorin market in Nigeria, contains a protein that

inhibits trypsin (CPTI) digestion by insects (Redden et al. 1984). This gene has recently been transferred to tobacco (*Nicotiana*) where the trypsin-inhibiting (insecticidal) gene is expressed and offers the same resistance against insects for tobacco as it does for cowpea (Newmark 1987a). It appears that this insecticidal (CPTI) gene can be moved to many crops to produce an *in vivo* insecticide. Another protein-based insecticide found in *Bacillis thuringiensis* (sold commercially as a dry powder: 'Dipel Dust' by Fertilome Corp., or BT), has been moved to tomato plants. The tomato plants show the same insect resistance as when dusted with BT (Fischhoff et al. 1987). The BT insecticidal gene has also been transferred to tobacco (Barton et al. 1987). Virus tolerance has been transferred to tomato plants by moving the gene that produces the viral protein coat from tobacco mosaic virus (TMV) to tomatos (Nelson et al. 1988).

Phosphinotricin, the active ingredient in Hoechst's *Basta* herbicide, acts by blocking the essential plant enzyme, glutamine synthetase. Recently, Plant Genetic Systems (Belgium) reported that a gene from *Streptomyces* produces an enzyme that acetylates phosphinotricin thereby inactivating it as an herbicide (Newmark 1987b). This gene has now been moved to tobacco, tomato, and potato plants and these plants are resistant to the *Basta* herbicide. Another example of herbicide resistance is the transfer of a *Roundup* resistance gene from the bacterium, *Escherichia coli,* to the chloroplasts of *Petunia* (della-Cioppa et al. 1987).

The accumulation of toxic metals such as Cadmium (Cd) in plant tissues that are consumed for food presents a significant problem, particularly as our soils and waters become more contaminated. One method to reduce the amount of Cd in seeds might be for the plant to store more Cd in the leaves. Metallothioneins are low molecular weight proteins that selectively bind the heavy metals Cu, Zn and Cd (Lefebvre et al. 1987). Metallothioneins are found in mammals. Lefebvre et al. (1987) transferred a metallothionein gene from Chinese hamster to turnip (*Brassica campestris*) and demonstrated the first case of a mammalian gene product being functional in plants. More recently, Maiti et al. (1988) not only transferred a metallothionein gene from mice to tobacco plants, but demonstrated that the gene was inherited in the seeds as a dominant Mendelian trait.

The improvement of the protein quality of foods is of considerable concern since our cereals are generally low in lysine and sulfur containing amino acids. The transfer of storage proteins with desirable amino acid compositions will play a significant role in new, balanced protein crops. One example is the transfer of the potato storage protein, patatin, to tobacco (Rosahl et al. 1987). Undoubtedly, we will find many more nutritionally balanced proteins from wild species that can be moved into our field crops by genetic engineering.

The aforementioned examples clearly emphasize man's ability to transfer genes directly from plants, microbes, and animals to plants. This promises to revolutionize the manner in which we use genes. The germplasm of the world can now be envisioned as a planetary gene pool, from which we can make withdrawals and enhance quite unrelated species.

The number of novel insecticides, biocides, medicines, etc. that are found in

nature is innumerable. Yet the principal areas of diversity among plants, the lowland tropical forests, will have been cut over or severely damaged within the next 20 years (Raven 1987). The Amazon River system, for example, contains eight times as many species as the Mississippi River system (Shulman 1986). Raven (1987) estimates that as many as 1.2 million species will become extinct in the next 20 years. The loss of plant species means a loss of potential plant derived pharmaceuticals, now estimated at two billion dollars per year in the United States alone (U.S. Congress 1987). It also means a loss of genetic diversity in our current and potential crop species. Our crops are extremely inbred for factors such as yield, uniform flowering and height, and cosmetic features of the products. This narrow genetic base has resulted in several disastrous crop failures. Ireland's potato famine of 1846, which resulted in famine and the emigration of a quarter of its population, was due to the fact that their potatoes had no resistance to the late blight fungus, *Phytophthora infestans* (Plucknett et al. 1987). This can be traced to the lack of genetic diversity in Irish potatoes, which had been multiplied using clonal materials from just two introductions from South America. These two non-resistant potato lines came from two separate introductions from South America to Spain in 1570 and to England in 1590 (Hawkes 1979).

A more recent example is that of the southern corn leaf blight fungus, (*Helminthosporium maydis*) in 1970 in the United States. Because almost all of the corn in the United States is of hybrid origin and contains the Texas cytoplasmic male sterile line, our fields of corn presented an unlimited habitat for the fungus. By the late summer of 1970, plant breeders were scouring corn (maize) germplasm collections in Argentina, Hungary, Yugoslavia and the United States for resistant sources (Plucknett et al. 1987). Nurseries and seed fields were used in Hawaii, Florida, the Caribbean, and Central and South America to incorporate the resistance into hybrid corn in time for planting in the spring of 1971 (Ullstrup 1972). Without these genetic resources, this technological feat would not have been possible.

The National Cancer Institute (NCI) is now spending eight million dollars over the next five years for a massive plant collecting effort in the tropics to find anti-cancer andanti-AIDS virus compounds (Booth 1987). The plant collectors will gather leaves and/or bark and air-dry the material for shipment to Frederick, Maryland where it will be extracted and assayed against 100 cancer cell lines and the AIDS virus. Yet, no genetic resources will be collected! When a promising compound is found, the plants will have to be recollected. For extensive testing (as well as commercial utilization), plantations must be established in the tropics and deserts to provide material.

Concurrent with the advancements in gene cloning and transfer, has been the development of technology for the removal and analyses of DNA. The DNA from the nucleus, mitochondrion, and chloroplast are now routinely extracted and immobilized onto nitro-cellulose sheets where the DNA can be probed with numerous cloned genes. The extraction of high molecular weight DNA from materials has been detailed in the works of Bendich et al. (1980), Murray and Thompson (1980),

Fluhr and Edelman (1981), Rivin et al. (1982), Zimmer and Newton (1982), Palmer (1982, 1986), Palmer and Zamir (1982), Palmer et al. (1985) and Rogers and Bendich (1985) to name but a few.

In the first paper published on the degradation of DNA in plant specimens (Rogers and Bendich 1985), DNA was obtained with a maximum length of 20 to 30 Kilobase pairs (Kbp) with average lengths of 0.1 to 1.0 Kbp from herbarium specimens ranging in age of 20 to 95 years old. *Juniperus osteosperma* seeds from pack rat middens (3,500; 27,000; and greater than 45,000 y bp) yielded DNA with maximum lengths of 10, 10, and 10 Kbp and average lengths of 7, 5, and 3 Kbp, respectively. Rogers and Bendich (1985) concluded, "The extent of DNA degradation for the herbarium specimens appeared to be related to the condition of the leaf rather than the year in which it was dried. The condition of the leaf would depend on the method of drying and the developmental stage of the plant...".

More recently, Doyle and Dickson (1987) reported on efforts to preserve *Solanum glutinosum* leaves in: formalin-acetic acid-ethanol (FAA); Carnoy's solution (ethanol:acetic acid, 3:1); 70% ethanol; chloroform:ethanol (4:3); brine solution (10% NaCl); and drying at 42°C. Essentially none of the chemical treatments preserved DNA for a week, except the chloroform:ethanol. However, the DNA was lost from the chloroform: ethanol treatment after 3 weeks. They did obtain good DNA from the dried leaves. The brine solution developed fungal contamination which likely lysed the cell membranes.

Pyle and Adams (1989) examined the use of acetic acid/ethanol (1:3), ethanol (95%), glycerol, Na azide (3%), Perfix preservative, pentachlorophenol/chloroform (1.5/98.5), paraformaldehyde (10%), guanidine thiocyanate (1%), NaCl (25%), Chlorox (5%), methanol:chloroform:propionic acid (1:1:1), glutaraldehyde (8%), formaldehyde (7.4%), trichloroacetic acid (10%), glutaraldehyde (2%), 0.5M Na cacodylate, and 10 mM EDTA to preserve spinach leaves. None of the aforementioned solutions preserved the DNA in spinach for seven days (Pyle and Adams 1989). The search for a "magic preservative" is apparently elusive.

Recent advances in the technology for the extraction and immobilization of DNA, however, coupled with the prospect of the loss of significant plant genetic resources throughout the world, warranted this study on the preservation of DNA for a few months under typical field conditions until the plant material can be shipped to a facility for DNA extraction. In addition, the stability of extracted genomic DNA during long-term storage of DNA is examined in this paper.

MATERIALS AND METHODS

Preparation of plant material.—Leaves from fresh spinach (*Spinacia oleracea*) were purchased locally. *Juniperus ashei, J. virginiana, Phorodendron tomentosum,* and *Quercus virginiana* were collected from native trees near the Baylor campus. *Pinus ponderosa* was obtained from Dryden, WA (Adams and Edmunds 1988). *Ginkgo biloba, Magnolia grandiflora,* and *Thuja orientalis* [= *Platyladus orientalis*] were collected from cultivated trees on the Baylor campus.

Approximately 0.5g fresh weight (FW) of leaf was used for each of the treatments (Table 1). Leaf drying (treatments 4 and 5, Table 1) was done in a conventional plant press dryer at 42°C for 24 - 48 h. Dessication was performed in a dessicator over Drierite (anhydrous CaSO4). For treatment seven, fresh spinach was placed on the lab bench and dried at room temperature. Microwave drying was for

TABLE 1.—Quality of DNA from spinach versus method of storage for spinach leaves. NT, Not Tested further. Good (G), mostly 50-20Kb; Fair (F) 20-2Kbp; Poor (P), 2Kb-500bp; Degraded (DG), <500bp.

Treatment Sampled after:	24 h.	1 mo.	2 mo.	5 mo.	8 mo.
1. Fresh	G	G	G	G	G
2. Frozen at −20′C	G	G	G	G	G
3. Kept refrigerated at 4°C	G	G	G	G	G
4. Dried 42°C, stored −20°C	G	G	G	G	G
5. Dried 42°C, stored 22°C	G	G	G	DG	DG
6. Desiccated, stored 22°C	G	G	G	P	DG
7. Dried 22°C, stored 22°C	G	G	G	DG	DG
8. Blanched in boiling water	DG	NT	NT	NT	NT
9. Microwaved, 3 min.	DG	NT	NT	NT	NT

three min. at a setting of high (700 watts). For the plastic bag storage tests, spinach leaf pieces were placed inside pint-size heavy duty Ziploc freezer bags (Dow Chemical) into which a preservative was then added. These preservatives are listed in Table 2. The bags were placed inside a plant dryer for seven days and the temperature kept at 35°C.

Liquid treatments, including the control with tap water, were added to the bags using an atomizer. The atomizer was sprayed 25 times into the bag (about 2 ml of liquid). For the dry powder treatments, 0.25 teaspoon of powder was placed inside the bag and shaken to distribute the powder over the leaf surface. Naphthalene was added to the bag in the form of a single mothball. The following products were used: Na Azide (Sigma S-2002); Na Cacodylate (Cacodylic acid sodium salt-trihydrate, Kodak 15404); Copper Oleate solution (American Brand copper fungicide); Sulfur powder (Hi-Yield brand, 90% sulfur); Maneb powder (Hi-Yield Brand, zinc ion and manganese ethylene bisdithiocarbamate); Phenol (Mallinckrodt 0025); Streptomycin powder (Fertilome Brand, fire blight treatment).

DNA extraction and analyses.—The hot CTAB procedure (Doyle and Dickson 1987, Doyle and Doyle 1987) was used with minor changes as noted herein. Fresh spinach leaf material (0.5 g FW) was weighed and placed in a preheated (60°C) mortar and pestle with preheated (60°C) 3.5 ml 2x CTAB buffer (2x CTAB = 2% (w/v) hexadecyltrimethyammonium bromide (Sigma H5882)), 1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 0.2% (w/v) 2-mercaptoethanol (Sigma M3148). The leaves were ground and the homogenate transferred to a polyallomer centrifuge tube via a wide-tipped Pasteur pipet. The mortar and pestle were washed with 0.5 ml of 2x CTAB buffer and the wash added to the centrifuge tube. The centrifuge tube was then incubated for 30-45 min. at 60°C. After incubation, an equal volume of 24:1 chloroform:isoamyl alcohol was added and mixed by gently shaking, then centrifuged (Centra 4B, 819 rotor) 10 min. at 8,500x g. The aqueous layer was removed using a wide-tipped Pasteur pipet, and transferred to a clean centrifuge tube to which 0.66 volume cold (-15°C) isopropanol was added and mixed. The DNA was pelleted by centrifugation for one min. at 350x g. Longer, more forceful spins were used as necessary to get the DNA to stick to the wall of the tube. The DNA pellet was washed with buffer (8 ml, 76% EtOH, 10mM NH4OAc) at room temperature from 20 min. to overnight. The DNA was then pelleted by high speed centrifugation for 10 min. at 8,500x g and the wash buffer carefully decanted. The DNA pellet was air dried for 30 min. then redissolved in 500 μl of resuspension buffer (10 mM NH4OAc, 0.25 mM EDTA). RNase A (Sigma R5000) was added to a final concentration of 10 μg per ml and incubated 30 min. at 37°C as per manufacturers instructions. Two volumes (1 ml) of dI water was added along with 848 μl of 6.92 M NH4OAc (2.5 M final conc.) and the DNA was then precipitated by the addition of 2.5 volumes (5.87 ml) of cold 95% ethanol and centrifugation for 10 min. at 8,500x g. The DNA pellet was air dried at room temperature overnight and the DNA resuspended in 0.5 ml of 1x TE (1mM Tris HCl, 0.1 mM EDTA, pH 7.2) buffer.

TABLE 2.—Comparisons of fungicides and a bactericide for short storage of fresh spinach leaves for DNA extraction after seven days at 35°C. Good (G), mostly 50-20Kb; Fair (F), 20-2Kbp; Poor (P), 2Kb-500bp; Degraded (DG), <500bp.

Treatment	DNA size
1. Fresh spinach leaves	G
2. Control (sprayed with tap water)	DG
Fungicides:	
3. Chlorox (5% soln in tap water)	G
4. Na Azide (3% soln in tap water)	DG
5. Na Cacodylate (1% soln in tap water)	DG
6. Copper oleate solution	F
7. Sulfur powder	F-P
8. Maneb Mn & Zn powder	F
9. Phenol	DG
10. Naphthalene	F
11. 0.5M EDTA	DG
Bactericide:	
12. Streptomycin Sulfate powder	G

For gel electrophoresis, DNA was mixed in various concentrations with 1x TBE and 0.20 volume of loading buffer (15% Ficoll w/v (Sigma F-2637) or 40% sucrose w/v, 0.05% bromphenol blue, and 0.10 M EDTA in 1x TBE). Ten µl of this mixture was loaded onto a 0.6% agarose gel (Sigma A-6013), submerged in running buffer (1x TBE, 0.5 µg/ml ethidium bromide) and electrophoresed for 30 min. at 100 V (10 V/cm), using a Biorad Mini-Sub cell and model 100/200 power supply. Concentrations of the extracted DNA's were judged by comparison with unrestricted lambda DNA (Sigma D-0144) loaded in various known concentrations. Restrictability was checked using EcoRI, Hind III, Kpn I, and Sal I (Sigma R2132, R1882, R9506, and R0754, respectively). When restricted DNA's were electrophoresed, lambda DNA restricted with Hind III (Sigma R-1882) was co-run to provide size standards. Gels were photographed under short wave UV light using a Polaroid direct screen camera (DS34).

RESULTS AND DISCUSSION

The results from the treatments are shown in Table 1. The DNA was well preserved in air dried and dessicated leaves for two months but severely degraded after five months storage (Table 1). Freezing the leaves gave good, high molecular weight DNA but storage and transport while frozen in the tropics is difficult and sometimes not possible. The dried leaves, stored at -20°C, still yielded high molecular weight DNA after eight months. The use of the hot (55°-60°C) CTAB extraction buffer prompted us to consider heating the leaves quickly (blanching in boiling water) to denature DNases that degrade the DNA. This apparently did not denature the DNases, because no DNA was obtained from this treatment (Table 1.). Subsequently, we have found that DNA restriction enzymes have varying resistance to heat denaturation. Many DNases are not denatured until temperatures of

TABLE 3.—Effects of storage on the size of extracted cpDNA (from fresh spinach foliage) in 10X TE buffer (10mM TRIS; 1mM EDTA, pH 7.2). NT, Not Tested further. Good (G), mostly 50-20Kb; Fair (F), 20-2Kbp; Poor (P), 2Kb-500bp; Degraded (DG) <500bp.

Treatment Sampled after:	1 mo.	2 mo.	5 mo.	8 mo.
1. Frozen −20° C	G	G	G	G
2. Refrigerated 4° C	G	G	G	G
3. Room temperature, 22° C	G	G	P	NT
4. Incubator 37° C	G	P	NT	NT
5. Incubator 55° C	DG	DG	NT	NT
6. Incubator 100° G	DG	NT	NT	NT

over 60°C and up to 100°C are reached (U. S. Biochemical Corp., 1988 Catalog, Cleveland, OH). Because DNA melts at about 70°-75°C, the DNA is lost before the DNases have been denatured. This might be the reason that blanching was not successful.

The microwave drying was tried as an alternative method of drying. The DNA was totally degraded; however, additional experimentation with power levels and time could result in dried specimens with good DNA. Thus, only the dried, refrigerated, and frozen foliage gave high molecular weight DNA from spinach (an easy species from which to obtain DNA).

The hot CTAB extraction methods (Doyle and Doyle 1987) were also tried on several species that are high in tannins and other secondary compounds that interefere with DNA extraction. The application of the hot CTAB procedure to fresh leaves of *Ginkgo biloba, Juniperus ashei, J. virginiana, Magnolia grandiflora, Phorodendron tomentosum, Pinus ponderosa, Quercus virginiana,* and *Thuja orientalis* yielded high molecular weight DNA, but the dried leaves often resulted in appreciable degradation. The quality of the DNA was examined by restriction enzymes. In each case where DNA of 30-50 kbp size was obtained, it restricted by EcoRI, Hind III, Kpn I and Sal I.

The results of the plastic bag storage trials showed that seven days storage the chlorox treatment yielded DNA comparable to DNA from fresh leaves (Table 2). The DNA from the chlorox-preserved sample was still much less concentrated than DNA from fresh spinach. Tap water only, Na cacodylate solution, phenol, and 0.5 M ETDA solution treatments did not yield any detectable DNA whensampled after seven days storage (Table 2). The Na azide (3 % solution) treated leaves had a poor DNA yield with considerable degradation (Table 2). The copper oleate solution seems to have promise as these leaves resulted in good yields of DNA of high molecular weight.

The sulfur powder, and Maneb Mn and Zn powder treatments resulted in only fair yields with moderate degradation (Table 2). The mothball (naphthalene) treatment was used to generate a non-oxidizing atmosphere by the release of naphthalene vapors inside the bag. This resulted in a fair DNA yield with little degradation. The Streptomycin sulfate powder treated leaves had a fair to good yield of DNA of high molecular weight (Table 2). Streptomycin sulfate might be very

useful if used in combination with a fungicide to prevent the growth of bacteria on the specimen.

All the storage methods involving powders yielded fair or good DNA. The leaves felt dry upon removal from the bags. It is possible that preservation by dessication may have been as important as any biocidal action of the preservatives. additional powders and combinations will be considered as a future study.

Storage of extracted DNA (in 1x TE buffer, pH 8.0) at various temperatures resulted in expected degradation at elevated temperatures (Table 3). However, even refrigerated samples (4°C) showed no degradation after 8 months storage (Table 3). More sensitive assays for DNA degradation are needed to detect any changes for the 4°C and -20°C storage treatments.

CONCLUSIONS

In summary, even though we have presented some constraints, the protocols for extracting and storage of DNA are available. The principal impediment to the expansion of DNA bank depositories appears to be a lack of vision by governments and private foundations. In addition, people still believe that somehow, miraculously, mankind will preserve all the genetic diversity on earth. Alas, DNA depositories appear to be the only genetic insurance available today.

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