THE POTENTIAL USE OF ARABITOL DEHYDROGENASE AS A PLANT SELECTABLE MARKER

by

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(Under the direction of Wayne Parrott)

ABSTRACT

The arabitol dehydrogenase gene was cloned from *Escherichia coli* strain C, modified for plant expression, and transformed into tobacco using *Agrobacterium tumefaciens*. This study indicates that arabitol dehydrogenase could serve as an effective means of plant selection as an alternative to antibiotic resistance markers.

INDEX WORDS: Plant transformation, Selectable markers, Antibiotic resistance.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Markers for Plant Transformation

In the past decade, there has been a surge in the development and use of biotechnology. From the development of new medicines and the potential benefits of gene therapy to the use of transgenic crops, biotechnology is becoming a greater presence in peoples' lives. In the field of agricultural biotechnology, more than 100 million acres of genetically modified crops were planted in the United States (Kucinich, 1999), reflecting the availability of several new transgenic crops available on the market conferring herbicide resistance, insect resistance and modified seed qualities, including high-oleic soybean and high-laureate canola.

Transgenic crops are produced by the introduction of genes from other organisms using the tools of molecular biology (Wullems et al., 1981; Barton, 1983). In developing these plants, selectable markers are linked to desired traits and used to screen for cells which carry the new transgene. There are currently two main selection systems: positive and negative. Negative selection kills the cells which do not contain the introduced DNA. Positive selection gives transformed cells the ability to grow using a specific carbon, nitrogen or growth regulator as the selection agent (Joersbo and Okkels, 1996; Bojsen et al., 1998; Haldrup et al., 1999).

NEGATIVE SELECTION: ANTIBIOTICS

Genes for antibiotic resistance have been the major source of negative selectable markers. Antibiotics have been mostly derived from fungi which use them as a defense against pathogens (Ohmae et al., 1979). One of the first selectable markers used in plants was the neomycin phosphotransferase II gene (*neo* or *NPTII*) (Bevan et al., 1983) derived from the Tn5 bacterial transposon from *Escherichia coli* (Barton and Chilton, 1983). This gene conveys resistance to the amino glycoside antibiotic, kanamycin, by encoding for an aminoglycoside phosphotransferase enzyme which inactivates the toxin (Wallis et al., 1996). Kanamycin is produced by the bacterium, *Streptomyces kanamyceticus*. The use of this resistance gene in plants was accomplished by using the combination of the resistance gene coding sequence with the promoter and terminator from the nopaline synthase or *nos* gene from T-DNA of *Agrobacterium tumefaciens* (Wullems et al., 1981). Tobacco plant cells successfully transformed by the chimeric gene using *A. tumefaciens* were able to grow in the presence of a kanamycin concentration toxic to those plant cells which had not been transformed with the *neo* gene (Bevan et al., 1983).

Hygromycin B resistance is another one of the earliest selectable markers developed (Gritz and Davies, 1983). The antibiotic was isolated from *Streptomyces hygrocopicus,* and its original use was as a vermifuge in poultry and swine livestock operations. The antibiotic's mode of action is as a protein synthesis inhibitor in prokaryotes and in eukaryotes by interfering with tRNA recognition (Zheng et al., 1981). Strains of *E. coli* resistant to the antibiotic were observed, and the resistance was determined to be of plasmid origin (Ohmae et al., 1979). The hygromycin B

phosphotransferase gene *hph* was identified, cloned from *E. coli* and used as a selectable marker to transform susceptible *E. coli* strains and *Saccharomyces cerevisiae* (Gritz and Davies, 1983). To transform the fungus, *hph* was fused to the promoter of the *cyc1* gene isolated from the fungus itself. This system of using *hph* to transform an organism was modified to successfully transform plant cells (Waldron et al., 1985; van den Elzen et al., 1985). The use of hygromycin B as a method of selection in plant transformation has become a commonly used system (Halfter et al., 1992), especially for those crops not affected by kanamycin.

A screening form of selection in plant cell transformation was developed using streptomycin resistance (Jones et al., 1987). Streptomycin does not kill plant cells, but rather bleaches and retards their growth when introduced into the culture medium. The gene for resistance to streptomycin was discovered in the *E. coli* transposon Tn5. The gene was identified as the streptomycin phosphotransferase or *spt* gene. A screening system to identify transgenic plants was developed using the *spt* gene driven by the promoter for agropine biosynthesis from *Agrobacterium* strain LBA 4404 and was successfully utilized to transform tobacco cells (Jones et al., 1987). Transformants exhibited a green phenotype on streptomycin-containing medium, which was in contrast to the bleached, untransformed cells.

Another selectable marker for plant cell transformation is phleomycin resistance. Phleomycin and bleomycin belong to the bleomycin family of antibiotics, which cleave the DNA of susceptible organisms. The resistance gene, *Ble*, encodes for a protein which binds to bleomycin and prevents its ability to cleave DNA (Yuasa and Sugiyama, 1995).

The gene was isolated from both *Streptoalloteichus hindustanus* and the Tn5 transposon of *E. coli* (Perez et al., 1989). Both genes were ligated to either a *nos* or a cauliflower mosaic virus (CaMV) 35S promoter to permit expression in plant cells. Transformed tobacco plants displayed resistance to both phleomycin and bleomycin, but the gene from *S. hindustanus* was observed to be more effective (Perez et al., 1989).

Sulfonamides or sulfa drugs as used in the medical field are antibacterial compounds which inhibit dihydropteroate synthase (DHPS) in folic acid synthesis, and resistance to the drug is conferred in bacteria by R plasmids (Guerineau et al., 1990). A gene on the plasmid, the *sul*I gene, codes for a form of the enzyme insensitive to sulfonamides. A gene from pR46 of *E. coli* was cloned and used to develop a chimeric gene which included the pea ribulose biphosphate carboxylase/oxygenase transit peptide sequence (Guerineau et al., 1990). This allowed for the product to be transported to the chloroplast stroma and processed there. This gene was demonstrated to be an efficient selectable marker for the selection of transgenic shoots from tobacco leaf explants. The *sul*I gene was used to transform potato cells and proved to be a highly efficient means of selection in that species (Wallis et al., 1996). The aforementioned system was developed to overcome the ineffectiveness of hygromycin B resistance selection in potato. This serves as an example of the complexities of transformation systems and that markers are not always interchangeable from one species to another.

Problems with Antibiotic Selection

Though the current concern over the use of genetically modified organisms (GMOs) stems from a variety of issues, one reason is the concern over the antibiotic

selection markers used in the development of transgenic crops (European Parliament, 2001). United States federal guidelines (FDA, 1998) currently state that antibiotic resistance marker genes present in transgenic plants pose no threat to consumers. These guidelines relate to genes which are under the control of eukaryotic promoters and expressed in the transgenic plants. Furthermore, the products of antibiotic resistance genes expressed in plants must not be toxic, allergenic or compromise the therapeutic efficiency of oral antibiotics. Antibiotics which cannot be used as selectable markers in plant systems according to the USFDA are:

"1. Important medicines

2. Prescribed regularly

3. Orally administered

4. Unique in terms of pathogen it controls

5. Invoke selective pressure on the pathogen

6. No or low levels of resistance in naturally occurring populations of bacteria"

The supporting evidence on which the agency bases its policy is that antibiotic resistance genes as selectable markers in plants are degraded for the most part in food processing or in the gastrointestinal tract, and transfer to gut microflora is inconsequential. Therefore, currently used markers are not a reason for concern and can be used according to current protocols and safety measures.

However, European governments have a different stance on the issue, and insist that antibiotic selection markers pose a potential threat to the safety of humans by increasing antibiotic resistance in bacterial pathogens. Plants derived from

microprojectile bombardment might also contain antibiotic resistance DNA sequences used to increase plasmid DNA in bacteria prior to bombardment, but such genes do not contain a plant promoter and therefore are not expressed in plant cells or in growing plants. The *bla* gene (β-lactamase gene for ampicillin resistance) has attracted the most attention in this regard. The *bla* gene was cloned from *Salmonella paratyphi* B in London in 1963 (Datta and Kontamichalou 1965). The *bla* gene codes for β-lactamase, an enzyme which binds to and inactivates certain penicillin and cephalosporin antibiotics. This is the same gene which the FDA has deemed safe in transgenic plants (FDA, 1998). Though this form of the *bla* gene is very specific to the type of antibiotic to which it is resistant to (narrow spectrum of resistance), the EU points to the possibility of mutations within the gene, thus conferring an increase in the spectrum of antibiotics to which the bacterium would be resistant. They also note that because mouth microflora are naturally competent, they could be transformed by plant DNA. Because of the contact between mouth bacteria and pathogens of the respiratory tract, which currently do not exhibit high-level β-lactamase-mediated resistance to penicillin antibiotics, respiratory pathogens obtaining resistance to penicillin antibiotics is deemed a legitimate concern.

The European Union has banned the use of antibiotic markers in GM crops beginning in 2004 and will ban all antibiotic use with trangenic plants in laboratories in 2008 (European Parliament, 2001).

Several β-lactamase genes are found in human and animal gastrointestinal ecosystems and 65% of human isolates contained *bla* genes in profusion (Casin et al., 1999). Why these naturally occurring *bla* genes are not considered to pose the same

threats is an issue that has never been explained. Also, there are many different combinations of antibiotics available to control β-lactamase-producing strains (Salyers, 1996). β-lactam inhibitors and binding proteins that cause resistance problems in hospitals today are the result of modern genes, not the ancestral form of the *bla* gene whose sequence is found in some transgenic plants.

The *neo* gene was found to be so safe that there is no need to deny or restrict the gene's usage as a selectable marker in transgenic plants (Flavell et al., 1992). One reason is because bacteria resistant to the aforementioned antibiotics exist in abundance in nature. The average person has one trillion bacteria inside his or her gut that are resistant to kanamycin (Flavell et al., 1992). Hence, even if a transgene for kanamycin resistance was transferred from a plant cell to a gut bacterium, the gene frequency in the gut bacteria would remain unaltered.

Nonetheless European governments have taken steps to eliminate the use of antibiotic resistance genes for the selection of transgenic plant cells, and this has been a major reason for the halting of marketing and the introduction of genetically modified crops within the European Union (European Parliament, 2001). In the United States, companies such as Gerber (all transgenics), Frito Lay (corn and potatoes) and McDonalds (potatoes and meat from animals fed transgenic grain) have made a policy of eliminating the use of transgenic crops by not purchasing certain commodities which have been tested to be transgenic, and members of Congress have initiated actions against GMOs (Kucinich, 1999). Alternatives to antibiotics for selection of transgenic plant cells must be used so that the products are commercially viable and socially acceptable. Even if the

threat to human health is not real, the negative perception of antibiotic selection is a real issue.

Yet, the reasons for eliminating antibiotic selection go beyond the perceived threats to the effectiveness of antibiotics on human pathogens. Kanamycin does not work well in cereal crops, which have high levels of natural tolerance (Zhou et al., 1995). Furthermore, plant cells dying from antibiotic toxicity release growth inhibitors and toxins which affect the transformed cells and hinder their growth (Haldrup et al., 1998). Such detrimental effects on transformed cells limits the efficiency of transformation of negative systems.

Another detrimental characteristic of antibiotic selection is the effect on the antibiotics on the transformed plant cells. The commonly used antibiotic selective agents kanamycin and hygromycin cause genome-wide DNA hypermethylation (Schmitt et al., 1997). This nonreversible phenomenon leads to gene silencing and can cause serious problems when reporter or other important genes are not expressed, as it can hinder both the selection of transgenic cells and the plant regeneration process.

HERBICIDES/FUNGICIDES

Selectable markers that do not depend on antibiotics have been in use for many years. The fungicide blasticidin produced by *Streptomyces griseochromogenes* can produce phytotoxicity in susceptible plants. A resistant bacterium, *Bacillus cereus* K55-S1, was identified. The gene for resistance (*bsr*), codes for blasticidin deaminase, which inactivates the fungicide via hydrolytic deamination. *Bsr* was cloned and introduced into tobacco as a selectable marker for plant transformation (Kamakura et al., 1990).

Still later, systems were developed using herbicide resistance as a means of selection. One of the first was phosphinothricin or glufosinate (its synthetic, racemic form) resistance conferred by the *bar* gene, from *S. hygroscopicus* (De Block et al., 1987). Phosphinothricin is the herbicidal component of bialaphos, a compound produced by several *Streptomyces* species. Both herbicides are glutamine synthetase inhibitors which prevent the detoxification of ammonia (Wehrmann et al., 1996). *Bar* codes for a phosphinothricin acetyltransferase which detoxifies the herbicide (De Block et al., 1987). The phosphinothricin acetyltransferase gene, *pat*, from *S. viridochromogenese* was transformed into tobacco (Wohlleben et al., 1988). The *bar* gene was used in a binary vector and successfully used to transform *Arabidopsis thaliana* (Bouchez et al., 1993). The *bar* and *pat* genes are very similar in structure and affinity for phosphinothricin and therefore can both be used as selectable markers.

Another system based upon herbicide resistance was developed using glyphosate tolerance. Glyphosate is a non-selective herbicide which inhibits 3-enolpyruvylshikimate-5-phosphate synthase (EPSPS), which is an enzyme in aromatic amino acid biosynthesis (Kamakura et al., 1990; Zhou et al., 1995). Glyphosate tolerance is conferred by two genes. One is the *CP4* gene, isolated from *Agrobacterium* strain CP4, which produces a form of EPSPS which is tolerant to glyphosate and can induce glyphosate tolerance when transformed and expressed in plants (Barry et al., 1992; Kishore et al., 1992). The glyphosate oxidoreductase gene (*GOX*), cloned from *Agrobacterium,* was found to detoxify glyphosate into aminomethyl phosphonic acid (Barry, et al. 1992). When these genes were placed in one plasmid and introduced into

wheat using particle bombardment, transformed calluses could be selected on a medium supplemented with glyphosate, and regenerated plants were shown to be glyphosate-tolerant (Zhou et al., 1995).

Sulfonylurea is an acetolactate synthase (ALS) inhibitor which blocks the biosynthesis of valine, leucine, and isoleucine (Shin et al., 2000). The acetolactate synthase gene (*csr1-1*) isolated from the sulfonylurea herbicide-resistant *Arabidopsis thaliana* mutant *csr*-1 was placed behind a 35S promoter to transform rice protoplasts (Zj et al., 1992). The acetolactate synthase enzyme coded by *crs1-1* is resistant to ALS inhibitors. The use of ALS selection has also been shown to be efficient for cell colony stage selection in poplar transgenic breeding (Chupeau et al., 1994) and in sugar-beet transformation (D'Halluin et al., 1992).

As with antibiotic resistance, the negative effects on transformed cells by the dying non-transformants reduces transformation efficiency. Many transformants are lost because they are adversely affected by dying untransformed cells (Joersbo and Okkels, 1996). Also, herbicide resistance might be transferred to weedy relatives of crops via outcrossing (Rieger et al., 1999). The development of herbicide-resistant weeds could theoretically become a problem in weed management strategies and could cause public concern over introducing transgenes into the environment. Because of the inherent problems with negative selection, positive selection systems are being developed.

POSITIVE SELECTION

One of the first systems to utilize positive selection was the β-glucuronidase gene, or *gus*A gene system. The GUS enzyme catalyses the hydrolysis of *β*-D-glucuronides into

D-glucuronic acid and thus will hydrolyze aglycones or polysaccharides which contain a D-glucuronic acid linkage group (Gilissen et al., 1998). The *gus*A gene was first isolated from *E. coli* and used as a plant scoring marker (Jefferson et al., 1987). The gene is used to study and monitor gene expression and tissue specificity (Gallagher, 1992). The first use of *gus*A as a positive selection system was by transforming tobacco plant cells and placing them on a medium supplemented with benzyladenine-3-N-glucuronide (Joersbo and Okkels, 1996). Only the plant cells which were transformed with the *gus*A gene were able to convert the benzyladenine-3-N-glucuronide into usable cytokinin, and hence acquired the ability to regenerate. This system was shown to have higher transformation frequencies than kanamycin-based selection (Joersbo and Okkels, 1996), but the *gusA* system is not usable for those crops which do not require cytokinin to regenerate from cell culture (e.g. soybean and maize).

A selection system using an inducible isopentenyl transferase gene (*ipt*) system for plant transformation was developed and found to be highly efficient (Kunkel et al., 1999). *Ipt* encodes for an enzyme which catalyzes the reaction that forms the first intermediate in cytokinin biosynthesis. However, use of this system, which uses the *ipt* gene from *Agrobacterium tumefaciens* to regenerate shoots in transformed plants, is complicated because it requires a dexamethasone (Dex)-inducible system which places *ipt* downstream from the binding site of a transcription factor that is activated by Dex (Aoyma and Chua, 1997; Gilissen et al., 1998). Additionally, the cultivation period to obtain normal regenerants is lengthy and selection is based on a phenotypic characteristic

which can be variable in nature (Kunkel et al., 1999). This system is also limited to those plant species that need cytokinin to regenerate.

CARBON-SOURCE SELECTION

A xylose-based positive selection system was developed using the xylose isomerase gene (*xyl*A) from *Thermoanaerbacterium thermosulfurogenes*, which metabolizes xylose into xylulose, which plant cells can use as a carbohydrate source (Bojsen et al., 1998; Haldrup et al., 1998). Those plant cells that are not transformed do not grow. Using the same gene isolated from *Streptomyces rubiniosus*, the efficiency of this system was found to be ten-fold higher than that of kanamycin selection in potato (Haldrup et al., 1998).

Another selection system using a specific carbohydrate for selection is mannose selection. This system uses the mannose-6-phosphate isomerase (*pmi*) gene from the mannose-metabolizing operon in *E. coli* (Bojsen et al., 1998). Mannose is added to medium as the carbohydrate source. Plants convert the mannose into mannose-6-phosphate, a toxic compound, which they cannot further metabolize. The transformed cells convert the mannose-6-phosphate into fructose-6-phosphate, which is then metabolized.

ARABITOL SELECTION

It should be feasible to develop additional positive selection systems. Different *E. coli* strains can use a multitude of carbohydrate sources, due to a series of operons that can be located within the *E. coli* genome (Reiner, 1975). One of these in particular is the ability of *E. coli* strain C to grow on D-arabitol, whereas the laboratory K-12 strains

cannot (Reiner, 1975; Scangos and Reiner, 1978). Since most plants cannot metabolize most sugar alcohols including D-arabitol (Stein et al., 1997), there is an opportunity to develop positive selection systems based on sugar alcohols.

The genes for arabitol metabolism are located in an operon which is adjacent to an operon for ribitol metabolism. The operon has been cloned and sequenced from *Klebsiella pneumoniae* (designated as the arabinitol operon) and includes a transporter (*dal*T), kinase (*dal*K), dehydrogenase (*dal*D) and a repressor (*dal*R) (Heuel et al., 1998).

However, for use as a plant marker, the gene from *E. coli* strain C would be needed because the concept of placing a gene from a human pathogen (*K. pneumoniae)* into a plant would not be socially acceptable. In *E. coli* strain C, the arabitol genes are *atl*T*, atl*D*, atl*K*,* and *atl*R (Heuel et al., 1998). The arabitol dehydrogenase gene, *atl*D, converts D-arabitol into D-xylulose.

Plants have the ability to grow on D-xylulose (Haldrup et al., 1998), so if a plant cell could be transformed with *atl*D, such a cell would then be able to grow in a medium containing D-arabitol whereas an untransformed plant cell would not. We have also verified the ability of soybean embryos to grow on D-xylulose. The arabitol dehydrogenase enzyme also converts D-mannitol into fructose (Linn, 1961), which can be utilized by plant cells as a carbon source (Viola, 1996; Kanabus et al., 1986). We have verified the ability of soybean embryos to grow on fructose. D-mannitol has been used as a substrate for mannose selection of plant cells in a dual selection system using phosphomannose isomerase and mannitol dehydrogenase (Trulson et al., 2000).

The affinity of the *K. pneumoniae* arabitol dehydrogenase for D-mannitol has been estimated to be 30% to 45% relative to its affinity for D-arabitol (Heuel et al., 1998; Linn, 1961). Because of this, a plant cell which has been transformed with *atl*D would also acquire some ability to use D-mannitol as a carbohydrate source. Therefore, a system could potentially be developed using the *atl*D as a selectable marker and using either D-arabitol or D-mannitol as the selection agent.

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CHAPTER 2

THE POTENTIAL USE OF ARABITOL DEHYDROGENASE AS A PLANT SELECTABLE MARKER[1]

[1] Kane, P.M.; LaFayette, P.R.; Parrott, W.A. To be submitted to *In Vitro Cell Dev. Biol. - Plant*

The potential use of arabitol dehydrogenase (*atl*D) as a plant selectable marker

Patrick M. Kane, Peter R. LaFayette, and Wayne A. Parrott

Summary

The arabitol dehydrogenase gene was cloned from *Escherichia coli* strain C. The gene was subsequently mutagenized to remove a predicted intron splice site. Tobacco was transformed with the modified form of the gene using *Agrobacterium*-mediated transformation. This gave tobacco the ability to regenerate on medium containing D-arabitol as the sole carbon source. This study indicates that D-arabitol could serve as an effective means of plant selection as an alternative to antibiotics.

Key words: transformation, non-antibiotic selection, positive selection, biotechnology.

NTRODUCTION

In developing transgenic plants, selectable markers are linked to desired traits and used to screen for cells which carry the new transgene. There are currently two main selection systems: positive and negative. Negative selection kills the cells which do not contain the introduced DNA, and includes antibiotic- and herbicide-based selection. Positive selection gives transformed cells the ability to grow using a specific carbon, nitrogen or growth regulator as the selection agent (Joersbo and Okkels, 1996; Bojsen et al., 1998; Haldrup et al., 1998a). Plant cells dying from antibiotic toxicity release growth inhibitors and toxins which are thought to negatively affect transformed cells and hinder their growth (Haldrup et al., 1998a), thus limiting the transformation efficiency of negative systems. The commonly used antibiotic selective agents, kanamycin and hygromycin, cause genome-wide DNA hypermethylation (Schmitt et al., 1997). This

nonreversible phenomenon leads to gene silencing and hinders both the selection of transgenic cells and the plant regeneration process.

Socio-political issues also affect selection systems. The European Union has enacted a ban on antibiotic resistance genes for the selection of transgenic plant cells effective the end of 2004, and thus any future genetically enhanced plants and food products sold in the EU will have to contain alternative selectable markers or have the markers removed (European Parliament, 2001). If herbicide resistance is used, resistance might transfer to weedy relatives of crops via outcrossing (Rieger et al., 1999).

Different *E. coli* strains can use a multitude of carbohydrate sources, due to a series of operons that can be located within the *E. coli* genome (Reiner, 1975). One of these in particular is the ability of *E. coli* strain C to grow on D-arabitol (Reiner, 1975; Scangos and Reiner, 1978). Since most plants cannot metabolize most sugar alcohols, including D-arabitol (Stein et al., 1997), there is an opportunity to develop positive selection systems based on sugar alcohols. The *dal* operon (GenBank AF045245) has been cloned and sequenced from *Klebsiella pneumoniae,* and includes a transporter (*dal*T), kinase (*dal*K), dehydrogenase (*dal*D) and a repressor (*dal*R) (Heuel et al., 1997). However, for use as a plant marker, the gene from *E. coli* strain C is preferable because the use of genes from a human pathogens (*K. pneumoniae)* faces regulatory hurdles. In *E. coli* strain C, the arabitol genes are located in the *atl* operon (Fig. 2.1), and include *atl*T*, atl*D*, atl*K*,* and *atl*R (Heuel et al., 1998). The arabitol dehydrogenase gene, *atl*D, converts arabitol into xylulose (Fig. 2.2). Plants can grow on D-xylulose (Haldrup et al., 1998b), so if a plant cell could express arabitol dehydrogenase, then such a cell would be

able to grow in a medium containing D-arabitol, whereas an untransformed plant cell would not proliferate. Arabitol dehydrogenase also converts D-mannitol into fructose (Linn, 1961; Hartley, 1984; Stein et al., 1997) which is utilized by plant cells as a carbon source (Viola, 1996; Kanabus et al., 1986).

Because of the inherent problems, both biological and political, in using antibiotic and herbicide resistance genes as plant selectable markers, we investigated the potential of using arabitol dehydrogenase from the non-virulent enteric bacterium, *E. coli* strain C, as a plant selectable marker.

MATERIALS AND METHODS

Cloning the arabitol operon

Cells of *E. coli* strain C (LaFayette and Parrott, 2001) were grown on Luria Bertani (LB) agar plates. Subsequently a single colony was used to innoculate 16- x 100-mm culture tubes containing 2 ml of LB broth. DNA isolation was performed using CTAB extraction (Ausubel et al., 1987), and *Pst*I digests were performed with 3 μg DNA in a 30-μl reaction according to the manufacturer's instructions (New England Biolabs, Beverly, MA). Plasmid Bluescript™ (Stratagene, La Jolla, CA) was digested with *Pst*I (1 μg DNA in a total volume of 20 μl) and dephosphorylated with calf intestinal phosphatase (CIP) (New England Biolabs). Fifteen μl of the genomic digest and 7.5 μl pBluescript™ digest were mixed and concentrated (Maniatis et al., 1982), then resuspended to a final volume of 10 μl. Ligation was done using a FastLink™ ligation kit (Epicentre, Madison, WI) according to the manufacturer's protocol. Electrocompetent *E. coli* DH10B™ (Invitrogen, Carlsbad, CA) were transformed via electroporation, using

a BIO-RAD MicroPulser™ (BIO-RAD, Hercules, CA) according to the manufacturer's protocol. Each transformed culture was placed into 25 ml of D-arabitol-supplemented 2B minimal medium (LaFayette and Parrott, 2001) and incubated in a 125-ml silicon-capped flask at 37 °C, shaking at 225 rpm. *E. coli* transformed with a plasmid containing the arabitol operon can replicate in the arabitol medium, whereas those not containing the operon do not replicate. After noticeable growth was observed, a 1:100 dilution into fresh D-arabitol-modified 2B medium was performed and repeated. The bacteria were streaked onto 2B arabitol plates to obtain single colonies containing the arabitol operon insert. Plasmids were isolated using the Quantum Prep® Plasmid Miniprep Kit (BIO-RAD) using the manufacturer's protocol. *Pst*I digestion and subsequent gel electrophoresis revealed three *Pst*I fragments within the clone. These were subcloned individually into *Pst*I-digested pBluescript™ and transformed into *E. coli* DH5α™ (Invitrogen), but none were able to grow in 2B arabitol medium, indicating none had an intact *atl* operon. The largest fragment, approximately 4.5-kb, was sequenced via the EZ::TN™ <KAN-2> Insertion Kit using the manufacturer's suggested protocols (Epicentre). The sequence of the dehydrogenase, *atl*D, was assembled using GeneRunner 3.00 (Hastings Software, Inc., Hastings-on-Hudson, NY) after comparing sequence runs with the sequence of the arabitol operon from *K. pneumoniae* via BLAST analysis (Altschul et al., 1997). A primer walking strategy was used to sequence the coding and complementary strands of the gene. Primers AtlD-F and AtlD-R (Table 2.1) were constructed from the 5' and 3' ends outside of the coding region, and used to amplify *atl*D via PCR using *Pwo* polymerase (Roche, Basel, Switzerland) according to the

manufacturer's suggested protocol. This fragment was phosphorylated with T4 polynucleotide kinase (NEB) and cloned into the CIP-treated *Eco*RV site of pBluescript™ forming pAtlD, and sequenced. Subsequently, the entire arabitol operon was sequenced using primer walking from the plasmid obtained from *E. coli* DH10B™ cells able to grow on D-arabitol. The sequence was deposited in GenBank as accession number AF378082.

Sequence Modification and Vector Construction

Putative intron splice sites, including both a donor and acceptor splice sequence, were discovered within the sequence of *atl*D using NetGene 2.0 (Hebsgaard et al., 1996). To remove these sites, mutagenesis of the gene was done via overlap PCR (Ho et al., 1989). Mutagenic primers (Table 2) were designed, and then PAGE-purified by the manufacturer (IDT, Coralville, IA) after synthesis. Primers AtlSynR and AtlD-F were used in one reaction, and primers AtlSynF and AtlD-R in another. Both reactions were as follows: 200 ng plasmid pAtlD, 1 μM of each primer, 2.5 U *Taq* polymerase (Perkin Elmer, Boston, MA), 200 μM dNTPs, 10X PCR Buffer and 2.5 mM $MgCl_2$. The PCR reactions consisted of a 2-min initial denaturing at 94 °C followed by 25 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and a final 7-min extension at 72 °C. The resulting PCR 902- and 521-bp products were gel-purified, and 1 μl each used in an overlap PCR reaction using *Pwo* polymerase with primers ATLD-F and ATLD-R according to the manufacturer's protocol. The PCR reaction consisted of 25 cycles of 94 °C for 1 min, 50 °C for 45 s, 72 °C for 70 s, followed by a 7-min extension at 72 °C. The mutagenized gene, hereafter designated *atl*D-1, was cloned into pBluescript™ to

form pATLD-1 as described for *atl*D, and sequenced to confirm the presence of the expected mutations. To construct a suitable plant vector (Fig. 2.3), *atl*D-1 was excised from pATLD-1 using an *Eco*RI/*Hin*dIII digest and ligated into *Eco*RI/*Hin*dIII digested pUPC-6 (courtesy of Joe Nairn, School of Forest Resources, University of Georgia) to form pUP-A1, thus placing *atl*D-1 under the control of the Ubi3 promoter and Ubi3 terminator (Garbarino and Belknap, 1994). The *npt*II gene was excised from pMKan (Thompson et al., *in press*) via a *Bgl*II/*Bam*HI digest, blunted with T4 DNA polymerase and inserted into the blunted *Xho*I site of pCAMBIA 1305.2 (CAMBIA, Canberra, Australia), thus replacing *hph* with *npt*II and forming p35KG+. Plasmid UP-A1 was digested with *Spe*I/*Stu*I to release the Ubi3P-*atl*D-1-Ubi3T construct, which was subsequently blunted and inserted into the blunted *Xba*I site of p35KG+ to form plant transformation vector pK.

Plant transformation

A. tumefaciens strain EHA105 (Hood et al., 1993) was transformed by electroporation with plasmid pK using a Micropulser™ (BIO-RAD) according to the manufacturer's protocol and subsequently used to transform *Nicotiana tabacum* cv. KY160 by leaf disc transformation (Horsch et al., 1985). *Agrobacterium* cells were grown overnight in YM medium (Vincent,1970) supplemented with rifampicin and kanamycin at 50 mg l^{-1} each. Leaf sections of no more than 1 cm^2 were cut from 30-day-old tobacco leaves from aseptically grown plants and placed into the YM-bacteria solution. The pieces were sectioned under the same medium to a size of 0.25 cm^2 and placed onto Tobacco Organogenesis Medium (TOM), which consists of Murashige and

Skoog salts (1962); B5 vitamins (Gamborg et al., 1968); 2.5 mg l^{-1} BAP; 1 mg l^{-1} IAA; 30 g l^{-1} sucrose; and 2 g l^{-1} GELRITE™ (Sigma, St. Louis, MO) for 2 days of co-cultivation. Sixteen explants were placed on each plate, for a total of 6 plates. Controls included two plates of tissue not subjected to transformation. The co-cultivated pieces were transferred to TOM, supplemented with 500 mg l^{-1} of cefoxitin (Merck, West Point, PA) and 300 mg l^{-1} of kanamycin and subcultured weekly. One control plate was subcultured to TOM on a weekly basis and the other control plate was subcultured to TOM supplemented with 300 mg l^{-1} of kanamycin. After one month, regenerating shoots were placed on T^- rooting medium, which is the growth-regulator-free version of TOM. T^- rooting medium was supplemented with 300 mg l^{-1} of kanamycin to eliminate any escapes. All tissue was cultured under 40 μE m^2 s^{-1} for 23 h d^{-1} provided by cool white flourescent tubes at 23 °C. Those shoots that rooted in T^- were transferred to soil pots inside GA-7 boxes (Magenta Corp., Chicago, IL). After a week, the plants were transferred to soil pots in greenhouse conditions.

Transgene analysis and expression

For RT-PCR and Northern analysis, total plant RNA was isolated from both transgenic and non-transgenic tobacco plants using an RNeasy™ Mini Kit (Qiagen, Santa Clarita, CA) and quantified by spectroscopy at a wavelength of 260 nm. RT reactions were performed with 1 μg RNA using the FirstStrand™ RT-PCR kit (Invitrogen). The resulting cDNA was subjected to PCR using primers ATLD-R and AtlD-f2a using a 2-min initial denaturing at 94 °C, followed by 40 cycles of 94 °C for 1 min, 45 °C for 45 s, 72 °C for 1 min, and a final 7-min extension at 72 °C.

RNA samples (10 μg) were denatured and electrophoresed in a 1% agarose gel according to Pelle and Murphy (1993). The RNA was then transferred to a Hybond N^+membrane (Amersham, Piscataway, NJ) via downward blotting (Chomczynski, 1992) in 5X SSC (1X SSC = 150 mM NaCl, 15 mM sodium citrate), 10mM $NaPO_4$ and fixed using UV cross-linking. PCR was used to generate templates for hybridization probes for *npt*II and *atl*D-1. Primer pairs were as follows (Table 2.1): *npt*II (nptII A & nptII B); *atl*D-1 (ATLD-R and ATLD-f2a). The PCR reactions consisted of a 4-min initial denaturing at 94 °C followed by 45 cycles of 94 °C for 1 min, 47 °C for 45 s, 72 °C for 1 min, and a final 7-min extension at 72 °C. The PCR products were individually purified using the Concert™ Nucleic Acid Purification System (Invitrogen) and labeled using the North2South ™ Direct Labeling and Detection Kit (Pierce, Rockford, IL). Lanes were hybridized at 55 °C with a probe for either *npt*II or *atl*D-1 using a North2South™ Direct Labeling and Detection Kit (Pierce) according to the manufacturer's protocol.

Shoot induction

Twenty tobacco explants (0.25 cm^2) from a single tobacco line, transgenic for both *npt*II and *atl*D-1 and 10 non-transgenic explants were placed onto each of the following media: TOM; TOM supplemented with kanamycin at 300 g l^{-1} ; TOM modified to contain mannitol (16 g l^{-1}) or arabitol (13.3 g l^{-1}), replacing sucrose at equimolar amounts. Each explant was subcultured on a weekly basis for a month to mimic selection conditions. Then the numbers of transferable shoots per explant were counted. The data were tested for heterogeneity of variance (Bartlett and Kendall, 1946), and then analyzed by PROC ANOVA and LSD MEANS using SAS version 8.0 (SAS Institute, Cary, NC).

Substrate analysis

To test the activity of the arabitol dehydrogenase enzyme for D-arabitol relative to D-mannitol, substrate assays were performed. Crude protein extracts of four transgenic tobacco lines and one non-transgenic line of KY160 were isolated. Fresh, young leaf tissue (1 g) was removed and placed at 0-4 °C. Approximately 1 g (wet wt.) of tissue was isolated per line. The tissue was homogenized with a mortar and pestle in a 5 ml solution of 20 mM Tris-HCL (pH 8.5) and 1.4 mM 2-mercaptoethanol at 0-4 °C and centrifuged for 15 min at 20,000 g at 4 °C. The supernatant was removed and placed on ice. Protein concentrations were measured by the protein dye binding method (Bradford, 1976). Arabitol dehydrogenase was assayed by following the reduction of NAD at 340 nm. The assay mixture consisted of 50 mM Tris-HCL (pH 9.5), 1 mM NAD, 50 mM of either D-arabitol or D-mannitol, and 10 μg crude enzyme extract (Stein et al., 1997). To establish baselines for reduction, A_{340} was measured over a 5-min period prior to the addition of arabitol or mannitol. The net change at A_{340} with D-arabitol or D-mannitol was measured for each transgenic line and the ratios were calculated as an estimate of the relative activity of arabitol dehydrogenase for D-mannitol compared with D-arabitol.

RESULTS AND DISCUSSION

Cloning and analysis

The arabitol operon (GenBank AF378082) of *E. coli* is 5344-bp in length. The operon consists of a repressor (*atl*R) 942-bp long, a dehydrogenase (*atl*D) 1368-bp long, an arabitol-induced xylulose kinase (*atl*K) 1464-bp long, and a 1278-bp transporter (*atl*T). As with the arabinitol operon (*dal*) of *K. pneumoniae* (Heuel et al., 1997), the

repressor is transcribed in an orientation opposite the rest of the operon. Overall, the operons of *E. coli* C and *K. pneumoniae* are 75% identical at the DNA level. *Atl*R is 75% identical at the DNA level to *dal*R and 82% identical at the protein level. The dehydrogenase, *atl*D, is 77% identical to *dal*D at the DNA level and 84% identical at the protein level. The xylulose kinase, *atl*K, is 75% identical to *dal*K at the DNA level and 83% at the protein level. The transporter gene, *atl*T, is 77% identical to *dal*T at the DNA level and 84% identical at the protein level.

Analysis of the *atl*D sequence found two possible start codons, one upstream from the start codon for *dal*D. The most suitable start codon for plant genes was determined using NetStart 1.0 software (Pederson & Nielsen 1997). Accordingly, the second 'ATG' (homologous to the start codon in *dal*D) was the most suitable start codon for a plant gene, generating a score of 0.764 compared to 0.388 for the upstream methionine. The gene was annotated as such when deposited to GenBank (AF359520).

Non-transgenic tobacco tissue did not produce shoots on kanamycin, mannitol, or arabitol-containing media. Shoot proliferation from transgenic explants (Fig. 2.7) occurred in media containing sucrose or D-arabitol, but not D-mannitol. To determine why mannitol was not able to support regeneration, enzyme activity measurements revealed that the activity of arabitol dehydrogenase for D-mannitol is approximately 15% that for D-arabitol. There was no net activity with either sugar alcohol in the non-transgenic extracts. The arabitol dehydrogenase from *K. pneumoniae* is estimated to have an enzymatic specificity for D-mannitol of 36% relative to D-arabitol (Linn, 1961). In comparison, the arabitol dehydrogenase from the alga *Galdieria sulphuraria* is estimated

to have an enzymatic specificity for D-mannitol of only 6% relative to that for D-arabitol (Stein et al., 1997).

D-arabitol supported plant regeneration in lines transgenic for *atl*D-1 and *npt*II, but generated significantly fewer shoots than on sucrose supplemented with kanamycin (300 mg l^{-1}) (Fig. 2.7). The arabitol transgene was being transcribed as shown by RT-PCR (Fig. 2.4) and at the correct size, as indicated by northern analysis (Figs. 2.5 and 2.6). Since transcription of *atl*D-1 does not appear to be limiting, the lower regeneration frequency on arabitol may be the result of poor translation due to the prevalence of prokaryotic codons within the sequence of *atl*D-1. Analysis of the gene using GENSCAN (Burge and Karlin, 1997) generated an expected protein expression score of 0.63 (out of a possible 1.00), while the *npt*II gene produced a score of 0.99. This may indicate that the expression of the *npt*II gene is much higher than that of *atl*D-1. It is possible that a synthetic form of the *atl*D gene, with codons optimized for plant expression, could greatly enhance expression and improve the efficiency of selection.

The results indicated that the arabitol dehydrogenase gene could be used as a selectable marker in the future, however more work needs to be done. One possibility is the need for sequences of arabitol and sucrose and/or mixtures of arabitol and sucrose for plant selection, as was done with mannose selection in maize (Negrotto, et al., 2000). When the correct sequence of events and selection medium for transformation have been worked out, arabitol dehydrogenase could serve as a highly effective marker and a viable alternative to antibiotic resistance genes for plant transformation.

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FIGURES AND TABLES

Fig. 2.1: Relative sizes and location of genes within the arabitol operon of *E. coli* strain C. Total size of the operon is 5344 bp in length.

Fig. 2.1 Arabitol operon of *E. coli* strain C.

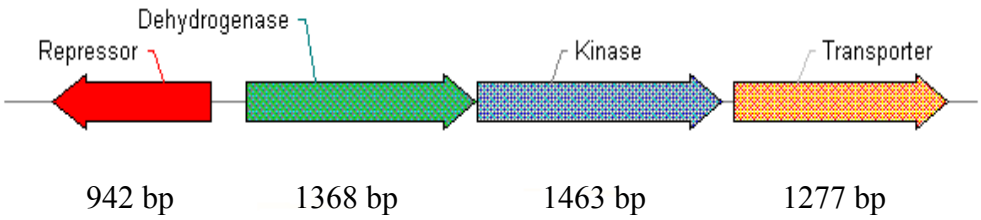


Fig. 2.2: D-arabitol metabolism in *E. coli* strain C by genes from the arabitol operon. D-arabitol is converted to xylulose by arabitol dehydrogenase (*atl*D). Next, an arabitol-induced xylulose ATP kinase (*atl*K) converts xylulose to xylulose-5-P which then continues on into the pentose phosphate pathway without the need of further arabitol-specific genes.

Fig. 2.2 Metabolism of arabitol in *E. coli* strain C.

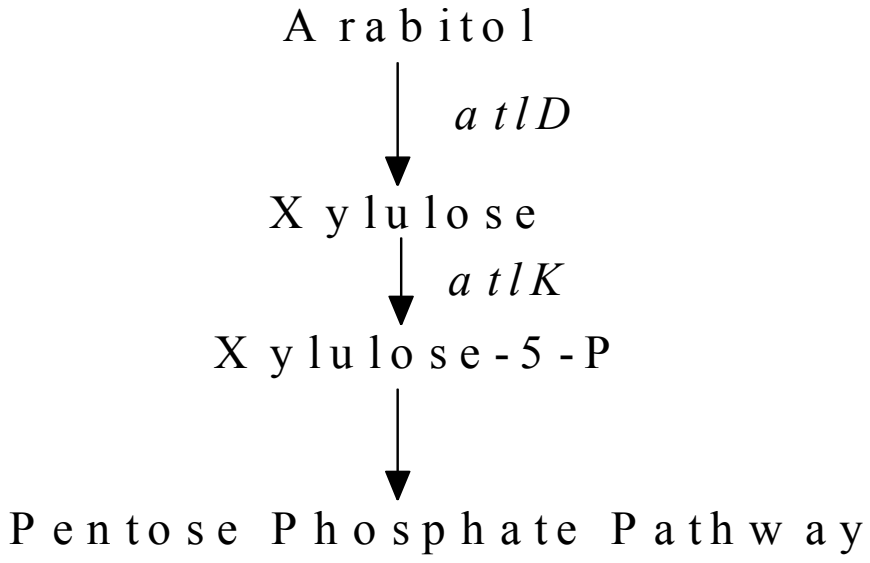


Table 2.1 Primers used in PCR amplification, probe generation and gene mutagenesis.

Primer	Sequence
ATLD-F	5'GAGAACGAAACAATGAACG
ATLD-R	5'GATACATAACCGCCTCCTG
AtlSynF	5'GTACTTTGCTGGATATATTTCTGACCGATCAAAG TTCCAGCCCAGCGATGCAAC
AtlSynR	5'CAGAAATATATCCACGAAAGTACAATGACTGATT TTATCTATCAAATTGCTGACC
AtlDrp-2	5'AAAGTTCCAGCCCAAGCG
AtlD-f2a	5'GGTGAACGTTTCCATGAT
nptII A	5'CCATTTTCCACCATGATATTCG
nptII B	5'AGAGGCTATTCGGCTATGACT

Fig. 2.3: Construction of transformation vector pK. First, *atl*D-1 was excised from pATLD-1 using an *Eco*RI/*Hin*dIII digest and ligated into *Eco*RI/*Hin*dIII digested pUPC-6 to form pUP-A1 placing *atl*D-1 under the control of the Ubi3 promoter and Ubi3 terminator. The *npt*II gene was excised from pMKan via a *Bgl*II/*Bam*HI digest, blunted with T4 DNA polymerase and inserted into the blunted *Xho*I site of pCAMBIA 1305.2, thus replacing *hph* with *npt*II and forming p35KG+. Plasmid UP-A1 was digested with *Spe*I/*Stu*I to release the [Ubi3P-*atl*D-1-Ubi3T] construct, which was subsequently blunted and inserted into the blunted *Xba*I site of p35KG+ to form plant transformation vector pK.

Fig. 2.3 Vector construction

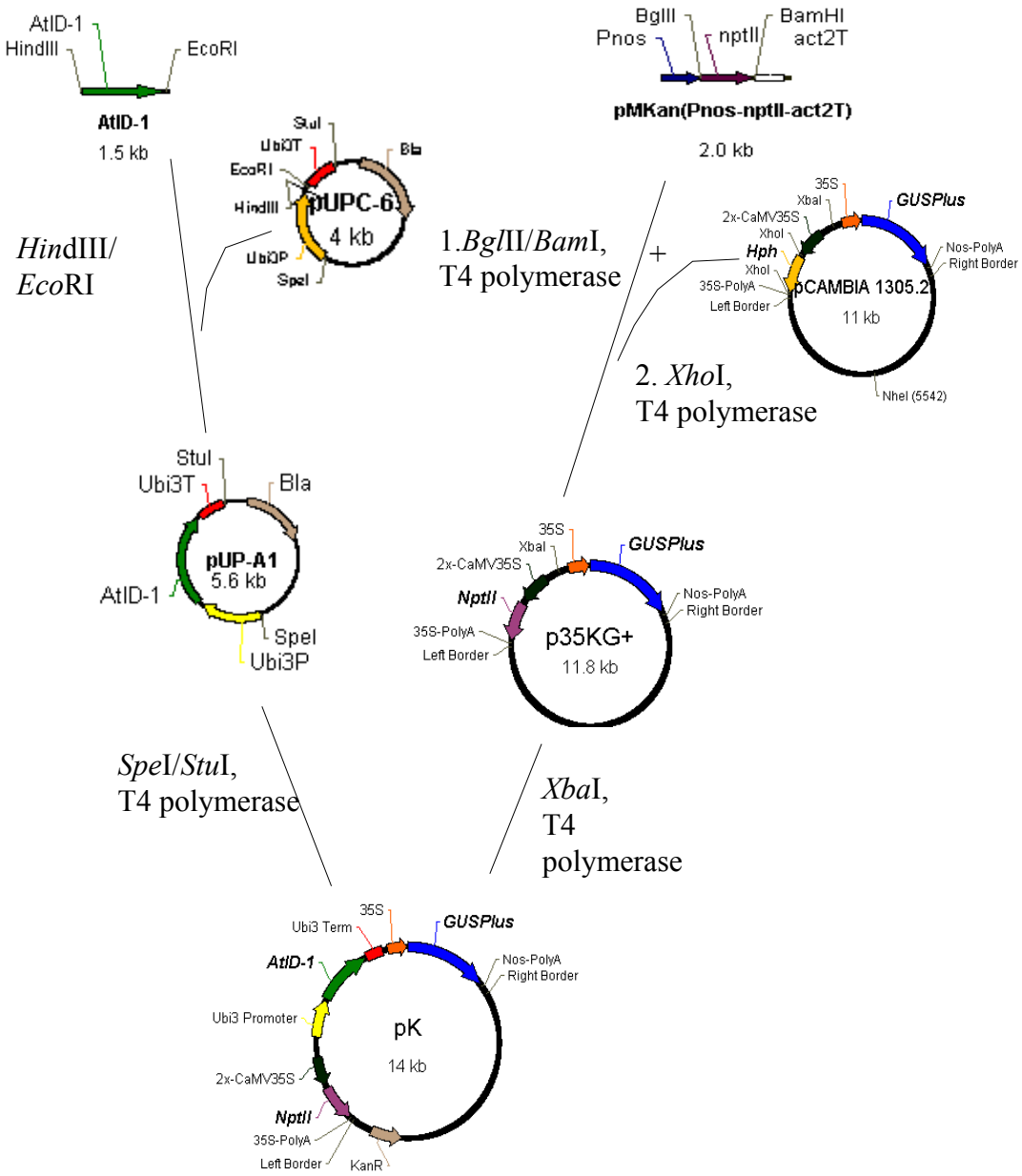


Fig. 2.4: An ethidium bromide-stained gel of RT-PCR products obtained with *atl*D-1-specific primers ATLD-R and AtlDf-2a, 900 bp apart. The templates for each reaction correspond to the lanes on the gel as follows: (1) cDNA generated from transgenic tobacco line 1-3 RNA; (2) PCR control reaction containing no template; (3) control reaction containing no reverse transcriptase; (4) plasmid pK, which was used as the transformation vector

Fig. 2.4 RT-PCR analysis of *atl*D-1 transcript.

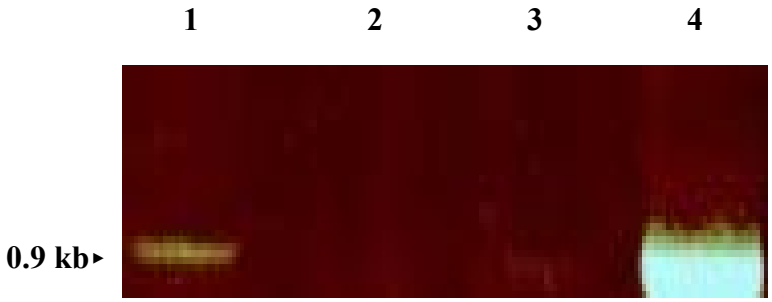


Fig. 2.5: Northern analysis. The blot on the left was hybridized with the *atl*D-1 probe. No transcript was present in the non-transgenic control and the correct-sized transcript was present in the transgenic line 1-3. A duplicate blot hybridized with an *npt*II probe is shown on the right. The *npt*II transcript was present and at the correct size in the transgenic line, but not present in the non-transgenic control. The lower panels are ethidium bromide-stained gels showing equal loading of RNA samples.

Fig. 2.5 Northern analysis indicating transcription of *atl*D-1 and *npt*II in transgenic tobacco.

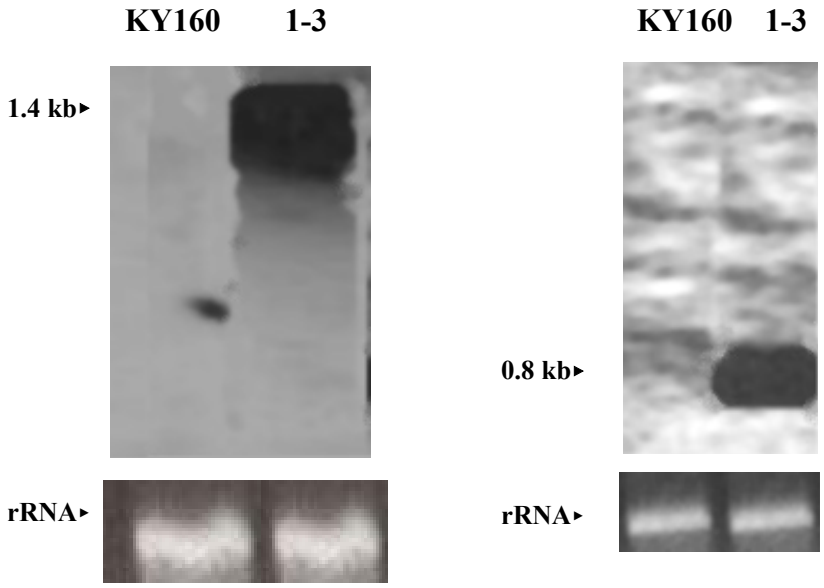


Fig. 2.6: Northern analysis of the transcription of *atl*D-1 in transgenic lines 1-6, 1-9, 2-1, and 3-1. KY160 RNA was used as a control to detect non-specific binding of the *atl*D-1 probe. The *atl*D-1 transcript was present at the correct size in all transgenic lines tested and absent in the KY160 lane. The lower panel is an ethidium bromide-stained gel showing equal loading of RNA samples.

Fig. 2.6 Northern analysis demonstrating transcription of *atl*D-1 across several lines.

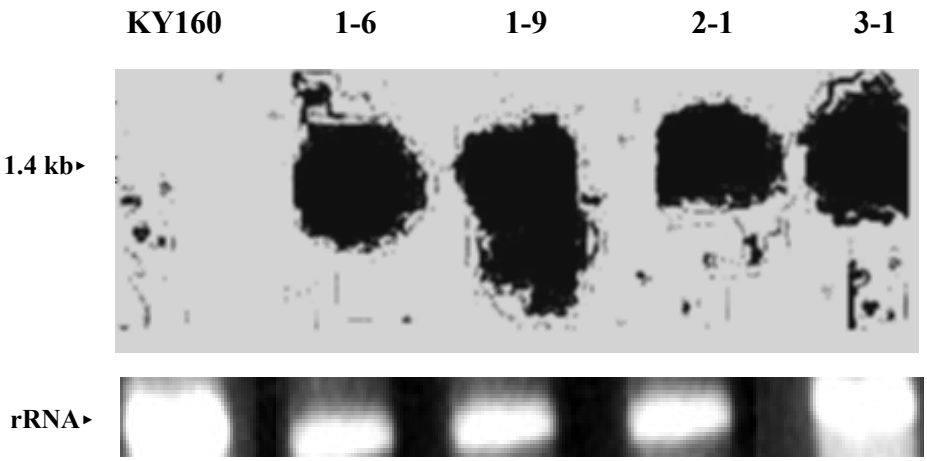
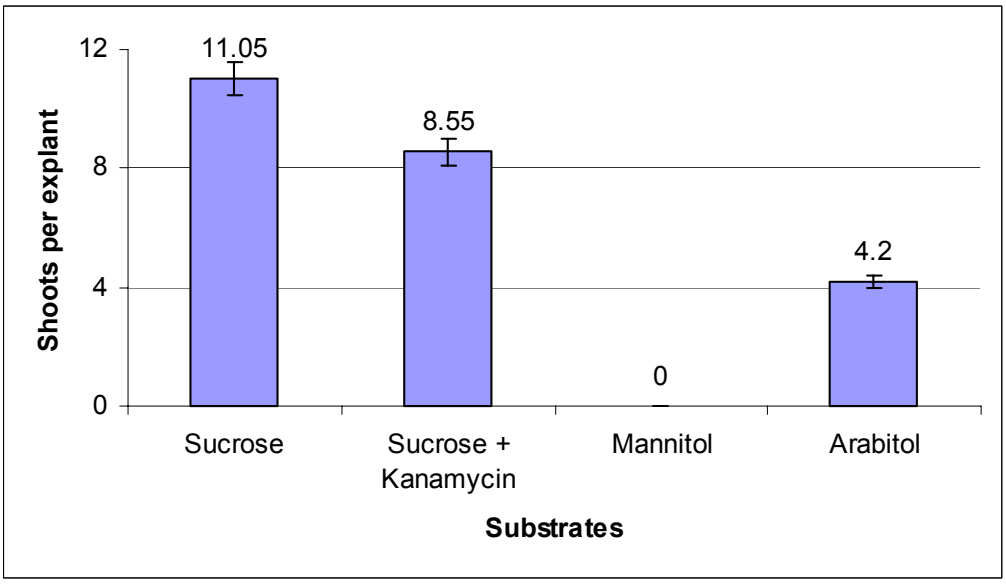


Fig. 2.7: Ability of tobacco explants to undergo shoot induction on modified shoot induction media. Treatments consisting of placing explants from a single event transgenic for both *atl*D-1 and *npt*II onto TOM with one of the following carbon-sources: sucrose; sucrose supplemented with kanamycin at 300 mg l^{-1}; D-mannitol or D-arabitol equimolar to sucrose. Data were analyzed using PROC ANOVA followed by LSD MEANS (pairwise T-tests), all using SAS 8.0. Regeneration frequencies for transgenic tobacco explants are displayed in terms of the average number of transferable shoots per explant after 30 d for each treatment. The treatment means were significantly different from one another at $p<0.01$.

Fig. 2.7. Ability of transgenic tobacco to undergo shoot induction on various carbon sources.



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APPENDICIES

A - ARABITOL SELECTION

CARBON-SOURCE EVALUATIONS

Soybean

To determine if a selection system based upon the conversion of mannitol to fructose would be feasible, soybean (*Glycine max* L. Merr cv. Jack) embryos 20 days after subculturing on MSD20 (Wright et al., 1991) were grown on carbon-source-modified MSD20 medium. Sucrose, mannitol and fructose were added in molar equivalence to sucrose (30 g l^{-1}) to 100- X 15-mm Petri dishes. MSD20 medium was autoclaved and filter-sterilized carbon sources added before solidification. Embryogenic tissue was spaced evenly with a total of 5 per plate and exposed to 20 µE m^2 s^{-1} provided by cool white flourescent light, 23 h d^{-1}, at 26 °C for 4 weeks. Three replications were performed per treatment, and the number of live embryo clusters counted at the end of the study (Table A.1). Since no embryos grew on mannitol, but thrived on fructose, mannitol was determined to be a potential selection agent for transformation.

Table A.1 Ability of carbon-sources to support growth of soybean embryos.

	No. of live embryo clusters		
Rep	**Sucrose**	**Fructose**	**Mannitol**
1	5	5	0
2	5	5	0
3	5	5	0
Total	15	15	0

A total of 5 soybean somatic embryos were subcultured to each plate containing MSD20 medium, or MSD20 carbon-source-modified to contain fructose or mannitol in equimolar amounts to sucrose (30 g l^{-1}). The above table displays the number of live embryo clusters after 30 d for each plate and demonstrates the inability of soybean embryos to proliferate on mannitol.

Tobacco

Since tobacco regenerates faster than soybean and is a model plant for transformation, it was first decided to test arabitol as a selectable marker in tobacco. First, however, carbon-source studies needed to be performed as was done with soybean. Leaf discs 1 cm in diameter were punched from 30-day-old plants of *Nicotiana tabacum* cv. KY 160 using a cork borer. Each treatment consisted of three discs placed upon various media, all versions of TOM shoot induction medium. The treatments were as follows: sucrose 1X (30 g l^{-1}); 0.5X sucrose (15 g l^{-1}), 0.25X sucrose (7.5 g l^{-1}), 0.1X sucrose (3 g l^{-1}), 0.01X sucrose (0.3 g l^{-1}); no carbon-source; fructose, mannitol, or arabitol replacing sucrose (30 g l^{-1}) in equimolar amounts. All sucrose treatments were supplemented with mannitol to keep osmolarity constant. After 30 days, the discs were freeze-dried and their dry mass measured for analysis. Data were analyzed using PROC ANOVA and then MEANS LSD using SAS version 8.0 (SAS Institute, Cary, NC). The results are summarized in Table A.2 and indicate that arabitol and mannitol do not support tobacco regeneration and thus could serve as selection agents.

Table A.2 Comparison of carbon-sources to induce shoot growth of non-transgenic tobacco explants *Nicotiana tabacum* cv. KY 160.

Treatment	Mean of 3 (g)	MEANS LSD*
Fructose	0.0686	A
No carbon	0.0040	D
Sucrose 0.5X	0.1170	B
Sucrose 0.25X	0.0791	A
Sucrose 1X	0.1276	B
Mannitol	0.0036	D
Arabitol	0.0027	D
Sucrose 0.1X	0.0708	C
Sucrose 0.01X	0.0104	D
		*Treatments with the same letter are not significantly different at p=0.05.

The above displays the results of carbon-source study of tobacco explants. Treatments were as follows: sucrose 1X (30 g l^{-1}); 0.5X sucrose (15 g l^{-1}), 0.25X sucrose (7.5 g l^{-1}), 0.1X sucrose (3 g l^{-1}), 0.01X sucrose (0.3 g l^{-1}); no carbon-source; fructose, mannitol, or arabitol replacing sucrose in equimolar amounts. After 30 days, 3 discs per treatment were freeze-dried and dry mass measured for analysis. Data were analyzed using PROC ANOVA and then MEANS LSD using SAS version 8.0 (SAS Institute, Cary, NC).

TOBACCO TRANSFORMATION

Transformation with the native altD gene

A. tumefaciens strain EHA105 (Hood et al., 1993) was transformed by electroporation with plasmid pAGUA, which contained *atl*D under the control of the ubi3 promoter and terminator (Garbarino and Belknap, 1994) as well as an Act2P-GUS*Plus*-Act2T construct, using a Micropulser™ (BIO-RAD) according to the manufacturer's protocol and subsequently used to transform *Nicotiana tabacum* cv. KY160 by leaf disc transformation (Horsch et al., 1985). *Agrobacterium* cells were grown overnight in YM medium (Vincent,1970) supplemented with rifampicin and kanamycin at 50 mg l^{-1} each. Leaf discs of 1 cm in diameter were cut from 30-day-old tobacco leaves from aseptically grown plants and placed into the YM-bacteria solution. The pieces were lanced lightly under the same solution, blotted, and 10 placed onto each plate of shoot induction medium, which consisted of Murashige and Skoog salts (1962); B5 vitamins (Gamborg et al., 1968); 0.3125 mg l^{-1} BAP[2]; 1 mg l^{-1} IAA; 30 g l^{-1} sucrose; 2 g l^{-1} GELRITE™ (Sigma, St. Louis, MO) for 2 days of co-cultivation. Controls included three plates of tissue not subjected to transformation. The co-cultivated pieces were transferred to carbon-source modified medium containing arabitol or mannitol equimolar to sucrose (30 g l^{-1}), supplemented with 500 mg l^{-1} of cefoxitin and subcultured weekly. A total of three plates of tissue were selected on arabitol and three on mannitol. One control plate was subcultured on a weekly basis and the other two control plates were subcultured to arabitol and mannitol. After 2 months, no regenerating shoots were visible except on the

[2] This amount was based upon a lab protocol received from the University of Kentucky. The correct amount was later determined to be 2.5 mg l^{-1} BAP.

sucrose-control plate, indicating selection with *atl*D had failed. This prompted further analysis of the gene to help determine the cause of failure.

Transformation using the mutagenized atlD gene

Putative intron-splice sites were discovered using prediction server software. After removing these sites using primer mutagenesis to form gene *atl*D-1, another round of tobacco transformation was performed. Transformation was performed as before, but this time using plant vector pK, which contains the ubi3P-*atl*D-1-ubi3T construct, as well as the 35SP-GUS*Plus*-NosT construct and the 2X-35SP-*npt*II-35ST construct. In this experiment, leaf discs were co-cultivated as before. Selection media (same as used with *atl*D) were supplemented with kanamycin at 300 mg l^{-1} and cefoxitin at 500 mg l^{-1}; or carbon-source modified containing arabitol or mannitol equimolar to sucrose (30 g l^{-1}) and 500 mg l^{-1} of cefoxitin. Two plates with 20 discs total were subjected to co-cultivation for each treatment. Controls consisted of a plate of 10 discs not co-cultivated, but subcultured to induction medium, medium supplemented with kanamycin, 300 mg l^{-1}; or carbon-source modified with arabitol or mannitol. All discs were subcultured on a weekly basis. After one month of selection, shoots developed on discs subjected to kanamycin selection and small buds developed on shoots subjected to mannitol selection. The mannitol-selected buds were transferred to sucrose for a one week recovery period and then subcultured back to mannitol for two weeks. At this time, all surviving shoots were transferred to T^- rooting medium, which contains no plant growth regulators. The rooting medium was supplemented with kanamycin at 300 mg l^{-1}. A total of five shoots was recovered from mannitol selection, none from arabitol, and two from kanamycin.

This suggested that there was a possibility of using mannitol as a selection agent with *atl*D-1 as the selectable marker. However, all shoots were vitrified and only one plant was recovered by micrografting onto KY160 root stock. Because of the relatively low frequencies of transformation, I traveled to the University of Kentucky to study tobacco transformation and determined that the correct amount of BAP is 2.5 mg l^{-1}. The incorrect amount had been given to the laboratory in a protocol received from collaborators at the University of Kentucky. Once the medium had been changed to contain the correct amounts of plant growth regulators, shoot induction under selection produced normal shoots and plants and much higher frequencies of transformation. Nonetheless, the incorrect ratio of growth regulators may have led to recovery of shoots with mannitol but not arabitol.

SOYBEAN TRANSFORMATION

For the induction of soybean somatic embryos, cotyledons were excised from immature zygotic embryos of *Glycine max* L. Merr. cv. Jack, and placed on MSD40 medium (Finer and Nagasawa, 1998) with the flat side up. After 6 weeks of induction, the embryos were transferred to MSD20 medium and subsequently subcultured every four weeks. At the time of subculturing, selection for "raspberry" appearing globular-stage embryos under light microscopy was performed. Plates of MSD20 tissue were subcultured to fresh MSD20 arranged in a tight circle of approximately 2 cm in diameter in the center of the Petri dish. After four days, the five plates were shot once with plant vector pK using standard shooting parameters (Christou et al., 1991) and another two plates were shot using a vector containing the hygromcyin resistance gene, *hph*, under the

direction of the 35S promoter. The embryos used were 5 months old at the time of shooting. Approximately 0.25 g of tissue was placed into a 125-ml Erlenmeyer flasks containing 25 ml of FNL medium (Samoylov et al., 1998b) shaking at 125 rpm at 26 °C. Each plate shot yielded enough tissue for two liquid flasks. After one week, the media were replaced with FNL medium modified to contain various levels of sucrose and mannitol. Two flasks each contained one of the following media: FNL (sucrose at the standard level of 10 g l^{-1}); FNL (sucrose at 7.5 g l^{-1}); FNL (sucrose at 5.0 g l^{-1}); FNL (sucrose at 2.5 g l^{-1}); FNL containing mannitol completely replacing sucrose at an equimolar amount. For 7.5, 5, and 2.5% sucrose mixtures, mannitol was added to maintain the proper osmolarity. Tissue undergoing antibiotic selection was placed into FNL medium supplemented with 20 mg l^{-1} hygromycin. Controls consisted of non-transformed embryos placed into flasks containing FNL; FNL supplemented with 20 mg l^{-1} hygromycin; or FNL carbon-source modified to contain mannitol (16 g l^{-1}) equimolar to sucrose (30 g l^{-1}). The media were replaced at weekly intervals. After five weeks of selection, no selection was occurring in flasks containing any sucrose and the tissue in medium containing only mannitol was chlorotic. Several green embryonic clumps were visible in the flasks undergoing hygromycin selection while the non-transgenic tissue was visibly necrotic. Embryos from flasks selected on mannitol (16 g l^{-1}) were then placed in FNL with medium replacement occurring at weekly intervals. After 3 weeks of subculturing, green embryos became apparent in flasks from the mannitol treatment and were subsequently subcultured to individual flasks containing FNL. Several of the embryos began to proliferate. After 2 more weeks of medium replacement, GUS assays

(Jefferson et al., 1987) were performed on these embryonic clumps. All were negative. The FNL medium became contaminated and as such no plants were recovered, including from the controls. The results of this study led us to believe that the *atl*D-1 gene was not able to act as a selectable marker with mannitol as the selection agent.

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APPENDIX B - OTHER SELECTABLE MARKERS

INTRODUCTION

Since other non-antibiotic markers have been developed, to test arabitol without analyzing other markers would be short-sided. Therefore, I tested the use of cyanamide and mercuric chloride as selection agents of soybean somatic embryos in biolistics transformation and liquid selection.

Cyanamide

Cyanamide hydratase was identified from the soil fungus *Myrothecium verrucaria* (Maier-Greiner and Klaus, 1991). The enzyme converts the herbicide/fungicide cyanamide into the fertilizer urea. The gene has been successfully used as a selectable marker in wheat (Weeks, et al., 2000).

Using non-transgenic soybean somatic embryogenic MSD20 tissue, a kill-curve was generated. Two flasks of tissue (each 75-100 embryos) were subjected to FNL supplemented with 25, 50, 75, and 100 mg l^{-1} cyanamide (Sigma) with media replaced weekly for 2 months. Only tissue subjected to 100 mg l^{-1} (2.4 mM) was completely killed. This indicated that cyanamide might be used to select soybean somatic embryos.

The *cah*+ gene was excised from pCAM (courtesy of J. T. Weeks, ARS, USDA, Univeristy of Nebraska-Lincoln) using *Pst*I, blunted and inserted into pUPC-6. This added the potato ubiquitin promoter and terminator. The ubi3-cah+-ubi3term construct was excised from pCyan and inserted into pCAMBIA 1305.2 to form pCSS (Fig. B.1A).

Mercuric Chloride

The *mer*A77 gene (courtesy of Rich Meagher, Department of Genetics, UGA) is a synthetic gene developed from *mer*A, an *E. coli* mercury reductase gene (Rugh et al., 1996). Mercury reductase converts ionic mercury (Hg^{2+}) into non-ionic mercury (Hg^0).

Non-transgenic soybean somatic embryogenic MSD20 tissue (75-100 embryos per flask) were placed in FNL medium supplemented with mercuric chloride ($HgCl_2$) (Sigma) in concentrations of 5, 10, 15, 20, and 30 μM with media replaced weekly for 2 months. The level of 5 μM had killed most of the tissue, whereas levels of 10 μM or greater caused complete death. This indicated $HgCl_2$ might be able to serve as a selection agent for soybean transformation.

Plasmid pRLS16 contained the merA77 gene under the control of the actin 2 promoter and terminator (An et al., 1996). This construct was transferred to pCAMBIA 1305.2 (CAMBIA, Canberra, Australia) to form pMerSoy (Fig. B.1B).

SOYBEAN TRANSFORMATIONS

Soybean somatic embryos were obtained as before. Plates of MSD20 tissue were subcultured to fresh MSD20 arranged in a tight circle of approximate 2 cm in diameter in the center of a Petri dish. After 4 days, 5 plates were transformed with pCSS (see Fig. B1.A) and 4 plates transformed with pMerSoy (Fig. B1.B) using biolistics at a rate of 6.5 μg DNA per plate of 75-100 embryos (Christou et al., 1991). After a week of recovery on MSD20, 3 plates each were subjected to 8 weeks of selection in FNL supplemented with either 2.4 mM cyanamide, 5 μM $HgCl_2$, or hygromycin at 20 mg l^{-1}. In total, there were three replications (plates) for each treatment. Controls consisted of non-transgenic tissue placed into flasks containing either FNL, or FNL supplemented as with selection. After 8

weeks of selection, surviving embryos were counted (Table B.1) and placed into FNL. There were no live embryos in any of the negative controls and none in cyanamide or $HgCl_2$-selected tissue. After 4 weeks, proliferating embryos (from hygromycin selection) were placed into SHAM medium (Samoylov et al., 1998b) for 6 weeks. The embryos were then desiccated for one week in Petri plates before being transferred to MSOL for germination. After developing roots, plants were transferred to soil. Due to a contamination event, only two lines transformed with pCSS (none with pMerSoy) were recovered.

It is believed that urea produced by *cah*+ resulted in a toxic level of available nitrogen in the medium. Nitrogen content will therefore have to be lowered to adjust for the greater amount available from embryos converting the cyanamide into urea.

In regards to mercury selection, it is believed that stress caused by shooting could be affecting the ability of embryos to proliferate in the presence of $HgCl_2$. Mock transformation of embryos could provide a greater means of mimicking the conditions occurring in bombardment and thus allow mercury to be used as an effective selection agent.

Fig. B.1: Construction of the vectors used for soybean transformation.

A) Vector pCAM was digested with *Pst*I to release the *cah*+ gene. The ends of this digest were blunted with T4 DNA polymerase (T4P) and inserted into the multiple cloning site of vector pUPC-6 (courtesy of Joe Nairn, School of Forest Resources, University of Georgia) which had been previously digested with *Bam*HI and blunted. The resulting [ubi3P-*cah*+- ubi3T] construct was released from the plasmid via a *Spe*I/*Stu*I digestion and subsequently blunted. Plant vector pCAMBIA 1305.2 (CAMBIA, Canberra, Australia) was digested with *Xba*I, blunted, and ligated with the [ubi3P-*cah*+-ubi3T] construct to form pCSS. This added the hygromycin resistance gene *hph*, as well as the GUS*Plus* reporter gene.

B) Plasmid RLS-16 (courtesy of Rich Meagher, Department of Genetics, University of Georgia) was digested with *Kpn*I/*Sac*II to release the [Aac2P-*mer*A77-Aac2T] construct, which was subsequently blunted with T4P. (Vector pCAMBIA 1305.2 was digested with *Xba*I, blunted, and ligated with the [Aac2P-*mer*A77-Aac2T] construct to form pMerSoy. This added the hygromycin resistance gene *hph*, as well as the GUS*Plus* reporter gene.

Fig. B.1 Vector construction.

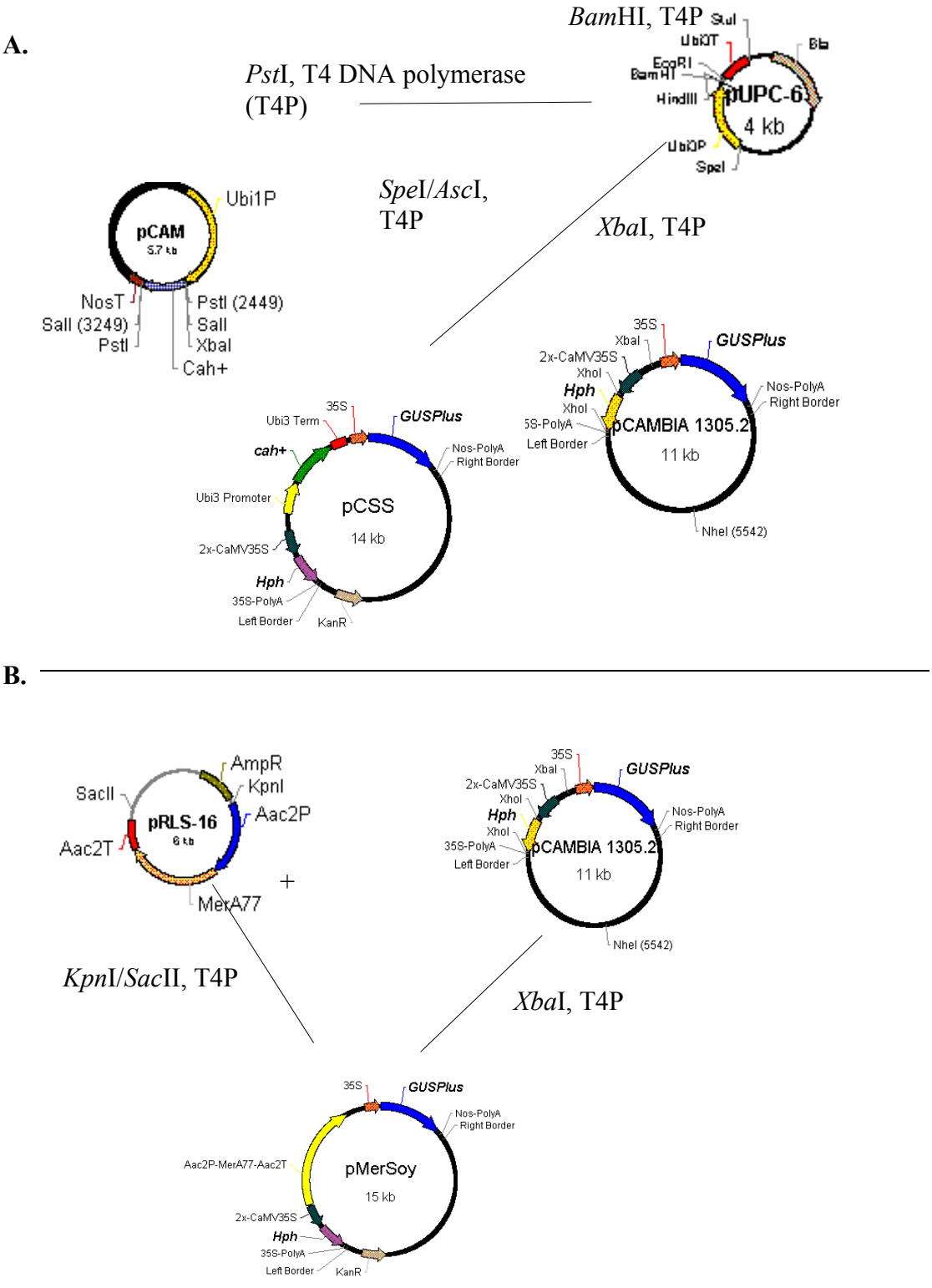

Table B.1 Number of transformation events recovered following bombardment with different selection agents.

Rep	Hygromycin	$HgCl_2$	Cyanamide
1	14	0	0
2	11	0	0
3	11	0	0
Total	36	0	0

Table summarizing the results of a side-by-side analysis of mercuric chloride and cyanamide as selectable markers versus hygromycin selection. Displayed is the total number of surviving embryos after 8 weeks of selection per replication. One replication was all tissue deriving from one plate subjected to bombardment.

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