INTERSPECIFIC SOMATIC HYBRIDIZATION BETWEEN *SOLANUM TUBEROSUM* L. AND *S. BULBOCASTANUM* DUN. AS A MEANS OF TRANSFERRING NEMATODE RESISTANCE

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Abstract

Interspecific somatic hybrids were produced between tetraploid *Solanum tuberosum* and a nematode-resistant accession of the diploid species *Solanum bulbocastanum* by protoplast fusion. Hybrid cells were selected using dual fluorescent labeling of protoplast preparations prior to fusion. Hybridity of regenerated plants was confirmed with a combination of morphological assessment, chromosome counting and isozyme analysis. Somatic hybrids had the same level of resistance to infection by race 1 of the nematode *Meloidogyne chitwoodi* as the *S. bulbocastanum* parent used in the fusion. Some of the somatic hybrids were fertile as females when crossed with tetraploid *S. tuberosum* breeding lines. Thus, these hybrids can be used in a potato improvement program to incorporate a valuable pest resistance.

Compendio

Híbridos somáticos interespecíficos fueron producidos, por fusión de protoplastos, entre el tetraploide *Solanum tuberosum* y una entrada de la especie diploide *Solanum bulbocastanum* resistente a los nematodos. Se seleccionaron células híbridas utilizando la marcación con sondas fluorescentes duales en las preparaciones de protoplastos antes de la fusión. Se confirmó la hibridación de las plantas regeneradas con una combinación de evaluación morfológica, contaje de cromosomas y análisis de isozimas.

Los híbridos somáticos tuvieron el mismo nivel de resistencia a la infección por la raza 1 del nematodo *Meloidogyne chitwoodi* como el de los padres *S. bulbocastanum* utilizados en la fusión. Algunos de los híbridos somáticos fueron fértiles cuando se les usó como madres en cruzamientos con líneas de mejoramiento del tetraploide *S. tuberosum*. Por lo tanto, estos híbridos pueden ser usados en un programa relacionado al mejoramiento de la papa, para incorporar resistencia valiosa a la plaga.

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Accepted for publication November 30, 1992.
ADDITIONAL KEY WORDS: Protoplast fusion, potato, pest resistance, breeding, *Meloidogyne chitwoodi*.

Introduction

Somatic hybridization may provide a means to incorporate traits from sexually incompatible wild species into crop plants. Protoplast fusion in the genus *Solanum* has been used to produce somatic hybrids by several research groups (*e.g.* 7, 9, 10, 15, 30). We have produced several interspecific somatic hybrid combinations of *Solanum* in an attempt to evaluate the potential of somatic hybridization for incorporating disease resistance traits into potato breeding lines (3, 5, 6, 19, 20).

Columbia root-knot nematode, *Meloidogyne chitwoodi* Golden *et al.*, is an important pest of potato in the Pacific Northwest of the USA. The primary economic damage is caused by the invasion of tubers by second stage juveniles (J2) causing swelling or galls and discoloration of the flesh. Currently no durable resistance against this pest is found in commonly grown cultivars. Resistance to *Meloidogyne chitwoodi* was found in several accessions of *Solanum bulbocastanum* Dun. (8). Crosses of *S. bulbocastanum*, a 1EBN diploid species, were made successfully with *S. pinnatisectum* Dun. (35), *S. cardiophyllum* Lindl. (17) and with *S. acaule* Bitt. (21). Hybrids between *S. bulbocastanum* and *S. acaule* were successfully crossed with *S. tuberosum* (22). Other workers failed in attempts to cross *S. bulbocastanum* with wild or cultivated diploids (28). Our efforts to cross nematode resistant *S. bulbocastanum* with *S. acaule* and cultivated diploid potato failed. Our crosses with *S. cardiophyllum* were successful, but the three hybrids obtained were sterile. Owing to the difficulty in crossing the particular *S. bulbocastanum* clones selected for nematode resistance, research presented in this report was initiated to incorporate nematode resistance from *S. bulbocastanum* into *S. tuberosum* breeding lines through somatic means.

Materials and Methods

Plant Material—The nematode-resistant line of *Solanum bulbocastanum* PI 275187, selection #10, was identified in a previous study that used botanical seed obtained from the Potato Introduction Project, IR-1, Sturgeon Bay, WI (8). Clonal copies of the original seedling were established and maintained *in vitro*. The *S. tuberosum* Gp Tuberosum line PI 203900 was also obtained from the IR-1 project. It is a tetraploid potato used as a differential for identification of races of *Phytopthora infestans*. The line was chosen in this and previous studies (2) since protoplasts isolated from this genotype generally divide and regenerate at high frequency. Clonal copies derived from a single sprout of the cultivar Hilite Russet were also maintained *in vitro* on Prop medium (18).

Preliminary Experiments—The *S. bulbocastanum* accession used in this study did not respond well to tissue culture procedures. Protoplast yields were generally low and inconsistent. Regeneration from protoplasts was difficult, only 3 calli eventually produced shoots out of several thousand tested

on a range of media. In contrast, the *S. tuberosum* lines were extremely amenable in culture. Cell division rates of isolated protoplasts were 40-50% and regeneration frequency on a range of media was typically 80-90%. Preliminary fusion experiments with no selection gave only *S. tuberosum* plants. It was not possible to select hybrids based on cultural requirements as was used in earlier studies (2). We therefore elected to use dual fluorescent staining of protoplast preparations prior to fusion (16) to allow for the selection of hybrid cells as outlined below.

Protoplast Isolation— Leaf mesophyll protoplasts of *S. tuberosum* (PI 203900 and cv Hilite Russet) and *S. bulbocastanum* (275187, selection #10) were isolated from *in vitro* leaf material following Shepard's procedure (34) as modified by Haberlach *et al.* (18) with the following changes. After the initial centrifugation in Babcock bottles (enzyme medium) protoplast bands were transferred to a 100 ml Erlenmeyer flask containing 50 ml of rinse medium. 100 µl fluorescein diacetate (stock solution of 5 mg/ml in acetone) was added to *S. bulbocastanum* protoplasts and 150 µl rhodamine isothiocyanate (stock solution of 5 mg/ml in acetone) was added to *S. tuberosum* protoplasts. Flasks were placed in dark for one hour and then transferred to Babcock bottles and centrifuged. Protoplasts were centrifuged twice in fresh rinse medium. Protoplast concentration was determined with a hemocytometer and adjusted to $1x10^6$ per ml. This step resulted in two populations of protoplasts each uniformly labeled with different fluorescent stains.

Protoplast Fusion and Plating—The fusion procedure was essentially the PEG (polyethylene glycol) method (3) with the following modifications. Prior to fusion, protoplast populations were mixed 1 part *S. tuberosum* protoplasts : 2 parts *S. bulbocastanum* protoplasts to a total volume of 2 ml. Fusion using PEG was then carried out. Following the final incubation step of the fusion procedure, 25 ml of wash medium (0.3M sucrose and 0.05M $CaCl_2$ at pH 5.8) was added and the suspension was immediately centrifuged in a Babcock bottle at 1300 rpm (350 g) for 10 min. The protoplast band was transferred to fresh wash medium and centrifuged again. The protoplast band was removed, diluted to approximately $2x10^5$ protoplasts/ml in SKM medium (modified Kao and Michayaluk medium, 23) without BSA (bovine serum albumin) and placed in the dark. The following day, this suspension was diluted in half with SKM without BSA. Two days following this, the suspension was again diluted in half and then plated out as follows: 1 part protoplast solution, 1 part 1.4% agarose and 1 part 2X SKM without BSA were gently mixed and 3 ml aliquots of this suspension poured onto each SKM plate (10 ml with BSA). Plates were placed in dark for one week and then gradually moved to low light. Protoplasts from parental lines not subjected to the fusion procedure were treated similarly.

Selection and Regeneration of Heterokaryons— Fused cells were identified after plating using dual fluorescence. Plates were viewed using a Nikon inverted microscope (Diaphot-TMD) equipped with epifluorescence. *S. bulbocastanum* protoplasts stained with fluorescein diacetate are bright green when

viewed under UV light using filter cassette B-2A (blue excitation 450-490nm, barrier 520nm). *S.tuberosum* protoplasts stained with rhodamine fluoresce bright red using filter cassette G-2A (green excitation 510-560 nm, barrier 590 nm). Heterokaryons therefore fluoresce both green and red when viewed under the two appropriate conditions. The location of the fused cells on the plates was recorded using a Nikon object marker on an inverted microscope. The marker stamps a small ink circle (~1.5 mm) on the underside of the plate. Calli appearing within these circles were picked off as they appeared (2-3 weeks later) and transferred to CUL plates (18) with *trans*-zeatin (1.0 mg/L) replacing BAP. When calli were light green and at least 2 mm in diameter (2-6 weeks) they were transferred to differentiation medium SA4 (MS salts with NH_4NO_3 at half strength, 100mg myo-inositol, 2.5g sucrose, 35g mannitol, N&N vitamins, 2.0mg *trans*-zeatin, 0.5mg kinetin, 0.5mg GA3, 0.1mg IAA, 100mg caesin hydrolysate and 10g noble agar per liter, filter sterilized). Calli with shoot initials were further transferred to a high gibberellin medium (1) to enhance shoot development. Shoots were excised when they were approximately 1cm in height and established on Prop medium (18).

Chromosome Counting and Morphological Observations—Chromosome number was assessed using root tips treated with an 8-hydroxyquinoline solution (0.29g/L), fixed in a 3:1 (v:v) solution of methanol and acetic acid and stained with a solution of 1% acetocarmine. Somatic hybrids were assessed visually *in vitro* and under greenhouse conditions, and their phenotypes compared with parental lines. Additional observations were made on representative hybrids established in the field at Prosser, WA.

Isozyme Analysis—Tuber tissue samples (120mg), taken from the eyes of four dormant greenhouse-grown tubers from representative somatic hybrids and parental lines, were macerated in a 2% glutathione extraction buffer (0.1M Tris-HCl, pH 7.5). The crude protein was eluted onto 3x8 mm paper wicks, then separated by horizontal starch gel (10.5%) electrophoresis (31). Eight enzyme systems were investigated: malate dehydrogenase (MDH), phosphoglucose isomerase (PGI), 6-phosphogluconate dehydrogenase (6-PGDH), acid phosphatase (APS), glutamate oxaloacetate transaminase (GOT), phosphoglucomutase (PGM), peroxidase (PRX) and alcohol dehydrogenase (ADH). Standard staining protocols were used (36). The *Mdh-1, Mdh-2, 6-Pgdh-3, Pgi-1,* and *Aps-1* loci were resolved in a histidine-citrate buffer system, pH 5.7, while the *Got-1, Got-2, Pgm-1, Pgm-2, Prx-3* and *Adh-1* loci were resolved in a lithium-borate buffer system, pH 8.3 (11). Allelic designations are as described (32, 12).

Nematode screening—The nematode population used in this study was WAMc1 (*M. chitwoodi*, race 1). It was obtained from the collection maintained at Washington State University, Irrigated Agriculture Research and Extension Center at Prosser, Washington. Inoculum was derived from single

egg mass cultures, and prepared by collecting eggs after shaking tomato roots (*Lycopersicum esculentum*, Mill., cv. Columbian) with fully developed egg masses in a 0.5% w/v solution of NaOCl (24). Rooted cuttings or plantlets (approximately 7 cm in height) were transplanted into 10cm diameter plastic pots containing methyl bromide fumigated loamy sand (84% sand, 10% silt, and 6% clay). An aliquot of inoculum containing 5000 eggs was pipetted around the root systems of freshly transplanted cuttings. Potato and tomato, excellent hosts for both races of *M. chitwoodi* were used as standard hosts. Alfalfa, (*Medicago sativa* L., cv Thor), a non-host for race 1 was included to detect cross-contamination by race 2 (27). Pepper (*Capsicum annuum* L., cv California Wonder), a non-host for *M. chitwoodi*, was included to check for contamination of inocula with *M. hapla* Chitwood, another common root-knot nematode species in the Pacific Northwest. Plants were allowed to grow with regular watering and fertilization at 24 ±3 C for 55 days. At harvest, roots were washed free of soil and eggs were extracted for counting by shaking roots in 0.5% aqueous NaOCl. The reproductive efficiency [R_f = final number of eggs (P_f)/initial inoculum (P_i)] determines the fecundity of the nematode and is one measure of resistance of host crop species to *Meloidogyne* spp.(29). The reproductive factor, R_f, was reported as the geometric mean of $P_f/5000$ (*i.e.* P_f/P_i). Host suitability was categorized as "good host", "poor host", and "non-host" for R_f values of $R_f > 1.0$, $0.1 < R_f < 1.0$, and $R_f < 0.1$, respectively (33).

Crossability—The male fertile tetraploid breeding clones A84118.3, AC8045.1, A8469.1, and A8292.1 were obtained from J. Pavek, USDA/ARS, Aberdeen, ID, USA. Inflorescences were prepared for crossing by emasculating buds two days prior to anthesis. Pollen was vibrated from flowers into gelatin capsules which were stored in sealed containers with activated silica gel. Pollen was used on the same day of extraction and sometimes on two successive days while being stored in the interim at -12 C. Pollen was applied to stigmas of fully opened emasculated flowers by inserting the style into the gelatin capsule so that pollen coated the stigma. Pollen of the fusion hybrids was stained using the acetocarmine glycerol method (26). Fruits were allowed to develop on the plants for six weeks, removed and stored for four weeks to allow ripening. Seeds were expressed into tap water, debris was decanted and seeds were allowed to air dry on paper towels.

Results

Protoplast Fusion and Production of Somatic Hybrids—Prestaining of protoplast preparations reduced viability and subsequent division of *S. bulbocastanum* protoplasts by as much as 75%, as compared to unstained preparations. Viabilities of *S. bulbocastanum* protoplasts were reduced to zero using overnight staining of protoplast preparations or adding stain to the enzyme digest

medium. Viability of *S. tuberosum* PI 203900 was reduced 20-25%. There was no significant effect on viability of protoplasts isolated from *S. tuberosum* cv Hilite Russet.

Results from a total of four fusion experiments using protoplasts from *S. bulbocastanum* and cv Hilite Russet were as follows. Visual examination of preparations 1 day after fusion showed that approximately 1 out of 200 cells were dual-labelled. Overall viability at this point was typically 5-25%, of which approximately 95% of viable cells were *S. tuberosum*. After plating, 240 putative heterokaryons were circled, of which 105 developed into microcalli and were transferred to regeneration medium. Of these 37/105 regenerated shoots, but only 7/37 gave shoots which appeared phenotypically normal. Three to five representative individual plantlets were excised from each callus piece, rooted and chromosome number assessed. This data showed that 5/7 calli gave rise to plants with 72 chromosomes, the expected chromosome number of somatic hybrids, the remaining 2/7 produced plants which were at the tetraploid level. A total of 20 plants were established from these 5 calli. Plants had the appearance of somatic hybrids, but generally were not vigorous in culture and grew slowly in greenhouse trials. The reason for this is not clear and currently under investigation. These plants were not included in nematode or crossability studies.

Only one fusion experiment out of 3 attempts was successful using protoplasts from *S. bulbocastanum* and *S. tuberosum* PI 203900. Visual examination of protoplast preparations 2 days after fusion showed that approximately 1 out of 120 cells were dual-labelled. Overall viability of the preparation, however, was very low, approximately 4-5 %, of which 80-90% were *S. tuberosum*. Only 12 putative heterokaryons were circled after plating and 2/12 developed into microcalli. Of these 2, only 1 gave morphologically normal shoots with the expected chromosome number of 72. A total of 22 plants were excised from this callus for use in nematode screening and crossability studies.

Morphological Assessment—Under greenhouse and field conditions, *S. bulbocastanum* has undissected dark green rounded leaves with pigmented undersides, a tall erect habit with few heavily pigmented stems, small cream star-shaped flowers and typically sets small round tubers. Plants of PI 203900 have dissected light green leaves, green stems, a bushy habit, medium white-cream flowers and set a large number of medium-sized round tubers.

Somatic hybrids between *S. bulbocastanum* and PI 203900 clearly had phenotypes intermediate to parental types. There was also some obvious variation between individual hybrids. Since all these plants were derived from a single selected microcallus, the most probable reason for differences in phenotype is somaclonal variation induced after fusion. Leaf dissection, growth habit and stem pigmentation were particularly good intermediate characteristics to identify hybrid plants. These characteristics could not always be used to identify hybrids *in vitro*. Typically leaf dissection was not

always evident in test-tube plants and the degree of pigmentation was much more variable, both in *S. bulbocastanum* and the somatic hybrids.

Isozyme Analysis—Isozyme variation was detected at seven of the 11 isozyme loci examined. Of these five loci, *Pgm-1, Pgm-2, Adh-1, Got-2,* and *Aps-1* confirmed hybridity of the somatic hybrids. It was most evident in the PGM, GOT, and ADH enzyme systems (Figure 1). Tetra and triallelic somatic hybrids were identified at the *Pgm-1* and *Got-2* loci, respectively.

Nematode Resistance—Non-host reactions of *M. chitwoodi* on alfalfa (cv Thor), and non-host response on pepper (cv Californian Wonder) confirmed *Meloidogyne chitwoodi* race 1 identity and freedom from contamination by *M. chitwoodi* race 2 and *M. hapla.* All of the fusion hybrids and the resistant *S. bulbocastanum* showed non-host reactions. Either no eggs were present or the reproductive factor (R_f) was less than 0.01. Given that the cultivated tetraploid parent showed a clear good host reaction ($R_f = 3.9$) it is reasonable to conclude that resistance to *M. chitwoodi*, race 1, derived from *S. bulbocastanum* PI275187, selection #10 has been successfully transferred to the somatic hybrids and is fully expressed. Furthermore, resistance to the nematode appears to be dominant to susceptibility.

Crossability—Examination of the pollen of the somatic hybrids revealed that they were pollen sterile, as no acetocarmine stained grains were observed. A total of 290 pollinations were performed using somatic hybrids as pistillate parents. Approximately eleven per cent of the pollinations resulted in berry formation and there was an average of 28.7 seeds per berry.

Discussion

There are many procedures for the production of somatic hybrids in *Solanum* (14). In previous studies we selected for hybrids in culture using media preferences of parental lines coupled with increased vigor of hybrid cells (3, 4, 5). This was not possible with the particular lines used in this study. Dual fluorescent labeling was used as an alternative method. This was not a very efficient process. The main reason for recovery of so few hybrid calli was probably the difficulty of culturing protoplasts from the nematode resistant *S. bulbocastanum* accession used in this research. Our experience is that other *S. bulbocastanum* accessions (unfortunately not nematode resistant) are more amenable in culture (Helgeson, in preparation). Cell division and regeneration from isolated protoplasts were accomplished at reasonable frequencies (5-10%). This observation further illustrates the differences in tissue culture responses seen in individual lines within the same species (18). The *S. bulbocastanum* accession used in this study was also particularly sensitive to staining and to the effects of the fusion procedure. Detrimental effects of using fluorescent stains were not reported in tobacco (16), but have been observed previously in carrot (25). The lack of vigor in fusion hybrids between *S. bulbocastanum* and cv Hilite Russet is difficult

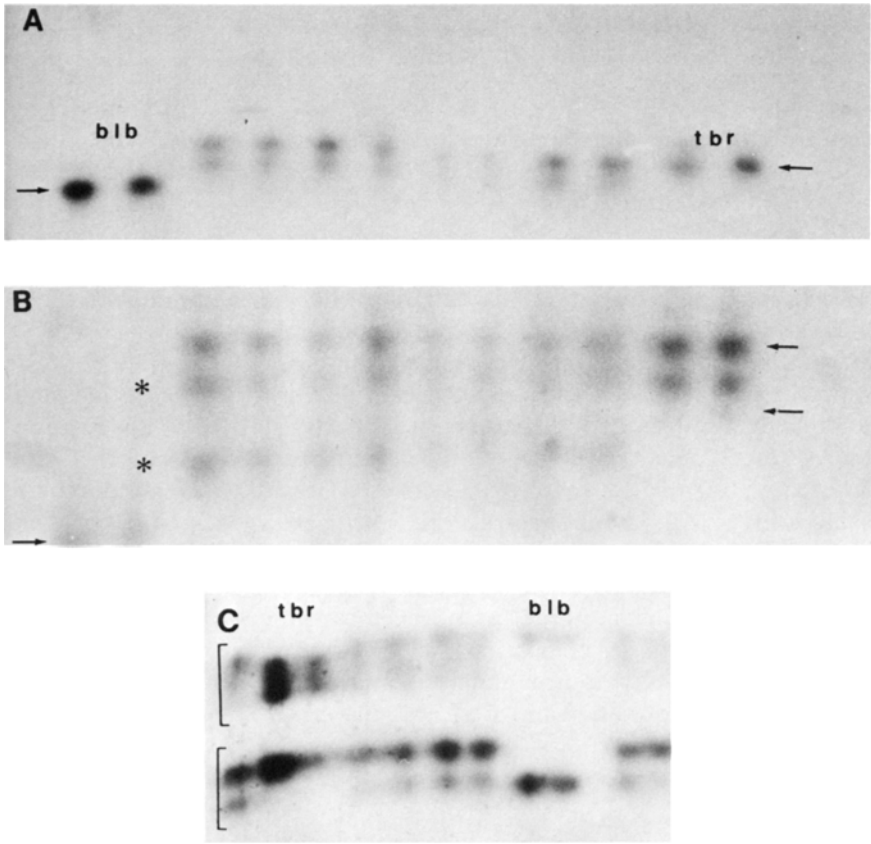

FIG. 1. Isozyme analysis to confirm hybridity of putative somatic hybrids.

A. The fusion hybrids are flanked by the two parents *S. bulbocastanum*; *blb*, and *S. tuberosum*; *tbr*, respectively (2 lanes each). The *blb* and *tbr* parents are homozygous for *Adh-1*^{3} and *Adh-1*^{2} (see arrows). The four hybrids are additive for this dimeric enzyme, however, due to the imbalance of dosage (4 doses for *tbr*, 2 doses for *blb*), *Adh-1*^{3} homodimer is not clearly visualized.

B. The same order of the parents and somatic hybrids is seen for the *Got-2* locus as for the *Adh-1* locus above. The arrows designate in a descending order the *Got-2*^{3}*Got-2*^{5} and *Got-2*^{8} allozymes. The *tbr* parent is heterozygous for the *Got-2* locus *(Got-2*^{3}*2*^{3}*2*^{3}*2*^{5}*)*. In comparison *blb* is homozygous for the *Got-2*^{8} allozyme which has negligible migration from the origin. The somatic hybrids are tri-allelic heterozygotes *(Got-2*^{3}*2*^{3}*2*^{3}*2*^{5}*2*^{8}*2*^{8}*)* as indicated by the intensity of the intra locus heterodimers (*).

C. The *PGM* enzyme (monomeric) resolves 2 loci: *Pgm-1* (upper) and *Pgm-2* (lower). For the *Pgm-1* locus, the *blb* parent codes for an unconfirmed fast-migrating allozyme, whereas the *tbr* parent is triallelic for the *Pgm-1*^{1}, *Pgm-1*^{2}, and *Pgm-1*^{3} allozymes. The hybrids show an additive pattern for the *Pgm-1* locus. The *Pgm-2* locus also demonstrates this additivity with the *tbr* and *blb* parents contributing the *Pgm-2*^{2} and *Pgm-2*^{3} allozymes, respectively.

to explain. Representative plants from fusion calli were shown to be at the expected hexaploid level and clearly had phenotypes intermediate to parental types. Somaclonal variation could be one explanation for poor growth. Another could be a basic incompatibility between these genotypes at the nuclear or cytoplasmic level.

Isozyme analyses were used as confirmation of somatic hybridity between *S. bulbocastanum* and *S. tuberosum* PI 203900. These analyses identified allelic diversity that was introduced to the *S. tuberosum* cultivated gene pool at the *Pgm-1* and *Got-2* loci for *S. bulbocastanum*. In addition, the *S. bulbocastanum* parent contributes the *Adh-1*^{3} and *Mdh-2*^{3} allozymes which are found in very low frequencies in cultivated North American potato cultivars (11). Previous linkage data (12) indicate that these isozyme loci are unlinked, and since chromosome counts support the hexaploid genome structure we can conclude that the chromosome complements from the two parents were expressed in the somatic hybrids.

The limited number of somatic hybrids produced in this study were shown to be nematode resistant. The potential usefulness of these fusion hybrid plants is dependent on their ability to cross sexually and to transmit their desirable characteristics. The demonstration of female fertility in somatic hybrids is therefore encouraging. This is in agreement with previous work using hexaploid somatic hybrids between *S. tuberosum* and another 1EBN wild species, *S. brevidens* (13). Future studies will examine the sexual transfer of nematode resistance.

Acknowledgments

This work was supported in part by a USDA-ARS specific cooperative agreement # 58-91H2-0-282 with the University of Wisconsin Biotechnology Center.

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