TRANSFORMATION OF BARLEY (*HORDEUM VULGARE*) USING THE WHEAT PUROINDOLINE GENE

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Yusuke Odake
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ABSTRACT

Feed and malting barley are major crops in Montana. Harrington, a two-rowed spring malting barley variety, is the most cultivated variety in the state. Barley was the last of the world's major cereals for which transformation methods were developed because in vitro-cultured barley rapidly loses regeneration ability or gives rise to albino plants during selection for transformed tissue. Previous research used the variety Golden Promise because it regenerates well under research conditions, though it is not commercially used. Transformation could be an important method to improve varieties in North American barley cultivars. The puroindoline proteins (PINA and PINB) can be isolated from wheat endosperm and are basic cysteine and tryptophan-rich proteins that might play a role in defense against pathogens. The puroindolines show antifungal activity both *in vitro* and *in vivo*. Milk stage seeds were harvested to obtain embryos. Embryos were placed on induction media [2.5 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) and 0.01 mg/L 6-benzylaminopurine (BAP)] for 12 to 51days until calli formed. The biolistic method was chosen for transmitting the gene of interest into the calli, using a Biolistic PDS-1000/He Particle Delivery System (BioRad, Hercules, CA) with 900 psi rupture disks. Approximately 4,500 calli were bombarded with two plasmids; *pinA* driven by the maize ubiquitin promoter (*Ubi1*) which is expressed constitutively, and the hygromycin phosphotransferase (*hph*), which confers hygromycin B resistance and is driven by the CaMV 35S promoter for selection. The bombarded calli were selected on medium containing 30 mg/L Hygromycin B for 9 to 21 days, and subcultured to intermediate medium (1.0mg/L 2,4-D and 0.5 mg/L BAP), followed by regeneration media (1.0-3.0 mg/L BAP). Thirty hygromycin B resistant calli regenerated and 5 died in magenta box. Thirty five putative transgenic plants derived from 25 calli grew in soil and were harvested. Some plants tested by polymerase chain reaction (PCR) tested positive with primers for *pinA* and *hph*. Southern blots were performed to detect the presence of *hph* in T_1 barley genomic DNA but were negative. Northern blots performed to detect the presence of *pinA* and *hph* transcribed RNA in the T_1leaf tissue were also negative, showing that no stably transformed plants were obtained.

CHAPTER 1

LITERATURE REVIEW

Barley (*Hordeum vulgare* L. emend. Bowden)

Barley is in the genus *Hordeum*, which belongs to the tribe Triticeae and to the grass family Poaceae. Modern cultivated barley was domesticated about ten thousand years ago in the Near East Fertile Cresent (Nevo, 1992), with *Hordeum spontaneum*, a two-rowed barley, as an ancestor (Zohary, 1969). Modern cultivar barley (2n=14 chromosomes; HH genomes) is suited to cool, dry land around the world, but cannot grow in warm, humid conditions (Nevo, 1992). It is the fifth-ranking cereal in the world, with a total annual yield of approximately 141 million metric tons (FAO Yearbook Production 2001).

Barley is mainly cultivated in Europe (40% of the total), Russia (13%), North America (11%), North Africa, Ethiopia, China, India, Canada, and Australia (Nevo, 1992; Schildbach, 1994). The major uses of barley are for feed and malt. The United States produced approximately 5 million tons in 2001 (FAO Yearbook Production 2001), which was used for feeding cows (47%), malt (32%), and export (17%)(Lemaux et al., 1999). In the United States, it is grown in cool and/or dry lands, such as Montana, North and South Dakota, California, Washington, Idaho, Colorado, Wyoming, Oregon, Minnesota, Wisconsin, Michigan, and Kansas (American Malting Barley Association, Inc., 2003). Barley was grown on 1.2 million acres in Montana in 2002, and currently that

acreage is increasing. Malting varieties are seeded more often than feed varieties in Montana (Stringer et al., 2002).

Harrington, a two-rowed, spring barley was developed at the University of Saskatchewan and licensed in 1981 (Harvey and Rossnagel, 1984). It is one of the varieties that is recommended by the American Malting Barley Association, Inc. (AMBA) and seeded by 47.7% of Montana farmers in 2002. It has been the most popular barley variety for nine years in a row (Stringer et al., 2002).

Barley Diseases

Since immigrants brought barley seeds into the United States, barley started to be cultivated on both sides of the continent, in California and New York. Over time, the cultivated area has expanded inland (Steffenson, 2003). At the same time, the pathogens of barley also increased in numbers. Barley has diverse infectious pathogens including viruses, bacteria, fungi, and nematodes (Mathre, 1997).

Fusarium Head Blight

Fusarium Head Blight (FHB), or scab disease, is caused by *F. graminearum* Schwabe [teleomorph: *Gibberella zeae* (Schwein.)] and *F. culmorum* (Wm.G. Sm.). It is seen commonly worldwide on spring wheat, durum, and barley. In the case of barley, *F. poae*, *F. avenaceum* [teleomorph: *Gibberella avenacea*], and *F. sporotrichioides* are reported to cause the disease as well (McMullen; Steffenson, 2003; Mathre, 1997). *F. graminearum* also causes severe economic impact as an ear rot pathogen of maize (*Zea mays*)(Sutton, 1982).

F. graminearum forms sporodochia, white or pale orange septated mycelium, and septate macroconidia. Chlamidospores also may appear. During the sexual stage, the ascus contains eight hyaline three-celled ascospores that evolve from the dark colored perithecium (Mathre, 1997).

The infection period for these pathogens is limited, occurring during the flowering (pollination) period to the soft dough (kernel development) stage. The environment must be conducive for infection, since they require approximately 36 to 72 hours of high humidity and warm (23-30ºC) temperature, though these conditions can vary slightly (Parry et al., 1995). The damage is not only a loss of yield and quality of the kernels but also contamination with mycotoxins, such as deoxynivalenol (DON, vomitoxin) and zearalenone (McMullen et al., 1997 and the references therein).

In the United States, Minnesota, Wisconsin, Iowa, Illinois, and the Dakotas have had the most severe damage from this disease, with extreme damage in the last decade (McMullen et al., 1997; Gilbert and Tekauz, 2000). The devastating damage in 1993 resulted in the loss of 1.6 million metric tons and $122 million in the United States. However, inland states, namely Montana, Idaho, Colorado, and Wyoming normally do not experience severe damage by FHB unless they have heavy precipitation or irrigation during the flowering period (Steffenson, 2003 and the references therein).

Biological treatments (i.e., antagonists of FHB by *Bacillus spp*., Baye et al., 2003), fungicide application, and cultural practices (i.e., crop rotation, plowing, seed treatment with fungicide, burning of field) are common for FHB treatment (Steffenson, 2003).

There are no significantly resistant varieties for FHB (Mathre, 1997). Some known resistance traits are affected by multiple genes, making it harder for breeders to develop resistant varieties (Dahleen et al., 2001). Moreover, there are at least 40 important traits that are required for commercial malting cultivars, indicating it may require a 15-year breeding program with a number of backcrosses to obtain an economically important disease-resistant variety (Dahleen et al., 2001; Steffenson, 2003).

Genetic Engineering of Plants

Humans have modified crops by traditional breeding processes for domestication, to obtain high yield and better quality for food products and to obtain varieties resistant against pests, bacteria, or fungal diseases. However, it is a time-consuming process to develop a variety that has the desired features, and the available genes can only come from sexually compatible plants. This limited transferability from sexually compatible plants results in reduced gene diversity. Researchers have developed asexual methods to allow going beyond the barrier of the species (varieties), and the current, valuable varieties can acquire alien, useful traits in a relatively short time. The technique of non-sexual DNA transfer is called ‘plant transformation’, in which the gene of interest, which can come from almost any kind of organism, is directly and forcibly inserted and incorporated into the undifferentiated (immature embryo) or protoplast plant genome. Successful plant transformation was first achieved in 1984 with tobacco (DeBlock et al., 1984) and over 120 species can now be transformed (Birch 1997).

The three most common and successful systems used for plant transformation include using *Agrobacterium*, particle bombardment, and direct gene transfer into protoplasts by electroporation**.**

Agrobacterium–mediated Transfer of DNA

Agrobacterium tumefaciens is a phytopathogenic bacterium that causes crown galls or tumors (Zupan, et al., 2000). It infects a wide variety of plants including 596 dicotyledonous (dicot), 42 gymnosperms, and 5 agronomically unimportant monocotyledonous (monocot) cereals (De Cleene and De Lay, 1976). Interestingly, it can deliver part of its DNA, the transfer DNA (T-DNA) region of its tumor inducing (Ti) plasmid to eukaryotic cells and the T-DNA region is able to be expressed in the host (Bundock et al., 1995). The T-DNA is a segment of DNA with two borders, the right border (RB) and left border (LB). It contains genes that code for tumor formation and biosynthesis of unusual amino acids (opines). Up to 35 virulence (*vir*) genes form the virulence region that is located outside of the T-DNA region. The transformation of the T-DNA region into host plants is conducted by expression of the *vir* genes. To control *vir* gene expression, there are at least six operons upstream of the genes that act as regulators to make sure the transformation event only occurs after infection of the host (Stachel and Nester, 1986).

Researchers have manipulated this so-called 'Nature's genetic engineer' by establishing *Agrobacterium*-mediated transformation of plants with genes from many other organisms. In *Agrobacterium*-mediated transformation, unwanted genes between

the RB and LB are removed to prevent tumor formation, and the gene-of-interest is inserted. A benefit of using this method in transformation is it can deliver a low copy number of the gene to the host, which may prevent gene silencing after incorporation of the gene (Matzke and Matzke, 1995). Also, it causes less stress to the target tissue compared with other transformation processes and the plant is thought to recover better after the transformation process. Since quite a few dicots were reported as hosts of *Agrobacterium*, it was originally thought that this method would only work for dicot plants but not other organisms (Van Wordragen and Dons, 1992). Bundock and Hooykaas (1996) proved that the *Agrobacterium* method could transfer DNA into the yeast *Saccharomyces cerevisiae*. Various researches have modified *Agrobacterium*, or the T-DNA plasmid by changing the T-DNA promoter, for example, to adapt to the new targets.

One difficulty of monocot transformation with this method has been overgrowth of *Agrobacterium* over the calli. As a result, it prevents the calli from obtaining oxygen necessary for respiration. Thus, calli could not survive (Jacobsen, 2000). One solution would be to use penicillin-based antibiotics that kill, or inhibit further growth of the *Agrobacterium* itself (Hellens et al., 2000).

The *Agrobacterium*-mediated transfer of DNA in cereals was first used with maize in 1991, rice in 1992, and finally wheat and barley in 1994 (Jacobsen, 2000). The first successful report of transformation of barley with *Agrobacterium* was published in 1997 by Tingay et al., followed by Wu et al. (1998).

Biolistic (Particle) Bombardment Method

The particle bombardment gene transfer method forcibly delivers DNA-coated particles or other molecules, such as RNA (Tanaka et al., 1995), into cells or tissues. The method, which is also referred to as biolistic, gene gun, or microprojectiles, was invented by Dr. John Sanford at Cornell University in 1984, and it broke the limitation of the cell type, species or type of targets to be modified. Since then, the method has been optimized for different targets and modified to make it more reliable for recombination. The current method uses helium gas as the explosive and DNA-coated microscopic gold pellets as the bullets. Rupture discs determine the pressure of helium gas and gold spheres are used because gold is stable in the tissues and it is not very toxic. The DNA-coated pellet is arranged on the macrocarrier screen (as a cartridge), and when high pressured helium is released, the rupture disc ruptures at a certain pressure onto the macrocarrier screen. The gold pellets passes through the stopping screen and penetrate the cell walls. DNA that is tightly bound to gold under non-aqueous condition is then released in the cell.

This physical gene insertion system requires precise optimization for each target tissue. Ritala et al. (1993) conducted foundation research of gene gun methods for barley. They found that helium-propelled shot and placing the gene gun chamber under vacuum gave better transient expression than a gunpowder system. Apparently, their suggestions were accepted by other labs involved with barley transformation. Conditions that determine the velocity of the microcarrier at the moment it penetrates the plant tissue, such as helium pressure and distance between the stopping screen and the target have to be determined empirically.

Electroporation and Polyethylene Glycol Treatment Method

These are the most simple and primitive methods that have been used for cereal transformation since the 1980's. Insertion of genes of interest occurs directly into the target protoplast by electric shock (electroporation) or osmotic shock (polyethylene glycol). Though these methods laid the foundation of cereal transformation, their use is limited due to low success rate of transformation and the appearance of more successful methods, namely the *Agrobacterium*-mediated and biolistic methods (Shillito, 1999).

Selection

Regardless of the method used, transformation is an uncertain event and usually a gene encoding a selectable marker is co-transformed with the gene of interest.

For example, Hygromycin B ($C_{20}H_{37}N_3O_{13}$) is an aminocyclitol antibiotic, which inhibits translocation (movements of ribosomal RNA on messenger RNA during translation) so that both prokaryotic and eukaryotic cells are affected due to the failure of protein synthesis (Gonzalez et al., 1978; Cabaňas et al., 1978). Hygromycin phosphotransferase, the product of the *hph* gene (gene source, *Streptomyces hygroscopicus*) neutralizes hygromycin B; thus, it can be used for selection during the transformation process. There are some common anti-microbial selectable markers for either bacteria or plant selection, such as ampicillin, kanamycin, gentamycin, and spectinomycin and the resistant genes β-lactamase (*bla*), neomycin phosphotransferase (*nptII*), gentamicin acetyltransferase, and aminoglycoside 3" adenyltransferase (*aad*), respectively (Hellens et al., 2000). A herbicide, glufosinate (L-phosphinothricin), or bialaphos [L-2-amino-4-

((hydroxy)methyl)(phosphinoyl)-butyryl-L-alanyl-L-alanine] and its resistant gene phosphinothricin acetyltransferase (*bar*; gene source, *Streptomyces hygroscopicus*) is also routinely used to select for recombinant plants (Scutt et al., 2002).

However, use of some of these antibiotics has been reported to cause side effects during the plant transformation process. Hygromycin, kanamycin, or cefotaxime, when used for selection and regeneration, caused DNA hypermethylation in tobacco, which is said to be one of the reasons for homologous gene silencing. There seems to be a correlation between the influence of DNA methylation and length and amount of exposure of plant tissue to the antibiotics (Schmitt et al., 1997). Hygromycin B may also inhibit the regeneration of barley calli, such as rooting and shooting after the screening process (personal communication, Xiao-Hong Yu, Department of Plant and Microbial Biology, University of California, Berkeley).

Another problem with genes encoding resistance to antibiotics is that they and their products (proteins) in recombinant crops concern consumers greatly, as they fear allergic reaction to the proteins or ingesting “anti-microbial poisons” (Scutt et al., 2002). Also, DNA from bacteria survives with a half-life of 6 minutes in artificial human guts, and thus, the author indicated that the resistant DNA could be ligated into another bacteria in the stomach (MacKenzie, 1999). Schubbert (1994) reported that ingested foreign DNA was detected in the blood of a mouse. The questions of how many of these ‘survivor genes’ would still have function, and what the chances would be for an occurrence of this type in a human are still being investigated. Consumers and the press tend to connect these reports to GMO products. Since these resistant genes are only

needed during the screening process and become needless after that, some research has been conducted to remove these genes after selection or to use non-antibiotic selection, such as reporter genes (described below) for making transgenic plants (Kuiper et al., 2001).

In the history of barley transformation, *hph* and *bar* are the selectable marker genes that have been most often used. A *hph* gene selection yields twice as many transgenic plants, with a 5.5% transformation efficiency (obtained transgenic plants per number of calli), than *bar* using the same transformation method (gunpowder-driven microparticle bombardment) in wheat (Ortiz et al., 1996). In that report, 5-10% of escapes were noted through a 4-6 week selection period on media containing 25mg/L hygromycin B. Though successful regenerative frequency varies with each experiment, effective transformation frequencies (ETF; obtained independent fertile plants per numbers of target) were from 0 to 5.6% (Lemaux et al., 1999). In recent research, the *bar* herbicide-resistant gene has been most commonly used (Bregitzer et al., 2003; Dahleen and Manoharan, 2003;Yu et al., 2003) for barley transformation.

Construct (Plasmid)

Plasmids that are used for transformation are called expression vectors, and they contain a replication origin (*ori*) for amplifying in bacteria cells, a selectable marker, and restriction sites. There are various well-known backbone plasmids that confer these essential genes including expression cassettes for different methods and targets. Appropriate restriction enzyme(s) need to be used for ligating gene(s) of interest into the

right place and right orientation in the plasmid. More than one plasmid can be used for transformation.

Promoter

Since the final goal in plant transformation is to have expression of the gene of interest, the coding sequence needs to be expressed (transcribed and translated) in the entire plant (systemic) or specific tissue organs, as desired. Promoters are located upstream of the transcribed genes where RNA polymerase II binds and have an important role in controlling transcription. Each promoter is unique and specific, thus, it is important to choose the right promoter in the plasmid construct.

Cauliflower mosaic virus (CaMV) 35S promoter (Odell et al., 1985) has been often used as a promoter that is expressed constitutively. However there are some reports that CaMV 35S promoter is less active in dicot plants than in monocot plants, and thus, it is used less often in dicot plant transformation. Hauptmann et al. (1987) compared the expression levels of the promoter in monocot and dicot protoplasts transformed by electroporation and found tenfold less activity in dicot cells. Another report said that a construct with the CaMV 35S promoter, which showed constitutive expression in tobacco, did not express in certain rice tissues, including the palea, stigma and anthers (Terada and Shimamoto, 1990). It has been suggested that the activity of the CaMV35S promoter depends on the quantity and/or quality of regulatory factors in monocots and dicots (Båga et al. 1999).

Some promoters that function in monocots (cereals) have been described since the early 1990's, such as the rice actin (*Act1*) promoter (McElroy et al., 1991), maize alcohol

dehydrogenase1 (*Adh1*) promoter (Fromm et al., 1990), sugarcane bacilliform badnavirus (ScBV) promoter (Tzafrir, 1998), and the maize ubiquitin (*Ubi1*and *Ubi2*) promoters (Christensen et al., 1992).

Actin is expressed in all plant cells, so expression using the *Act1* promoter is constitutive. The rice *Act1* promoter cassette has the 5' upstream region of actin, including the 5' non-coding exon 1, intron 1 and the 5'part of the first coding exon (McElroy et al., 1991). Expression assays with GUS showed that expression is systemic (Nehra et al., 1994). Funatsuki et al. (1995) used the *Act1* promoter to drive a selectable marker in barley.

Ubiquitin is highly conserved in living organisms and possesses diverse and important functions, such as controlling the cell cycle (Rechsteiner, 1991), DNA repair (Jentsch et al., 1987), and stress shock (Christensen et al., 1989). A 228bp portion of the maize ubiquitin gene, including surrounding sequence, was isolated and analyzed by Christensen et al. (1992). The *Ubi1* promoter was found upstream of the ubiquitin1 coding sequence. The promoter is activated and the downstream coding sequence is expressed when plants are young and stressed. Thus, this characteristic might be used for the transformation process, especially for the expression of selectable marker genes. Wan and Lemaux (1994) used *Ubi1* for expressing a selectable marker gene in barley, and the promoter has been widely used not only for expressing selectable marker genes but also a gene of interest in monocot transformation.

The *Adh1* promoter is also from the maize genome and has been used in monocot transformation. However, it was only activated in specific tissues including root and shoot meristems, endosperms, and pollen (Kyozuka et al., 1991).

Badnaviruses are dsDNA viruses, and some badnaviruses naturally infect monocots. Therefore, the activities of some promoters from badnavirus, *Commelina* yellow mottle virus (CoYMV, Medberry et al., 1992), the rice tungro bacilliform virus (RTBV, Bhattacharyya-Pakrasi et al., 1993), and sugarcane bacilliform badnavirus (ScBV) have been tested. Tzafrir (1998) conducted an expression assay with a chimeric β-glucuronidase reporter gene (*uidA*; *gus*) driven by the ScBV promoter in *Avena sativa* and *Arabidopsis thaliana*. They observed constitutive expression by the ScBV promoter and its intron and terminated by the *Agrobacterium tumefaciens* nopaline synthase 3' polyadenylation signal, *nos*. In two monocots, the promoters from CoYMV and ScBV were tissue specific or host specific. Since domesticated cultivars of the Poaceae family are severely infected, and ScBV causes systemic damage in their tissue, the ScBV promoter may be useful for transformation of barley.

In general, transgene expression is enhanced by introns in the 5'-untranslated region in cereals (McElroy et al., 1991). McElroy et al. (1991) reported that the absence of the first intron of the *Act1* gene reduced expression of the reporter gene. In the same way, expression of the *Ubi1* promoter with the first intron is greater than without the intron (Cornejo et al., 1993).

The *Ubi1* promoter is the most commonly used promoter for both selectable and target genes in recent reports of barley transformations. However, expression of the gene

regulated by the *Ubi1* promoter is unstable in wheat (personal communication, Dr. Michael Giroux, MSU)**.**

A transient expression assay, such as a GUS experiment, is a useful way to analyze the efficiency of a new promoter in a specific tissue (Caplan et al., 1992, Li et al., 1997). The reporter gene, e.g., *uidA* driven by the promoter produces GUS protein, which cleaves X-gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt) when *uidA* is transcribed and translated in the tissue. Cleavage of the glucuronic acid results in the blue spot(s) in the plant tissue. Thus, promoter function and expression level are visually observed.

Terminator

Most heterogeneous nuclear RNA (hnRNA or pre-mRNA) and messenger RNA (mRNA) in eukaryotes have a poly(A)$^+$ tail, which is required to make RNA stable. The 100 to 200 adenine residues are not coded in the DNA template, meaning there is another procedure to terminate RNA synthesis. An immature RNA is cleaved at 10 to 30 nucleotides downstream of AAUAAA (TTATTT in DNA template), otherwise known as the poly(A) addition signal, and poly(A) polymerase adds poly(A) tail to the 3'-OH end (Garrett and Grisham, 1997). Most of the expression vectors used in plant transformation are terminated by the nopaline synthase 3' polyadenylation signal, *nos* derived from *Agrobacterium tumefaciens*.

Recombinant Barley

Genetic engineering allowed the introduction of transgenic maize and rice in 1988 and wheat and barley in 1994 (Vasil, 1999). Barley was the last major cereal in which DNA transfer was made, ten years after the first transgenic tobacco was obtained. Transformation of barley using the barley yellow dwarf virus coat protein (BYDVcp) driven by *Ubi1* promoter and first intron (Christensen at al., 1992) was reported by Wan and Lemaux in 1994.

Non-commercial varieties, such as Golden Promise and Igri, have often been used for lab research due to the relatively high regeneration ability of green plants and less genetic disruption after transformation events. None of the elite North American barley cultivars have such characteristics. Recent research has moved from development to practical experiments focusing on improvement of regeneration and reduction in the number of albino plants with elite barley varieties (Lemaux et al., 1999).

The difficulty of transformation of elite barley comes from the sensitivity of the barley genome to the transformation process. With all transformation methods, the target tissue (explant) is exposed to many stresses, e.g., physical gold pellets penetration, dehydration on osmotic treatment, and contact with antibiotics on selective media. As a result, high numbers of recombinant barley were albino (Cho et. al., 1998; Lemaux et. al., 1999), and somaclonal variation was observed from green recombinant barley (Choi et. al., 2000). Thus, researchers have paid attention to explant material. Explanted material is required to endure the stresses of a transformation process and must be a competent to accept the gene(s) of interest. Needless to say, explant material should be capable of

becoming a whole, intact and fertile barley plant (totipotency). In the early stages of experiments at the end of 80's and early 90's, various explants from somaclonal tissues, cell suspensions, protoplasts, and undifferentiated tissues were used to examine expression of selectable marker genes to determine the efficiency of DNA delivery systems, but all of these transformed plants were unstable. Successful barley transformation has been reported since 1994, the dawn of barley transformation. In those reports, immature zygotic embryos (IEs) were the most effective explant. Other than that, callus derived from IE, suspension culture, microspore, and meristem tissue have been used (for review Lemaux et al., 1999).

Meanwhile, improvements to the tissue culture media have greatly improved success in obtaining green and fertile plants. The improved media (MS) is based on that of Murashige and Skoog (1962), which has become the standard plant tissue culture media. Nuutila et al. (2000) reported that the addition of copper to MS media reduced albinism of barley tissue. Modification of callus induction and callus regeneration media has been performed by increasing micro-elements, copper and iron concentration and re-adjusting phytohormone balance or suitable concentration of hormones for each callus stage (Bregitzer et al., 1998a,b; Cho et al., 1998). For example, regeneration media made of a modified MS basal medium with a high sugar concentration that stabilizes green tissue, was used and obtained an ETF of 1.2% with transformation of Harrington (Cho et al., 1998). Also, utilization of osmoticum media containing high sugar concentration during the bombardment process helped reducing stress from gold penetration because of deflated callus surface tension. Consequently, more fertile plants may be obtained.

Phytopathology

Modern agriculture uses densely cultivated areas of one specific crop, and thus, there is a great risk of the crop being infected by phytopathogens. Without a knowledge of the environment, farmers should not be surprised that severe damage in the field may occur. Throughout history, we have been trying to understand the mechanisms of the interactions between domesticated crops and the environment, to tame the environment and crops so that we could obtain high and stable yields (Agrios, 1997)

The development of the field of chemistry in the 20th century devised various chemicals, such as antibiotics to kill fungi, bacteria, or insects, which brought a revolution to agriculture (Agrios, 1997). However, extremely effective chemicals are normally a double-edged sword. They are usually toxic to humans, and applying a large amount of them on the fields can demolish an ecosystem while killing the pathogens. Not only chemicals, but other the things that cause high selective pressure to pathogens often make new strains or varieties resistant to them (Agrios, 1997). Because of this, a more specific and more moderate approach of controlling diseases is desired.

Genetic Approach

The molecular basis for interactions between pathogens and plants have been one of the major concerns in modern phytopathology, and a numbers of genetic relationships have been revealed. One simple interaction of gene function and disease resistance is a physical trait. For example, the genetic function of stem solidness is one of the ways to have resistance to insect biting and lodging.

Plants also have an immune system to protect themselves from microbial infection (Zasloff, 2002). Researchers and farmers understood empirically that there were some varieties that naturally had more resistance to pathogens, and it was also known that most of the resistance traits followed Mendelian inheritance (Buchanan et al., 2000). Traditional breeding has added resistance traits to varieties that also have good agronomic characteristics. However, traditional breeding is normally a long process and it takes a long time to obtain the desired variety.

Harold H. Flor's gene-for-gene model using flax and the flax rust pathogen in 1955 was the first report that explained a genetic mechanism of disease (Buchanan et al., 2000). In his study, he found that plants that have the dominant resistance gene (*R*) were only resistant to pathogens that have the dominant avirlence gene (*Avr*) (incompatible). In compatible situations, the plant got disease from the pathogen. This interaction results in the appearance of pathogenesis-related (PR) proteins and the hypersensitive response (Buchanan et al., 2000).

In addition, some small peptides that are naturally produced by organisms have been reported to have antimicrobial properties, using different mechanisms than antibiotics. These proteins are called anti-microbial peptides. They strongly bind to the cell membranes (lipids) and disrupt the microbial membrane.

Learning about genes and their functions can be useful for understanding and creating plants resistant to the environment. Punja (2001) categorized these candidate genes for genetic engineering use by five different functions: 1) expression of a toxic or a growth-inhibiting protein; 2) expression of a protein that neutralizes substances, such as

oxalic acid, that attacks host; 3) expression of a protein that strengthen the plant's physical defense system; 4) expression of a regulatory signal protein that controls the plant defense systems; and 5) expression of a resistance gene (R).

Candidate Anti-disease Genes for Barley Transformation

Researchers have conducted barley transformation with several anti-disease genes. Leckband and Lörz (1998) noticed a grape stilbane (resveratrol) synthase gene, which expresses a type of phytoalexin. The barley cv. Igri was transformed by particle bombardment in their experiment. The leaf tissues of the homozygous transgenic line (T_1) had reduced colonization by *Botrytis cinerea*. Also, expression of hydrolytic enzymes that break down fungal cell walls, such as the endochitinase gene from *Trichoderma harzianum* was employed by Nuutila et al. (1999). In recent years, the thaumatin-like protein (*tlp*) gene; a rice chitinase (*chi*) gene; and trichothecene pathway genes from *F. sporotrichioides* has been employed for barley transformation to FHB resistance (Dahleen and Manoharen, 2003; Yu et al., 2003). The resistance levels of these transformants have not yet been reported.

Lipid-binding (Transfer) Proteins

Hydrophobic proteins that interact with lipids, such as lipid binding proteins and lipid transfer proteins, have been noticed due to their anti-microbial properties. These proteins have an α-helix structure, and they have been reported from many kinds of organisms (Oren and Shai, 1998). Seeds are thought to contain both lipid-binding and

anti-microbial proteins since germination and maturation occurs in wet conditions, which are also favorable for microorganisms (Jing et al., 2003; Blochet et al., 1993).

The first thionins, lipid-binding protein, were isolated in wheat seeds as a 'sulphur-bearing constituent' over half a century ago (Balls and Hale, 1940) and were noticed because of their 'bactericidal and fungicidal properties' (Stuart and Harris, 1942). The common characteristics of thionins are low molecular weight, basic, and cysteine-rich proteins that interact with membrane lipids (Blochet et al., 1993 and the references therein). Later, thionins were found in other plant tissues and are common in other plant species (Florack and Stiekema, 1994). In the same study, the thionins were categorized into four groups by features and the tissues in which they were expressed. According to this report, different groups of thionins can reside in one plant. For instance, there are five thionins in barley, two from endosperm, categorized as type 1, and three from leaf, categorized as type 2. Two homologous thionins in type 1, named α- and β-hordothionin, have 45 amino acid residues, contain four sulfide bonds, and are very basic (pI= 10.06). Type 2 thionins contained 46 amino acid residues with four sulfide bonds, with an approximate pI of 8.8. The anti-fungal properties of naturally-produced thionins from barley leaves were also reported (Bohlmann et al., 1988).

Recent research has found other lipid-binding proteins, referred to as non-specific lipid transfer proteins (ns-LTPs). Like thionins, ns-LTPs are ubiquitously expressed in plants, and heterogeneous ns-LTPs are present in other organisms (i.e., bacteria, yeast, plants and animals). As its name implies, lipid-transfer proteins are thought to interact with lipids transferring in or between cell membranes (Kader, 1996). However, details of

the biological functions are not well known (Douliez et al., 2000). Among plant tissues, ns-LTPs are highly expressed in kernels, and two types of ns-LTPs were extracted and analyzed from cereals (wheat, barley, maize, and rice). These ns-LTPs were named ns-LTP1 (9kDa type) and ns-LTP2 (7kDa type) (Douliez et al., 2000). The anti-microbial properties of these groups are not yet known. Dubreil et al. (1998) reported there was no significant fungal inhibition of ns-LTP1e_1 extracted from wheat endosperm. However, LTPs have important roles for the final product quality. For example, LTP(s) strongly affected the formation of beer foam (Sorensen et al., 1993).

Puroindolines

Two homologous proteins, puroindoline a (PINA) and puroindoline b (PINB) were isolated from wheat (*Triticum aestivum*) endosperm using a Triton X-114 phase partitioning procedure that only allows lipid-binding proteins to be extracted (Blochet et al., 1993). Puroindolines are categorized as lipid-binding proteins (Dubreil et al., 1998) or ns-LTPs (Blochet et al., 1993; Gautier et al., 1994). They are also known as friabilin (Greenwell et al., 1986; Gautier et al., 1994; Oda and Schofield, 1997; Morris 2002) or grain softness proteins (GSP) (Jolly et al., 1993). *PinA* and *pinB* have 60% nucleic acid sequence similarity (Gautier et al., 2000), and code for proteins with molecular masses of 13kDa (Gautier et al., 1994), with basic characteristics. In contrast with thionins and nsLTPs, puroindolines not only have five disulfide bonds but also tryptophan-rich domains (Gautier et al., 1994). These proteins were named puroindolines because of the unique tryptophan-rich region, which has an indole ring, and from wheat (puros in Greek) (Gautier et al., 1994). Puroindolines are α-helical proteins, as are ns-LTPs, and both are

similar structurally, although the mechanisms by which the puroindolines bind to lipids are similar to thionins (Charnet et al., 2003 and the references therein). Unlike thionins and nsLTPs, puroindolines are only expressed in seeds, specifically in the starchy endosperm and the aleurone cells of *Triticeae* and *Aveneae* tribe (Dubreil et al., 1998; Gautier et al., 2000). Digeon et al. (1999) conducted an expression assay in rice with the *pinB* gene promoter-GUS plasmid and showed that the promoter was seed-specific, primarily in epidermal cells, aleurone cells, and starchy endosperm. *PinA* and *pinB* are single copy loci and located in or closely linked to the *Hardness* (*Ha*) locus on the short arm of 5D genome in wheat, as located by RFLP markers (Sourdille et al., 1996). One of modern wheats (2n = 6x = 42, AABBDD) parents, durum wheat (*Triticum turgidum* L. ssp. *durum* Desf.Husr.) (AABB) lacks both *pinA* and *pinB* (Gautier et al., 1994), whereas the other parent, *T. tauschii* (DD) has two puroindolines (Gautier et al., 2000). Homologs of the puroindolines were found in other closely related cereals including, barley (hordoindoline), oat (*Avena sativa*, avenoindoline), and rye (*Secale cereale*, secaloindoline) but are absent in maize, rice, and sorghum (*Sorghum vulgare*) (Gautier et al., 2000).

Puroindolines have been of interest because of their two major functions, the determination of seed texture and antimicrobial property.

Grain texture, soft or hard, is an important feature in a wheat market, thus many molecular studies of the mechanisms regulating texture in seeds have been done. Friabilin is one of the starch granule proteins that determines wheat grain texture and quality (Greenwell et al., 1986). High friabilin content in wheat seeds results in a soft texture

(*Ha*), and low friabilin level in wheat seeds results in hard texture (*ha*). Because of the broad definition of 'friabilin', there has been some confusion and dispute about the products of *Ha* and mechanisms conferring grain hardness. Examination of the N-terminal (amino end) amino-acid sequences revealed that PINA and PINB compose friabilin (Gautier et al., 1994; Oda and Schofield, 1997; Morris 2002). Beecher et al. (2002) transformed wild-type *pinB* into a hard phenotype wheat that had a common, conserved mutation in *pinB*. The transformed wheat recovered the soft phenotype. *PinA* and/or *pinB* expression in transgenic rice, which lacks puroindolines, caused softer grain (Krishnamurthy and Giroux, 2001). Giroux and Morris (1998) reported that wheat grain was soft when both puroindolines were present, but hard when one or both of them was lacking.

A second function of puroindolines is antimicrobial activity. Puroindolines were extracted from the same phase as thionin was extracted, and hence, various experiments related to the genes and the proteins have been conducted *in vivo* and *in vitro*. Extracted PINA and PINB showed *in vitro* antifungal activity for four out of five fungal pathogens including *Fusarium culmorum* (Dubreil et al., 1998). Krishnamurthy et al. (2001) transformed *pinA* and/or *pinB* into rice cultivar M202, which is susceptible to the rice pathogens *Magnaporthe grisea* (rice blast) and *Rhizoctonia solani* (sheath blight). PINA and/or PIN B extracted from transgenic rice leaves inhibited growth of *R. solani* and *M. grisea*'s mycelia *in vitro* and the transgenic plants with either or both puroindolines reduced symptoms of both pathogens. Moreover, puroindolines enhanced antifungal

activity of α-purothionins (α-PTH), suggesting that collaboration of two proteins in seeds are important for anti-microbial activity (Dubreil et al., 1998).

The specific anti-microbial and molecular functions of puroindolines are unknown, although their anti-microbial function is considered to be promising. Recently, some reports have hinted at their mechanism of activity. Jing et al. (2003) focused on puroindoline's unique structure, the tryptophan-rich domain, puroA (FPVTWRWWKWWKG-NH_2) from *pinA* and puroB (FPVTWPTKWWKG-NH_2) from *pinB* to distinguish their cysteine-rich structure, where it is said to have lipid-binding functions. Synthesized 13-residue puroA caused membrane leakage of bacteria (*Escherichia coli*, *Staphylococcus aureus*), but no significant result for anti-bacterial activity was obtained with puroB. The three-dimensional structure of puroA showed there was a positively charged cluster, which was predicted for the functional site to bind the negatively charged bacterial membrane.

Another mechanism for many antimicrobial peptides is to make holes in microbial membranes by forming ion channels, which kills the microorganisms (Epand et al., 1999). In the same way, puroindolines were shown forming ion channels on *Xenopus* oocytes (Charnet et al., 2003).

Hordoindolines

Cultivated barley has hordoindolines, homologs of the puroindolines in wheat (Gautier et al., 2000). Puroindolines have two types, *pinA* and *pinB*, and hordoindolines have hordoindoline a (*hinA*) and hordoindoline b (*hinB*). They reside on the short arm of

chromosome 7 (homologous to Triticeae, 5H), the identical location to wheat 5D genome where *Ha* locus resides (Rouvès et al., 1996; Beecher et al., 2001; Beecher et al. 2002b). *HinB* is located close to the telomere of chromosome 7 and segregates with *hinA* and *Gsp-1*, a grain softness protein (Rouvès et al., 1996). Darlington et al. (2001) and Beecher et al. (2001) reported expression of these genes in the endosperm, whereas they were absent in the embryo. This result coincides with the location of puroindolines (Dubreil et al., 1998).

PinA and *hinA* have 90% identical nucleic acid sequence, and over 90% of the sequences are identical in *pinB* and *hinB* (Gautier et al., 2000; Beecher et al., 2001). To-date, there are three *hinA* alleles and *hinB* alleles in elite North American barley cultivars (Beecher et al., 2001). For instance, variety Harrington has the *hinA*-1, and *hinB*-1 alleles (Beecher et al., 2001). However, hordoindolines with slightly different sequences have been reported from different varieties, suggesting that mutations may occur at a higher frequencies than puroindolines since hordoindolines sequences are less conserved than puroindolines (Darlington et al., 2001).

The grain texture of barley is said to affect malting. Soft varieties are often used for brewing (Brennan et al., 1996). Because of the similarity of wheat and barley, the correlation between hordoindolines and kernel hardness has aroused the interest of brewers and researchers. Thomas et al. (1996) identified quantitative trait loci (QTLs) for barley hardness, measured by milling energy, on chromosome 7, the same location of hordoindolines. All seven soft-type varieties and four out of six hard type varieties contained *hinA* mRNA, suggesting a lack of correlation of *hinA* and grain softness

(Darlington et al., 2001). There needs to be more work to confirm a correlation between seed hardness and *hinB* or any effect of its mutation. Since hordoindolines reside within the starch texture trait locus, they may have a small role in barley grain texture. Moreover, added puroindolines stabilized beer foam (Clark and Wilde, 1994), suggesting that the existence of hordoindolines may be important in malting variety.

An anti-microbial property of the various hordoindoline types have not been reported. Since their tryptophan- and cysteine-rich regions are present, their antimicrobial property might be anticipated.

CHAPTER 2

MATERIALS AND METHODS

Barley Variety and Growing Conditions

Seed of two-rowed cultivar of barley, cv. Harrington, harvested in 2001 and 2002 was used in these experiments. Four to 5 seeds of Harrington were seeded in a 7" pot using pasteurized PGC Soil Mix (equal parts of Bozeman Silt Loam Soil: washed Concrete Sand: Canadian Sphagnum Peat Moss. AquaGro 2000 G wetting agent was blended in at one pound per cubic yard of soil mix.; http://ag.montana.edu/plantgrowth/specifications.htm) and grown in a green house at the MSU Plant Growth Center (PGC).

Plants were grown under 23ºC/20ºC day/night temperature and 14/10 hours light/dark periods. One thousand watt metal halide lamps were used for supplemental light during the 14 h photoperiod. Plants were watered as needed, and 100 ppm of water soluble fertilizer, Peters 20-20-20 NPK (Scotts Sierra Horticultural Products Company, Marysville, OH) was supplied three times a week. Insecticides were applied as needed.

Callus Induction and Regeneration

Milk stage barley kernels (12 day post anthesis [DPA]) (cv. Harrington) were harvested and sterilized for 20 min in 25% (v/v) bleach (5.25% sodium hypochlorite) on a shaker. Immature embryos (IEs) were removed from the sterile seeds with tweezers and

scalpel under a dissecting microscope, and placed onto a callus induction (CI) media, described below (Table 1), and the plates were incubated in the dark at 24ºC.

The different media used in these experiments were based on those described by Cho et al., 1998. All media namely Callus Induction (CI), Callus Regeneration (CR), Root induction Media (RM), and Osmoticum Media (OM) contained the following basic ingredients: MS (Murashige and Skoog) basal (Sigma, St. Louis, MO), 30 g/L maltose, 1.0 g/L thiamine-HCl, 0.25 g/L *myo*-inositol, 1.0 g/L casein hydrolysate, 0.69 g/L proline, 5 μM $CuSO_4$, and 2.5 g/L phytagel, pH 5.8. The OM media contained an additional 0.4 M total carbohydrates, 36.56 g/L sorbitol and 36.56 g/L mannitol.

Optimization of phytohormone levels, auxin [2,4-dichlorophenoxyacetic acid (2,4-D)], and cytokinin [6-benzylaminopurine (BAP)], in CI and CR media was done to get calli for bombardment and to have high callus regeneration ability. Five CI media (Table 1) and three CR media (Table 2), with different levels of phytohormones, were used. Callus Induction Frequency (CIF%), Callus Quality Frequency (CQF%), Green Callus Frequency (GCF%), and Callus Regeneration Frequency (CRF%) were determined from individual medium or combinations of media.

Table 1. Concentration of 2,4-D and BAP in Callus Induction (CI) medium.

	Callus Induction (CI) medium				
	CI 1	CI 2	CI 3	CI 4	CI 5
2,4-D (mg/L)	2.5	2.5	2.5	2.5	2.5
BAP (mg/L)	0	0.01	0.1	0.25	0.5

Table 2. Concentration of 2,4-D and BAP in Osmoticum Medium (OM) and Callus Regeneration (CR) medium.

	Osmoticum Medium (OM)	Callus Regeneration (CR) medium			
		CR 1	CR 2	CR 3	CR 4
2,4-D (mg/L)	2.5	1.0	0	0	0
BAP (mg/L)	0	0.5	1.0	2.0	3.0

To test callus regeneration ability after selection on hygromycin B (RocheDiagnostic GmbH Roche Molecular Biochemicals, Germany) containing medium, CI 2 media was supplied with 20, 30, 40, 50 mg/L hygromycin B, and Callus Regeneraton Frequency (CRF%) was recorded.

Plasmid Amplification

All plasmids were introduced into *Escherichia coli* (*E. coli*), MAX Efficiency DH5α Competent Cells (GibcoBRL, Rockville, MD). Plasmid minipreps were done using boiling methods (Sambrook et al., 1989) or maxiprep kits (Qiagen, Germany). The transformation of plasmids into *E.coli* was followed by manufacturer's protocol with the following changes: spectinomycin was used as an antibiotic to select for pMON410 plasmid and ampicillin was used for selection of the other plasmids, (p35Shph, pUbipinA, pRQ, and pScBVuidA).

To further purify DNA, approximately 10 μg RNase A was used and incubated at 4ºC for overnight, followed by phenol/chloroform extraction, and DNA precipitation with ammonium acetate and 100% ethanol. For p35Shph and pUbipinA, a maxiprep (Qiagen) was used to obtain a large amount of plasmids for particle bombardments.

Bombardment (Particle Preparation)

All DNA introductions into barley calli were done using a Biolistic PDS-1000/He Particle Delivery System (BioRad, Hercules, CA). An aliquot of sterile 3 mg microcarriers (1.0 μm gold pellet, BioRad) in 50 μL of 50% glycerol solution was prepared as described below. Thirty mg of gold microcarriers (gold pellet) were placed into a 2.0 mL tube (USA Scientific, Ocala, FL) and 1 mL of 70% ethanol was added. The tube was vigorously vortexed for 5 min, followed by 15 min gentle mixing on a rocker platform. The liquid phase was discarded after centrifugation at 1,000 x*g* for 1 min. The pellets were washed three times by 1min of vigorous vortexing in 1 mL of sterile ddH_2O. The pelleted gold was resuspended in 500 μL of sterile 50% glycerol (v/v) and vortexed vigorously for 5 min. The gold was then suspended in 50% glycerol and dispensed into 50 μL aliquots, which could be stored up to 2 months at 4ºC.

To precipitate plasmid DNA onto the microcarrier, the desired amount of plasmid dissolved in sterile 125 μM $CaCl_2$ and 2 μM spermidine was added to a tube containing the sterile microcarrier. The tube was vortexed vigorously for 5 min, followed by brief centrifugation. The aqueous phase was discarded and the pellet was washed first with 100 μL of 70% ethanol, then with 100 μL of 100% ethanol by repeated pipetting of the gold particle suspension. The tubes were centrifuged briefly, and the upper phase was discarded after each step. The pellet was resuspended in 60 μL of 100% ethanol and spread equally onto 5 pieces of macrocarrier. The DNA delivery system was used according to the manufacturer's instructions. All materials and chambers were sprayed

with 95% ethanol before and between uses. All target tissues were placed 6 cm away from the stopping screen in the gene gun chamber.

GUS Expression

Transient expression experiments with barley calli were done with two plasmids. Plasmid pRQ6 (Figure 1) contains *uidA* controlled by the cauliflower mosaic virus (CaMV) 35S promoter and terminated by the nopaline synthase *nos* 3' polyadenylation signal. The pScBVuidA (Figure 2) contains a pMON755i backbone with *uidA* controlled by ScBV promoter and terminated by nos. These plasmids were provided by Dr. Michael J. Giroux.

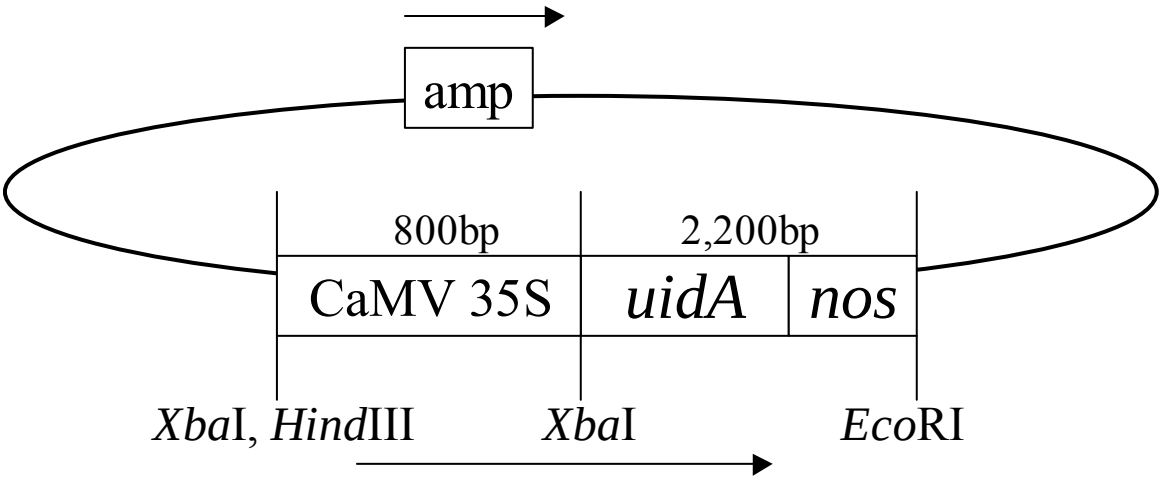

Figure 1. Plasmid pRQ (8,250 bp). Cauliflower mosaic virus 35S promoter (CaMV P35S), β-glucuronidase gene (*uidA*), and nopaline synthase terminator (*nos*) are shown.

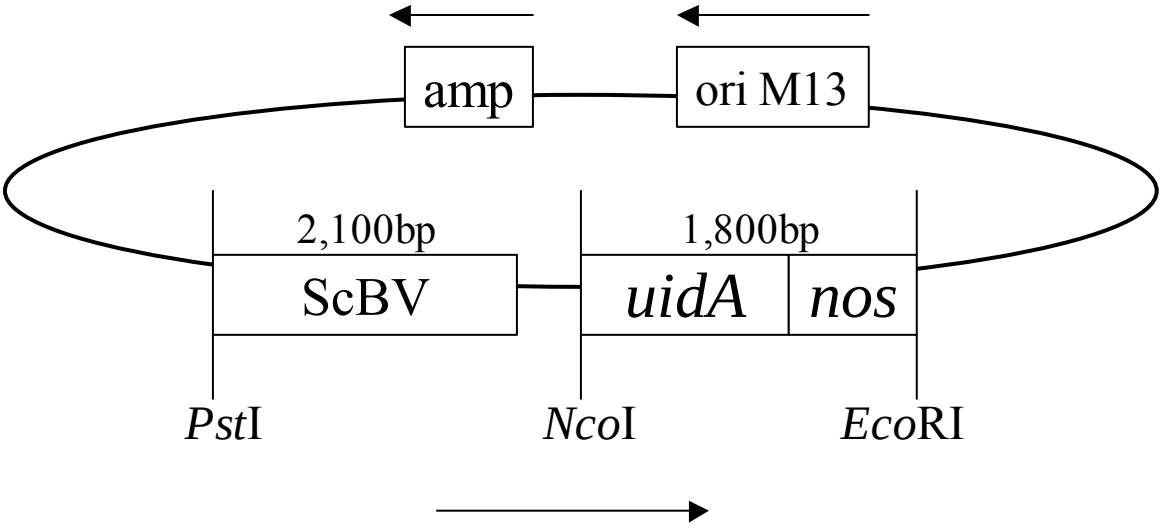

Figure 2. Plasmid pScBVuidA (7,300 bp). Sugarcane bacilliform badnavirus (ScBV) promoter, β-glucuronidase gene (*uidA*), and nopaline synthase terminator (*nos*) are shown.

Plasmid (7.5 μg) was precipitated on 3 mg of sterilized microcarriers. The particle delivery system was employed for single or double bombardment with 650, 900, 1,100 psi rupture disks (BioRad). Each set contained 10 to 30 calli (two to four weeks old), grown on either CI 2 or CI 3. Calli were placed on OM media 4 hours post bombardment and left on the media overnight in the dark at 24ºC.

The next day, histochemical staining for GUS was performed using 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid (X-gluc)(Research Organics, Cleveland, OH). Approximately 10 calli/mL were exposed to GUS assay buffer (50 mM $NaPO_4$ pH 7.0; 1 mM EDTA; 0.5 mM ferrocyanide, $K_4[Fe(CN)_6] \cdot 3H$; 0.5 mM ferricyanide, $K_3[Fe(CN)_6]$; 1 mg/mL X-gluc), and placed in a vacuum desiccator for 20 min to allow the reagent to infiltrate. Samples were incubated at 37ºC in the dark in the GUS assay buffer and positive sites on the calli were observed visually under a dissecting microscope every 5 h.

Transformation of Barley with the Puroindoline a Gene

pAHC17 (Christensen et al., 1996) is a vector cassette that contains a pUC8 backbone with the *Ubi1* promoter, the first intron upstream of a *Bam*HI endonucrease cutting site, and *nos*. Plasmid pUbipinA (Figure 3, provided by Dr. Michael J. Giroux), contains a pAHC17 backbone with the *pinA* gene, ligated into the *Bam*HI endonuclease restriction site (Krishnamurthy and Giroux, 2001).

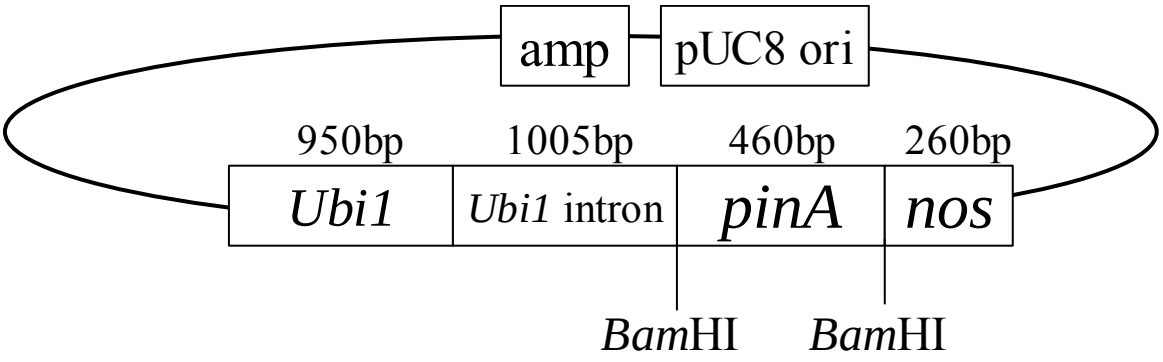

Figure 3. Plasmid pUbipinA (5,380 bp). The 5' untranslated exon, first intron of the maize ubiquitin (*Ubi1*), the puroindoline a gene (*pinA*), and nopaline synthase terminator (*nos*) are shown.

Plasmid pMON410 was digested with *Bam*HI to obtain *hph*, a selective antimicrobial resistance gene driven by CaMV 35S promoter and terminated by *nos*. The hygromycin fragment was ligated into a *Bam*HI digested pBluescript M13- to make a smaller vector, p35Shph (Figure 4).

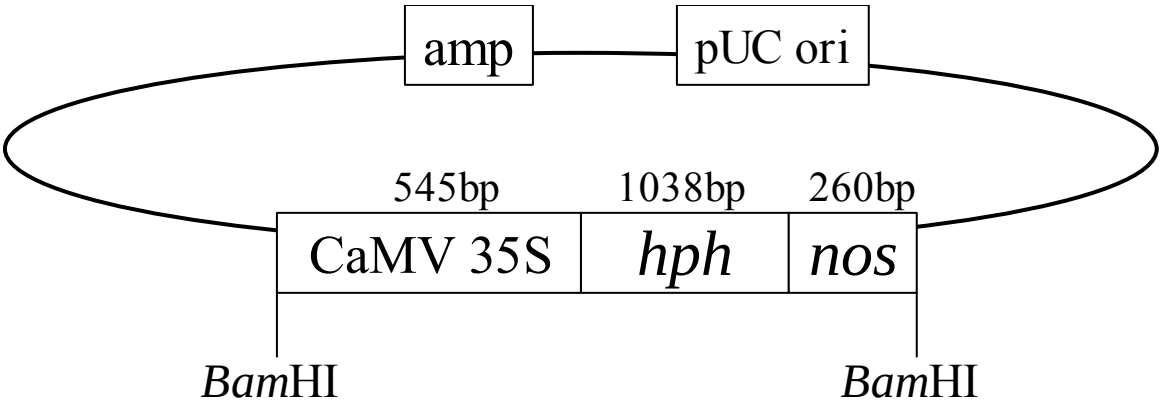

Figure 4. Plasmid p35Shph (4,803 bp). Cauliflower mosaic virus 35S promoter (CaMV P35S), hygromycin phosphotransferase (*hph*) gene, and nopaline synthase terminator (*nos*) are shown.

The plasmids were transformed into competent *E. coli* cells, and colonies were selected on LB agar containing ampicillin and X-gal. The orientation of the *hph* insertion was determined by the banding patterns observed after digestion with *Hpa*I, *Eco*RI, *Sma*I, and *Bam*HI.

pUbipinA was co-transformed with p35Shph. A particle delivery system was employed for bombardments using 900 psi rupture disks. Each bombardment was done with 0.6 mg microcarrier coated with 3, 6, or 7.5 µg pUbipinA and 1.5, or 1.875 µg p35Shph. Calli were bombarded with single or double shots.

Twelve to 51-day-old calli were used for this experiment. The calli were subcultured to high osmotic media and incubated at 24ºC in the dark for 4 hours post-bombardment, then brought back to 24ºC in the dark overnight after bombardment. Calli were then subcultured to CI 2 media for 0 to 6 days to recover from bombardment stress.

After that, calli were selected on CI 2 medium containing 30 mg/L hygromycin B for 9 to 21 days in the dark. Surviving calli were replaced onto CR media and placed in a dimly lit incubator at 24ºC for 16 h with 240 W fluorescent lights and dark at 21ºC for 8 h. A regenerated callus was left on the same media until it grew to approximately 2 cm long, turned green and/or roots appeared. These regenerating plantlets were placed in magenta boxes on RM, containing no phytohormone, to induce rooting and shoot elongation. Otherwise, calli were subcultured every 7 d up to CR 4, the highest BAP concentration to induce regeneration. The calli that had not regenerated after two months on CR 4 media were discarded.

The regenerated plantlets on rooting media were placed in moist soil (50:50 PGC Soil Mix/ Sunshine Mix #1) in small cups, kept in a shaded box covered with clear plastic wrap in a green house for a week until the plants adjusted to the altered environment. Finally, plants were transformed into 7" pots of PGC Soil Mix and grown with appropriate watering and supplements described above until mature (T_0).

DNA Extraction

To test the DNA of T_0 plants, young leaves were taken from each plant at the 3 to 4 leaf stage. Each blade was 3 to 4 cm long. Because 25% of the progeny should be homozygous recessive due to the segregation of the T_1 generation, 15 seeds from each T_0 parents were planted in pots in the greenhouse, and young leaf tissue (2 to 3 leaf stage) were taken, combined and stored at –80ºC until used. The Tris-CTAB method (Harwood et al., 2000) was used for DNA extraction. Approximately 300 to 500 mg of leaf tissue

was placed in a 2.0 mL tube and ground to powder under liquid nitrogen using an electrical grinder (Foredom Electric, Bethel, CT). One mL of extraction buffer (100 mM Tris-HCl, pH 7.5, 0.7 M NaCl, 40 mM EDTA, pH 8.0, 1% [w/v] Hexadecyltrimethylammonium bromide [CTAB], 0.4% [v/v] β-mercaptoethanol) was added and mixed thoroughly. After adding 100 μL chroloform/isoamyl (24:1), the mixture was vortexed vigorously and the tubes were incubated at 65ºC for 30 min. After the samples cooled to room temperature, another 400 μL of chloroform/isoamyl (24:1) was added to each tube and samples were vortexed thoroughly. Tubes containing samples were centrifuged at 16,000 x*g* for 5 min. The aqueous phase of each sample was transferred to a fresh tube containing 95% ethanol and incubated for 20 min at room temperature. DNA pellets were obtained by brief centrifugation and washed with 1.0 mL of 0.2 M sodium acetate in 75% ethanol for 10 min followed by a 5 min wash with 0.5 mL of 0.01 M ammonium acetate in 75% ethanol. After the DNA was dried, 50 to 100 μL of TE (10mM Tris-Cl, pH 8.0; 1 mM EDTA, pH 8.0) and 1 μL RNase A (10 μg/mL) was added to each tube. The quality and concentration of the DNA was determined spectrophotometrically (A260, A280) and on a 1% agarose gel staining with ethidium bromide. DNA samples were stored at –20ºC until used.

RNA Extraction

Barley leaf samples were harvested, stored and ground as with the DNA extraction procedure. One mL of TRIZOL reagent (Invitrogen, Carlsbad, CA) was added per 100 mg of finely ground leaf tissue. RNA extraction was performed according to

manufacturer's instructions. The RNA pellets were dissolved in 50-100 μL of diethyl pyrocarbonate (DEPC, Sigma) treated H_2O (0.01% v/v). The RNA concentration and quality was tested by gel electrophoresis and absorbance at 280 nm and 260 nm. The RNA solution was stored at –80ºC until used.

A positive sample of total RNA containing *pinA* RNA was isolated from wheat cv. Heron seeds, 21 DPA, which were obtained from Dr. Michael Giroux. Seeds were ground to a powder using a mortar and pestle, and stored at –80ºC. Seed powder (100 mg) was placed in a 2 mL centrifuge tube and 0.5 mL extraction buffer (50 mM Tris, pH 9.0, 200 mM NaCl, 1.0% Sarcosyl, 20 mM EDTA, 5 mM DTT in DEPC treated water) was added and vortexed. Phenol/chloroform/isoamyl alcohol (49:49:2) was added and vortexed. The samples were centrifuged at 13,800 x*g* for 5 min at 4°C. The aqueous phase was pipetted into a fresh 2.0 mL tube containing 1.0 mL TRIZOL reagent. The rest of the procedure was performed according to manufacturer's instructions. The RNA pellets were dissolved with 50 μL of DEPC-treated H_2O. The quality and concentration of RNA aliquot was tested by either OD^{260} and OD^{280} spectrophotometer readings or electrophoresis on a 1.2% formaldehyde (FA) gel, containing 220 mM formaldehyde and ethidium bromide. The RNA solution was stored at –80ºC until used.

Southern Hybridizations

Barley genomic DNA (10 μg) and 30 ng of p35Shph were digested with *Bam*HI (Takara Biomedicals, Shiga, Japan) at 37ºC for 3 h. Digested samples were loaded on a 1.0% agarose-TAE gel with ethidium bromide and electrophoresed for 3 h at 60 V. The

gel was photographed under UV light. Depurination (fragmentation) of the DNA in the gel was done with 0.125 M HCl for 10 min. The DNA was then denatured with 0.4 N NaOH for 30 min and neutralized with neutralization buffer (2 M NaCl, 0.5 M Trizma base, pH 7.5) for another 30 min. The gel was washed with ddH_2O between each step. DNA was transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech, Buckinghamshire, England) by capillary blotting with 10 X SSC buffer (0.15 M Tri-sodium citrate, 1.5 M NaCl, pH 7.5) buffer for 16 hours. DNA was fixed to the membrane by UV cross linking, (UV Cross-linker 2,400, Stratagen).

Southern hybridization was performed under high stringency conditions (65ºC) in a glass roller tube. The membrane was treated for 1 hour with hybridization buffer, which consisted of 2 g bovine serum albumin, 14 g sodium dodecyl sulfate (SDS), in 200 mL of 0.5 M Na_2HPO_4, pH 7.3.

A probe was prepared by PCR (Polymerase Chain Reaction, Eppendorf Scientific, Westbury, New York) using 0.1 μg of the forward and reverse primers hygR (5'ACCTGCCTGAAACCGAACTGCCCG3') and
hygR2 (5'AACCACGGCCTCCAGAAGAAGATG3'),
respectively. Approximately, 1 ng p35Shph was used as a template, and PCR reagents consisting of 0.2 mM of dNTPs, 1.25 U *Taq* DNA polymerase (Eppendorf), 5 X TaqMaster (Eppendorf), 10 X Taq Buffer including 1.5 mM Mg^{2+} (Eppendorf) were made to a final volume to 20 μL with ddH_2O. PCR was conducted using a program of 2 min initial denaturation step at 95ºC, 40 cycles of 95ºC for 30 s, 61ºC for 60 s, 72ºC for 60 s, followed by a 4 min final extension at 72ºC. The PCR product was purified with a

gel extraction kit (Qiagen), using the manufacturer's protocol. Fifty ng of the PCR fragment was used as a probe. Ready-To-Go DNA labeling beads (Amersham Biosciences, Piscataway, NJ) was used to label the probe with redivue deoxycytidine 5'-[α-^{32}P] triphosphated CTP (α-^{32}P-dCTP, 6000 Ci/mmol, Amersham, Sterling Heights, IL) using manufacturer's instruction. The solution was incubated for 20 min at 37ºC for incorporation of α-^{32}dCTPs. Unincorporated α-^{32}dCTPs were removed by gel filtration (Edge Bio Systems, Gaithersburg, MD) using manufacture's protocol. The ^{32}P labeled probe was then added to the glass roller tube with 25 mL of preheated fresh hybridization buffer (same as pre-hybridization buffer). After incubation at 65ºC overnight, the membranes were washed for 15 min each with 1 X SSC, 0.1% SDS pre-heated to 65ºC, followed by 0.1 X SSC, 0.1% SDS. Fuji Super RX film (Fuji film, Tokyo, Japan) was exposed to the membrane with an intensifying screen at –80ºC, and hybridized signals were visualized on the developed film.

The membrane was stripped as described by the manufacturer and reprobed to show the quality of extracted DNA. The PCR product of *hinA*, a naturally existing single copy gene in Harrington barley, was used as a probe. PCR was conducted as described in Beecher et al. (2001) with using 100 ng of wild-type Harrington genomic DNA as a template. Southern hybridization was conducted as previously described.

Northern Hybridizations

Northern hybridization was conducted under RNase free condition. All solutions were made with DEPC-treated water and plasticware and glassware were sprayed with

RNase AWAY (Molecular Bio Products, San Diego, CA) before use. Approximately 10 μg of barley total RNA and either 5 μg of wheat total RNA or 20 ng of p35Shph plasmid as positive controls were mixed with 3 μL of RNA loading dye, described in the Rneasy mini handbook (Qiagen), and made to 25 μL with DEPC water. Three μg of 0.24-9.5 kb RNA Ladder (Invitrogen) was used to estimate band sizes. All samples were placed in a 65ºC water bath for 8 min for denaturation. The samples were loaded on a 1.2% formaldehyde (FA) gel, containing 220 mM formaldehyde, followed by staining with ethidium bromide. The gel was electrophoresed for 3 hours at 60 V and blotted to a nylon membrane by capillary action with 10 X SSPE buffer (1.8 M NaCl, 0.1 M NaH_2PO_4, 0.01 M EDTA, pH 7.3) for 16 hours. After blotting, RNA was fixed to the membrane by UV cross linking, followed by a rinse with DEPC-treated H_2O.

Northern hybridization was performed with the same methods described for Southern blotting. The PCR products of the *hph* fragment (described above) or *pinA* fragment were used as probes. A *pinA* probe (380 bp) was prepared by PCR using primers, PAY5 (5'GCAGTTACGATGTTGCTGGC3'), and
YPA3 (5'TCACCAGTAATAGCCAATAGTGCC3'). Ten ng pUbipinA was used as a template. The amplification program was 2 min initial denaturation step at 94ºC, followed by 39 cycles of 30 s denaturation at 94ºC, 60 s annealing at 60ºC, 60 s elongation at 72ºC, followed by a 4 min final extension at 72ºC. Fifty ng of PCR products of both *pinA* and *hph* were used to probe individual membranes.

After a incubation at 65ºC overnight, the membranes were washed briefly with 2 X SSPE, 0.1% SDS preheated to 65ºC, followed by 0.2 X SSPE, 0.1 X SDS (15 min per wash).

After radioactive signals were visualized by X-ray film, membranes were stripped and reprobed with 50 ng of a rDNA PCR product. The PCR program using
ITC4 (5'GGAAGTAAAAGTCGTAACAAGG 3'),
ITC5 (5' TCCTCCGCTTATTGATATGC 3') primers, were a 3 min initial denaturation at 94ºC, followed by 40 cycles of 94ºC for 45 s, 50ºC for 30 s, 72ºC for 90 s, followed by a 5 min final extension at 72ºC (White et al., 1990).

CHAPTER 3

RESULTS

Optimization

To determine the media on which calli grew best, the callus induction frequency (CIF%), initial callus growth rate and quality of the callus (CQF%) were calculated for each medium (Table 3). Almost 70% of immature embryos (IEs) became calli, and calli grew almost 30 mg /day on all media. Interestingly, each set of data was higher for Harrington than for Golden promise (GP), which is said to be an easy variety for gene modification (data not shown). No significant difference was found in CIF% and initial callus growth rate between 5 different media used in the experiment. The CQF% results showed significant difference ($P<0.001$) between the 5 different media in the best quality calli. The least significant difference (LSD) indicated that medium CI 5 showed the highest quality calli but that did not differ from CI 2 (Table 3).

During the callus induction process, some distinct callus morphologies were observed, such as white, dark white, shiny, lusterless, watery, dry, smooth, or rugged. The evaluation of calli appropriate for transformation and most likely to regenerate green plants was determined using criteria described by Cho et al. (1998). Induced calli were picked randomly and classified into 4 grades, from ++++ for the highest quality (shiny, compact, slightly nodular, slightly brown-colored callus) to + for the lowest quality (soft, friable, white callus) in the period of 1 to 2 weeks after initial callus induction. Media CI 1 gave poor calli, which were frosting white with fast cell growth, reminding one of a

cancer. Instead, the small amount of BAP present in media CI 2 drastically reduced the number of poor quality calli and increased the numbers of better quality callus, suggesting that BAP inhibited unusual cell growth, and the correlation between auxin (2,4-D) and cytokinin (BAP) was critical for good callus formation in this cultivar.

Table 3. Callus Induction Frequency (CIF%), Callus Growth Rate (CGR, mg/embryo/day), and Callus Quality Frequency (CQF%) on CI media. Data were taken 10 days after placing IEs on media.

Medium	CIF%[a]	CGR (mg/embryo/day)[b]	CQF%[a]			
			+	++	+++	++++
CI 1	71.0±2.1	37.7±4.7	31.8±4.0	42.8±0.4	20.2±3.1	5.2±3.6
CI 2	69.8±7.5	33.6±0.5	9.8±3.9	22.8±4.0	27.2±2.7	40.2±5.8
CI 3	78.8±1.3	29.3±1.9	1.8±1.8	29.0±2.3	31.8±4.1	37.4±3.5
CI 4	76.6±2.8	34.8±2.8	1.4±1.4	21.4±3.8	40.4±3.9	37.0±2.4
CI 5	76.0±2.7	32.1±3.7	1.2±1.2	17.0±3.4	33.2±3.1	48.6±2.7
LSD (0.05)	ns[c]	ns	8.2	11.0	10.2	11.2
LSD (0.01)	ns	ns	11.2	14.9	14.0	15.3

[a] Means of 5 replications ± standard error.
[b] Means of 3 replications ± standard error.
[c] denotes no significant difference.

Induced calli from the five induction media were transferred to intermediate media (IM), either CI 4, CI 5, or CR 1, which contain high concentrations of both auxin and cytokinin. This step is reported to reduce stress for regenerating calli exposed to sudden auxin/cytokinin balance changes. The petri dishes containing media and calli were placed in a dim light incubator at 24°C. Green Callus Frequency (GCF%) was measured 10 days later (Table 4). The calli derived from CI 1 media showed low GCF%.

Interestingly, calli initially derived from CI 4 and CI 5 showed high performance when they were subcultured on CR 1. In most cases, calli grew best on CR 1, suggesting that a high auxin level in IM inhibited greening of calli.

Selected green tissues were then broken or cut in 2-3 pieces (4-5 mm size) and subcultured on CR 2 media, containing only BAP, for regeneration. The calli were exposed to the maximum light intensity in the incubator. The Callus Regeneration Frequency (CRF%) and the average number of green shoots per callus were measured every 1 week after 2 transfers (Table 4). Overall, both the GCF% and CRF% showed a significant difference of increasing quality of calli when cytokinin is present in CI media ($P<0.001$). Using CI 5 or CR 1 as IM rather than CI 4 increased the CRF% and the number of green shoots during the callus regeneration period. We determined the best medium for each step from these results. CI 2 medium was considered best for callus induction since it performed as well as CI 4 or CI 5 but with a minimum cytokinin level. For callus regeneration, CR 1 was chosen as best as an IM, followed by only BAP-containing media, CR 2, CR 3, or CR 4 to increase the regeneration ability of the calli.

Selection media that contained different concentrations of hygromycin B were also tested with non-transformed calli. The survival rate was observed 1 week after exposure of calli to the media. CI 2 medium with 30 mg/L hygromycin B killed approximately 80% of non-bombarded calli after 10 days, and was used for further experiments.

Table 4. Green Callus Frequency (GCF%) observed on Intermediate Media (IM); CI 4, CI 5, or CR 1. Callus Regeneration Frequencies (CRF%) were observed after 2 subcultures on CR 2 media containing only BAP. At the same time, numbers of regenerated green shoots per each callus were counted.

CI[a]	IM[b]	IM	CR 2	
		GCF%[c]	CRF%[c]	Green shoots/callus[d]
CI 1	CI 4	39.0±1.0	17.5±7.5	3.50±0.27
	CI 5	46.5±43.5	38.0±5.0	4.33±0.33
	CR 1	59.0±4.0	59.0±4.0	4.50±0.5
CI 2	CI 4	67.5±7.5	46.5±3.5	5.61±0.66
	CI 5	70.0±5.0	78.0±8.0	7.33±0.66
	CR 1	80.5±7.5	79.5±6.5	8.33±0.33
CI 3	CI 4	69.5±5.5	59.5±6.5	5.25±0.48
	CI 5	77.5±2.5	65.0±7.0	6.50±0.5
	CR 1	73.0±2.0	63.5±0.5	5.75±0.48
CI 4	CI 4	69.5±5.5	67.5±7.5	7.00±0.58
	CI 5	81.5±4.5	78.5±1.5	4.67±0.66
	CR 1	100.0±0.0	96.5±3.5	5.75±0.48
CI 5	CI 4	82.5±2.5	58.5±1.5	2.50±0.5
	CI 5	82.5±7.5	79.0±9.0	5.00±0.41
	CR 1	95.0±5.0	83.0±5.0	6.75±0.48
LSD (0.05)		14.5	17.2	
LSD (0.01)		20.0	23.7	

[a] Callus Induction media from which calli for the experiment were derived.
[b] Intermediate media used to protect calli from the stress of high BAP concentrations.
[c] Means of 5 replications ± standard error.
[d] Means of 2-4 replications ± standard error. Because variable number of replications were used, no LSD was calculated.

GUS Experiments

Experiments examining the transient expression of β-glucuronidase gene (*uidA*; *gus*) were performed before the bombardment experiments with the gene of interest to confirm that the transformation protocol worked and to select the optimal rupture disk. Two plasmids were employed; pRQ6 and pScBVuidA, driven by the cauliflower mosaic virus (CaMV 35S) and sugarcane bacilliform badnavirus (ScBV) promoters, respectively. Each experiment gave blue spots (transformation signal), showing that with the protocol used, Harrington calli could be transformed. The use of two different plasmids driven by different promoters also showed a significant differences in the number of blue spots on calli (Table 5).

The *uidA* gene driven by the ScBV promoter was transported to calli tissue by three different helium pressures, and the gene was transcribed and translated in the tissue (Table 6). According to the numbers of exhibited transformation signals (blue spots on calli), single shots with 650 psi were not enough pressure whereas double shoots with 650 psi increased the number of spots drastically. Since there was no significant difference with the different rupture disks, the 900 psi was used with both single and double shots.

Table 5. GUS experiment with pRQ6 or pScBVuidA plasmid. Average numbers of blue spots (spots/callus) on bombarded calli 6 and 24 h after bombardment. The calli were bombarded with 900 psi disks and single and double shots of DNA.

GUS Spots per callus					
Plasmid	Shot	After 6 h[a]	D/S[b]	After 24 h[a]	D/S[b]
pScBVuidA	Single	39.4±5.5	0.33**	33.4±3.2	0.46**
	Double	13±1.7		15.4±1.2	
pRQ6	Single	10.4±2.3*	0.71	14±2.9	0.67*
	Double	7.4±2.0*		9.4±2.5	
Comparison of pScBVuidA vs. pRQ6					
pScBVuidA vs. pRQ6	Single	0.26**		0.42**	
	Double	0.57**		0.61*	

[a] denote means of 5 repications ± standard error.
[b] Double shots/ Single shot
*, ** denote means significantly different at ,0.05 and 0.01, respectively.

Table 6. GUS experiment with plasmid pScBVuidA and three rupture disks (650, 900, 1100 psi). The ratio of calli that have at least 1 expressed signal, and an average of blue spots on bombarded calli.

		Average number of spots/callus	
Rupture disks	Shot(s)[a]	After 5 h[b]	After 9 h[b]
650 psi	Single	3.4±2.0	2.8±1.0
	Double	29.2±11.1	13.4±3.8
900 psi	Single	13.6±6.8	16.4±8.6
	Double	18.4±6.8	19.0± 13.7
1100 psi	Single	5.4±1.6	7.6±1.7
	Double	13.4±7.1	25.2±7.3

[a] Numbers of shots on calli.
[b] Means of 5 replications ± standard error.

Bombardment Experiment

A total of 16 bombardments were conducted (Table 7 and 8).

Table 7. Particle bombardment with plasmids pUbipinA and p35Shph.

Experimental number	Date of bombarded	Calli age (days)	CI 2 after bombardment (days)	Selective media (days)	pUbipinA (µg/shot)	p35Shph (µg/shot)	Total		
							Bombarded calli	Regenerated calli	% regeneration
G3	3/25/03	17	4	9	6.0	1.5	285	2	0.70
G4	4/2/03	22,24	6	16	6.0	1.5	243	3	1.23
G5	4/5/03	26	3	16	6.0	1.5	270	7	2.59
G6	4/5/03	27	2	21	6.0	1.5	240	0	0
G7	4/8/03	22	4	17	6.0	1.5	285	1	0.35
G8	4/13/03	27	3	13	6.0	1.5	141	2	1.42
G9	4/20/03	15	4	12	6.0	1.5	523	7	1.34
G10	5/7/03	44,51	4	10	6.0	1.5	293	2	0.68
G11	5/8/03	45	0	11	6.0	1.5	258	0	0
G12	5/13/03	38,50	2	11	6.0	1.5	217	0	0
G13	5/22/03	12	4	9	7.5	1.875	265	0	0
G14	7/6/03	21,27	4	12	6.0	1.5	312	1	0.32
G15	7/13/03	31	6	11	6.0	1.5	275	0	0
G16	7/19/03	22,38	6	10	6.0	1.5	295	0	0
G17	7/27/03	29,30	3	14	6.0	1.5	289	0	0
G18	8/3/03	29	1	10	3.0	1.5	268	0	0
Total							4,459	25	0.56

Table 8. Particle bombardment with double and single shots with plasmids pUbipinA and p35Shph.

Experiment number	Double shots				Single shot			
	Numbers of target plates	Numbers of target calli	Regenerated calli	Mature plants (individual/divided)	Numbers of target plate	Numbers of target calli	Regenerated calli	Mature plants (individual/divided)
G3	4	185	2	2/2	2	100	0	0
G4	4	156	2	2/4	2	87	1	1/1
G5	4	178	5	4/4	2	92	3	3/3
G6	4	161	0	0	2	79	0	0
G7	4	197	0	0	2	88	1	1/2
G8	2	98	2	2/2	1	43	0	0
G9	8	345	8	5/11	4	178	2	2/3
G10	3	106	3	2/2	4	187	0	0
G11	3	93	0	0	4	165	0	0
G12	4	148	0	0	2	69	0	0
G13	2	99	0	0	4	166	0	0
G14	4	210	0	0	2	102	1	1/1
G15	4	186	0	0	2	89	0	0
G16	4	189	0	0	2	106	0	0
G17	4	184	0	0	2	105	0	0
G18	4	171	0	0	2	97	0	0
Total		2,706	22	17/25		1,753	8	8/10

The G1 and G2 bombardment experiments were done to optimize conditions (data not shown). All calli for experiments G3-G18 were grown on CI 2 media. Calli were chosen carefully by the same criteria described previously for optimization. Calli with

rugged, stiff, brittle, and frosting white morphologies. Those calli that grew very fast were not used because they only increased in size but never regenerated or greened, according to preliminary experiments (data not shown). Calli that were 5-8 mm in diameter were obtained within the range of a 12 to 51 day induction period. Approximately 40 calli were placed in a 2 cm circle on OM medium, and single and double shot(s) were applied. The length of the recovering period on CI 2 medium before selection with hygromycin B varied according to the initial multiplication of calli which might inhibit regeneration after the selection. The hygromycin B selection period also varied to see the influence on the regeneration rate. There was a wide difference in regeneration rate between each set. Half of the sets (G6, G11 to G13, G15 to G18) did not have any regenerated calli. Correlations between each condition, i.e., calli age, recovery days after bombardment, the days on selective media were obtained. However, no significant correlation was obtained. A total of 2,706 calli were bombarded with double shots and 1,753 calli were single shots. As a result, 22 calli (0.8%) survived after selection from double shots. Five calli died during the regeneration period and 17 calli (0.62%) produced 25 plantlets. From the single shots, 8 calli (0.45%) survived selection and produced 10 complete plantlets. From the total of single and double shots, 25 calli (0.56%) produced 35 fertile T_0 plants which produced viable seeds. The history of these 35 T_0 plants is shown in Table 9.

Table 9. List of putative transgenic plants.

Plant number	Bombardment[a]	Calli age	Recovery days[b]	Selection days[c]	Regenerated days[d]	Regeneration media[e]
G3-1	D	17	4	9	44/31	CR 2
G3-2	D	17	4	9	128/115	CR 4
G4-1a	D	22	6	16	45/23	CR 2
G4-2a	D	22	6	16	50/28	CR 2
G4-2b*	D	22	6	16	50/28	CR 2
G4-2c*	D	22	6	16	50/28	CR 2
G4-3	S	22	6	16	64/42	CR 4
G5-1	S	26	3	16	40/21	CR 2
G5-2	S	26	3	16	40/21	CR 2
G5-3	S	26	3	16	40/21	CR 2
G5-4	D	26	3	16	61/42	CR 3
G5-5	D	26	3	16	61/42	CR 4
G5-6	D	26	3	16	61/42	CR 4
G5-7	D	26	3	16	61/42	CR 3
G7-1a	S	22	4	17	39/18	CR 2
G7-1b*	S	22	4	17	39/18	CR 2
G8-1	D	27	3	13	39/23	CR 2
G8-2	D	27	3	13	43/27	CR 4
G9-1a	S	15	4	12	27/11	CR 1
G9-1b*	S	15	4	12	27/11	CR 1
G9-2a	D	15	4	12	35/19	CR 2
G9-2b*	D	15	4	12	35/19	CR 2
G9-3a	D	15	4	12	35/19	CR 2
G9-3b*	D	15	4	12	35/19	CR 2
G9-3c*	D	15	4	12	35/19	CR 2
G9-4	D	15	4	12	35/19	CR 2
G9-5	S	15	4	12	35/19	CR 2
G9-6a	D	15	4	12	45/29	CR 3
G9-6b*	D	15	4	12	45/29	CR 3
G9-9a	D	15	4	12	58/42	CR 3
G9-9b*	D	15	4	12	58/42	CR 3
G9-9c*	D	15	4	12	58/42	CR 3

Table 8. continued.

G10-1	D	44	4	10	29/15	CR 2
G10-2	D	44	4	10	62/48	CR 4
G14-1	S	21	4	12	52/36	CR 4

[a] D indicates double shot, S indicates single shot.
[b] The period (days) that calli were placed on CI 2 media in the dark at 24°C after bombardment for recovery.
[c] The period (days) calli were exposed to 30 mg/L hygromycin B in CI 2 media.
[d] The period (days) that the plant took for recovering i.e., from bombardment to the day the plant was placed into RM (left) and after removed from selection media to RM (right).
[e] The media from which calli were regenerated.
* indicates that the plants were derived from the same callus that a regenerated.

Screening for Target Genes in Putative Transgenic Plants

Genomic DNA from the T_0 generation was first screened by PCR. Because the wild type Harrington has naturally occurring homologous sequences to the puroindolines (hordoindolines), both forward and reverse primers,

PA5 (5'ATGAAGGCCCTCTTCCTCA 3') and

PA3 (5' TCACCAGTAATAGCCAATAGTG 3'), as described in Gautier et al. (1994) also amplified the hordoindoline a (*hinA*) gene in barley. To solve this problem, three primers containing sequences distinct from *hinA* derived from the *pinA* gene were made.

PAY5 (5' GCAGTTACGATGTTGCTGGC 3'),

PAY3 (5' CGAGCCGACTGCAACACTCCC 3'), and

YPA3 (5' TCACCAGTAATAGCCAATAGTGCC 3').

Also the primer Ubi (5' GTCGATGCTCACCCTGTT 3') coding the ubiquitin1 (*Ubi1*) promoter region and primer no3 (5'GTAACATAGATGACACCGCG 3'), coding the nopaline synthase (*nos*) terminus region, were obtained from Dr. Michael Giroux. All

possible combinations were tested in a PCR gradient program with *pinA* transgenic wheat and wild-type Harrington. The PAY5 and YPA3 combination gave one specific band of 375 bp only for *pinA* with the first denaturation at 94°C for 1 min, followed by a 40 cycles of denaturation at 94°C for 30 s, annealing at 72°C for 1 min, and elongation at 72°C for 1 min, followed by final annealing at 72°C for 4 min. Primers from the *hph* sequence were also employed to screen for hygromycin B resistant transgenic plants (i.e., a 411 bp amplified DNA bands). Positive results were obtained in the original screen PCR, but could not be repeated. To clarify those results, Southern blots were done with a *hph* probe (Figure 5). Even after two weeks exposure, no significant signal was detected, suggesting that the *hph* gene was not integrated into the barley genome. Southern blots probed with *pinA* were not performed because of the presence in barley of the hordoindoline genes. Also, *pinA* was unlikely to have integrated into the genome in *hph* negative plants. Northern blots with *pinA* (Figure 6) and *hph* probes (Figure 7) were conducted to confirm the Southern blot results. No significant bands were found, meaning no *hph* or *pinA* were expressed in the putative transgenic barley plants.

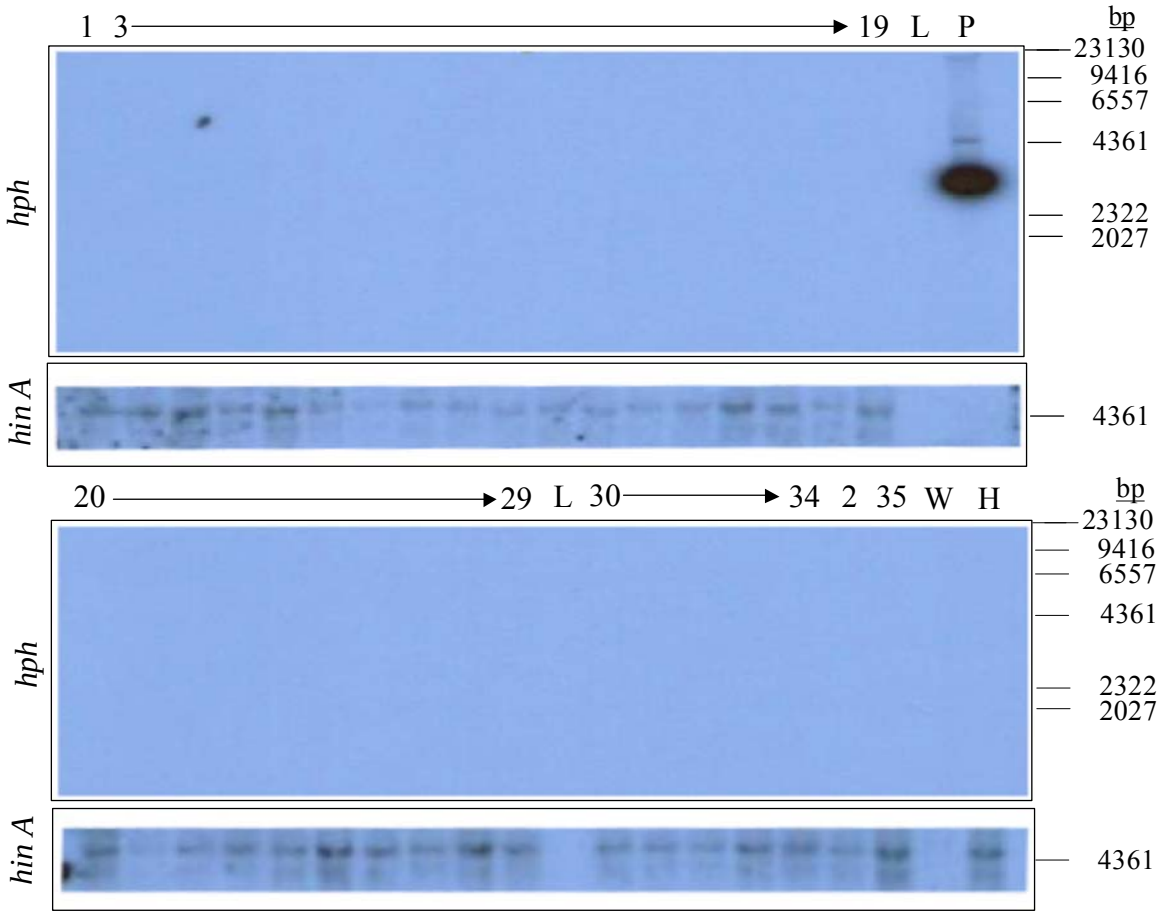

Figure 5. Southern blot analysis of 35 putative transgenic lines. 10 µg of genomic DNA was cut with *Bam*HI and probed with *hph*. L denotes the lane that was loaded 250 ng of Lambda *Hind*III ladder, W denotes 10 µg of DNA extracted from a *pinA* transgenic wheat leaf and H denotes 10 µg of DNA extracted from a wild-type Harrington leaf. 30 ng of p35Shph plasmid cut with *Bam*HI was loaded in P lane as a positive control. Equal loading of DNA in each lane was confirmed by reprobing with *hinA*.

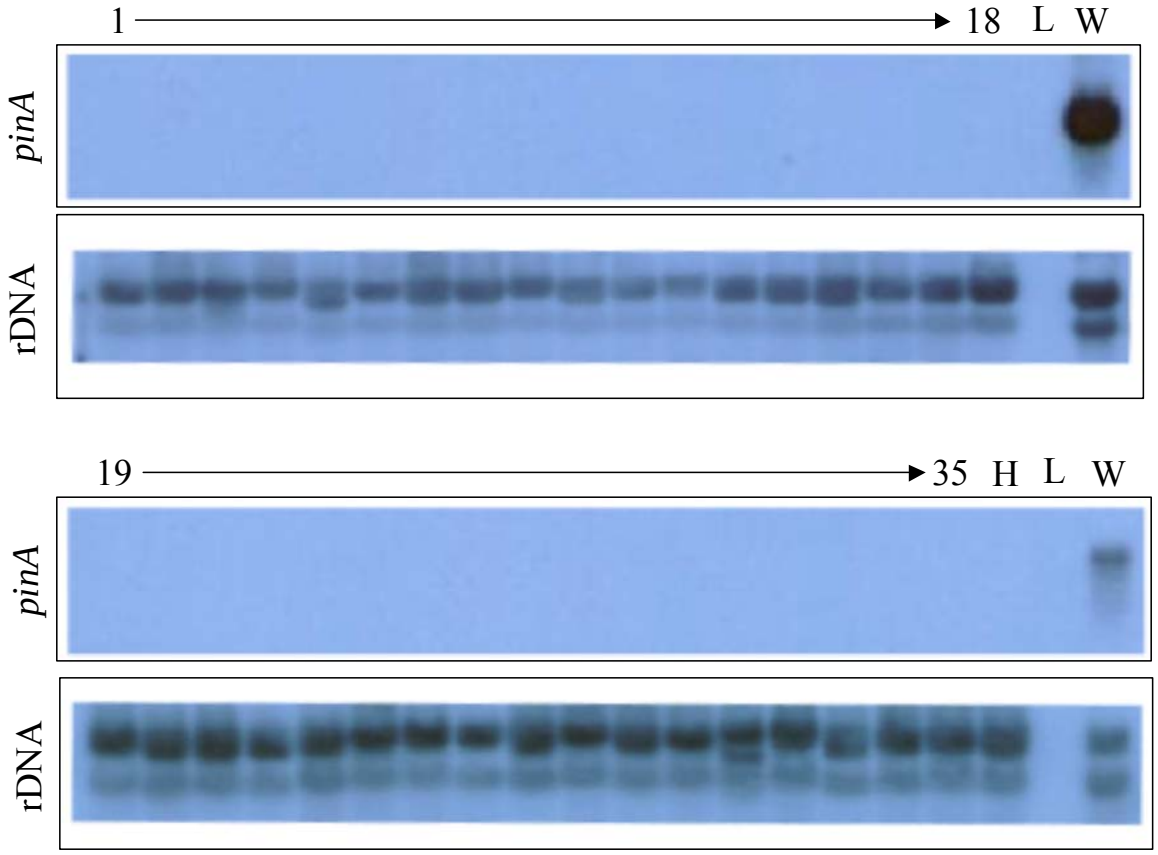

Figure 6. Northern blot of *pinA* expression in 35 putative transgenic lines. Each lane was loaded with 10 μg of total RNA from T_1 leaves. W denotes 2 μg of RNA extracted from wheat seed as a positive control, L denotes the lane loaded with 3 μg of 0.24-9.5 Kb RNA Ladder (Invitrogen, Carlsbad, CA), and H denotes 10 μg of DNA extracted from a wild-type Harrington leaf. Equal loading of RNA in each lane was confirmed by reprobing with ribosomal DNA (rDNA).

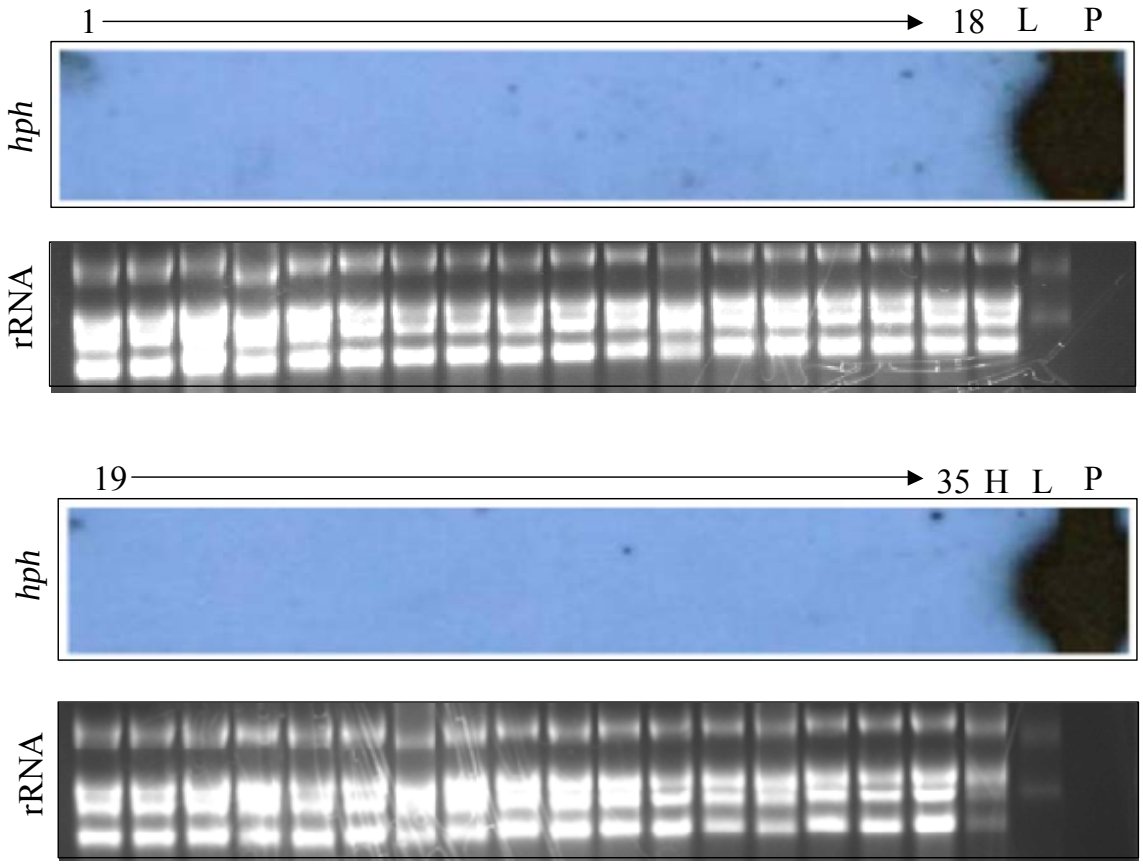

Figure 7. Northern blot of *hph* expression in 35 putative transgenic lines. Each lane was loaded with 10 μg of total RNA from T_1 leaves. 30 ng of uncut p35Shph plasmid was loaded in lane P as a positive control. L denotes that the lane was loaded with 3 μg of 0.24-9.5 Kb RNA Ladder (Invitrogen), H denotes 10 μg of DNA extracted from a wild-type Harrington leaf. Equal loading of RNA in each lane was confirmed by rRNA on an agarose gel stained with ethidium bromide.

CHAPTER 4

DISCUSSION

It is difficult for a plant breeding program to obtain resistant varieties for diseases to which a plant has no specific genetic resistance. Using plant transformation is an alternative approach to traditional breeding methods. However, transformation of barley has been slow compared to other cereals due to difficulties in regeneration of calli. Stable transformation of barley was first reported in 1994, ten years after the first tobacco transformation was reported. Currently, transformation is used to introduce novel genes in barley, especially elite malting varieties. In our research, an anti-microbial gene, puroindoline a (*pinA*) from wheat was employed and transformed into Harrington to introduce disease resistance, including resistance to Fusarium Head Blight.

The negative results seen in Southern and Northern blot analysis of the 35 putative *pinA* or *hph* transgenic plants, indicates that all surviving plants were escapes and that any delivered genes were not stably incorporated to the barley genome.

Before discussing the potential causes of the negative results, some points are clear. The success of the GUS experiments showed that the plasmid preparation methods, the DNA precipitation on the microcarrier, and the gene delivery method were successful, at least on a transient level. From this evidence, both p35Shph and pUbipinA were delivered to the cytoplasm of the calli. The gene delivery method and target used in this work should also be effective in barley transformation. According to published research, the target tissue (i.e. calli derived from immature embryos [IEs]) and gene delivery

system (i.e. biolistic bombardment), have been the most successful for barley transformation (Lemaux et al., 1999).

The process of transformation is largely divided into 4 processes: target tissue preparation, gene insertion, selection, and regeneration. I will focus on these four parts step by step to discuss possible reasons for my experimental results.

Preparing durable target tissue that can survive the stresses of the transformation process is the first step. However, there may be problems with the calli themselves and/or with the formation process that make them unsuitable for transformation. Therefore, the calli used for transformation may not have been vigorous enough to overcome the bombardment stress.

The criteria selected here for size and age of calli may have not have been correct. Cho et al. (1998) used 10 to 14 day-old calli regardless of size, while here, calli were grown to a 5-8 mm diameter which, in some cases took up to 51 days. During the callus induction process, the IEs from some heads grew much faster than others. The calli derived from slow growing IEs did not perform well as a target tissue. The influence of callus freshness on gene incorporation and other recovery processes after bombardment is not clear, since 4-month-old non-transgenic calli could regenerate (Cho et al., 1998). Zhang et al. (1999) succeeded in obtaining transgenic barley using 9-month-old shoot meristematic cultures (SMCs).

The heads harvested for use as IEs were kept moist at 4°C until embryos were excised, and this period might cause IEs weakening. Some of the stored heads were

infected with insects, especially mites, which might cause unknown negative effects on callus formation or quality.

The amount of DNA used for the gene delivery and incorporation process might not have been adequately optimized. For example, the amount of the DNA used might not have been sufficient for integration. For the biolistic bombardment method with Harrington tissue culture, Cho et al. (1998) used 25 μg of total plasmid DNA for 1 shot; we used 7.5 μg of pUbipinA and p35Shph for a single shot and 15 μg for a double shot. For the selectable marker gene, the microcarrier was only loaded with 1.5 μg of p35Shph for a single shot, and 3.0 μg for double shots. However, Ritala et al. (1994) succeeded in transforming barley cv. Golden Promise by the biolistic particle bombardment method with only 1.0 μg plasmid DNA coated on the microcarrier per single shot to IEs. Data reported in other papers that discuss successful transgenic barley recovery did not mention the amount of bombarded DNA used.

The gene recombination process is complex and, by chance, stable integration of DNA into barley genome did not happen with the numbers of calli used. Perhaps more calli would have resulted in a positive response.

Another potential problem might have been the plasmids used in this study, especially p35Shph. If *hph* was not expressed, the surviving calli from hygromycin B selection would have been false positives. The original plasmids, pUbipinA and pMON410 were used for rice transformation experiments (Krishnamurthy and Giroux, 2001). Both were integrated to the rice genome and both proteins were expressed successfully. The same plasmid, pUbipinA was employed for the barley transformation

experiments, so no problems would be expected. On the other hand, pMON410 was modified to reduce the plasmid size and replace the ori site that enhances amplification in *E. coli*. The outside of the *hph* coding sequence driven with 35S CaMV promoter and terminated by *nos* in pMON410 was replaced by *Bam*HI-digested pBluescript. The orientation and expected size were checked by the endonuclease cutting pattern, but a double check with sequencing was not done. Another possibility is that either one or both pUbipinA or p35Shph plasmids might be degraded. When the last 100bp *pinA* and first *nos* in pUbipinA were sequenced, a disruption of the *Bam*HI site where *pinA* fragment was ligated into pAHC17 was detected. A null mutation or disruption in the plasmid might happen. Sequencing these plasmids could reveal whether the sequence has been changed or not.

The CaMV 35S promoter may not work well in calli of cv. Harrington. The promoters that have been used in successful transformation of barley are *Ubi1* with its intron (Wan and Lemaux, 1994; Wu et al., 1998; Zhang et al., 1999; Nuuitila et al., 1999; Cho et al., 1998; Harwood et al., 2002), *Act1* (Jähne et al., 1994), *Act1* and its intron (Zhang et al., 1999), *Adh1* and the first intron (Wan and Lemaux, 1994; Ritala et al., 1994; Hagio et al., 1995; Jähne et al., 1994), and CaMV 35S (Nuutila et al., 1999; Wan and Lemaux, 1994, Wu et al., 1998; Ritala et al., 1994). A modified CaMV 35S promoter with the *Adh2* intron was also used by Wan and Lemaux, 1994, and the use of the CaMV 35S promoter with the *Adh1* intron was also reported (Hagio et al., 1995; Wu et al., 1998). Though the CaMV 35S promoter has been widely employed for barley transformation, especially during early efforts, the modification of this promoter, i.e.,

adding intron after the promoter, is currently done. Moreover, Cho et al. (1998) modified the CaMV 35S promoter in a plasmid containing *hph* and *nos* to the rice actin promoter (*Act1*) and its intron for their barley cv. Harrington transformation experiments.

The stress during microcarrier penetration is less likely to have caused serious damage in the experiments since others reported success using 600 or 1,100 psi rupture disks (Harwood et al., 2002; Zhang et al., 1999; Cho et al., 1998), and the 900 psi disks that we employed was in between those values. Even if the double shots caused more damage, calli bombarded with a single shot should still have survived.

During the selection period, only 0.56% of bombarded calli regenerated on selective media containing 30 mg/L hygromycin B, on which 20% of non-bombarded calli survived. This difference probably resulted from non-transgenic calli being exposed to the hygromycin B containing media for a shorter period of time than bombarded calli and because of addition stresses to the calli resulting from bombardment. Hygromycin B is a widely used antibiotic for plants, and is often used for barley transformation (Hagio et al., 1995; Cho et al., 1998). Since it has been reported that hygromycin B may cause gene silencing in transgenic plants (Schmitt et al., 1997), careful attention was paid to concentration and calli exposure time to reduce the stress that it caused. The hygromycin B concentration in selective media of successful barley transformation experiments varied from 10 mg/L (Hagio et al., 1995) to 50 mg/L (Wu et al., 1998). In most cases, 20 or 30 mg/L Hygromycin B media were used for barley transformation, although, the average concentration of Hygromycin B in selective media for other plant transformation experiments such as with rice (Krishnamurthy and Giroux, 2001) was 50 mg/L. In

experiments in which selection with 20- 30 mg/L hygromycin B in the media for barley transformation was used, less than 5% of the bombarded calli of cv. GP, Haruna Nijo, Dissa, and New Golden survived (Hagio et al., 1995), while 1.7% of Harrington calli survived (Cho and Lemaux, 1997).

In our experiments, 30 mg/L hygromycin B media was used, and the period for which calli were exposed to hygromycin B was shorter than in other published reports, so presumably our plants under less stress and should have had increased regeneration ability. Our result of 0.58% regeneration was quite low under these conditions if *hph* was actually expressed. This low rate might have been obtained because *hph* might be expressed transiently without inserting into the barley genome, and the low amount of residual hygromycin phosphotransferase kept them alive on the low hygromycin B-containing media.

Since at least 35 plants regenerated from calli, there were presumably no problem with plant regeneration or with shoot and root elongation. However, there were 5 hygromycin B-resistant calli that regenerated but died after growing a few cm. It is not clear why they died, but they might have been transformants that could not survive due to gene disruption or other genetic abnormalities resulting from the transformation process.

Overall, as Bregitzer et al. (1998b) suggested, optimization of barley transformation should be done at the cultivar level. Even using different target tissues from the same plant variety may need optimization. For instance, the biolistic bombardment transformation protocol developed for Harrington meristems may not be applicable for Harrington calli derived from IEs. The sensitivity of barley to the

transformation process causes these difficulties. The protocols used here for transformation of barley with the wheat *pinA* gene were based on a successful report of making stable transgenic Harrington (Cho et al., 1998). Since several conditions were changed from the original experiment, such as using a different promoter, it is hard to know if our negative result was caused by the protocol changes, or because barley transformation is so sensitive that the results change from one lab to another.

To determine the reasons for the negative results offered here, more optimization experiments, such as a GUS assay might be useful. For example, the effect of callus ages could be examined. Counting the number of transformation signals would tell the expression level of the *uidA* gene driven by different promoters, such as CaMV 35S, *Ubi1*, *Act1*, and ScBV in callus tissue. Apparently, an intron after each of these promoters enhances its expression level. Different amounts of plasmid DNA and different microcarrier velocities, i.e., the use of different rupture disks, may cause large differences. In addition to counting blue spots on calli, observing the process of transformation and callus regeneration might give useful data. Counting blue spots the day after bombardment only give information on expression level, but the survivor ratio and the affect of selection on different hygromycin B concentrations would give ideas about the damage that the calli sustained during bombardment. Transformation frequency (TF), i.e., the percentage of regenerated plants that are successfully transformed could be observed. More importantly, analysis of DNA and RNA from regenerated tissue with PCR, Southern blots, and Northern blots reveals the effective transformation frequency (ETF), i.e., the percentage of plants that have the gene of interest from each set of

different conditions. This number tells the frequency of integration of the gene in barley genome. Following these transformants in subsequent generations would give information about the stability of gene expression. While it is a very time consuming process to optimize each step, it would be necessary to develop a successful barley transformation protocol.

There are some modifications that might improve Harrington transformation. Recently, there have been some new protocols described. Bregitzer et al. (1998b) reported that 50 μM Cu instead 5 μM in the callus induction media is better specifically for Harrington. Also, the use of FHG medium (Cho et al., 1998) during regeneration may give higher TF and ETF (personal communication, Dr. Lynn S. Dahleen, USDA-ARS, Fargo, ND, 2003). We did not see a significant effect of these conditions with non-transgenic calli in our experiment, however, they might have a positive effect with transformed calli.

Although there have been some success in transformation of barley with hygromycin B selection (Hagio et al., 1995; Cho and Lemaux, 1997; Cho et al., 1998), and Ortiz et al. (1996) reported that *hph* performed better than *bar* in wheat transformation, the use of *bar* may allow better regeneration since it has been used successfully for barley transformation with glufosinate or bialaphos (Jacobsen et al., 2000).

In this work, no transgenic plants were obtained, but there would be many experiments that could be done once Harrington is successfully transformed with *pinA*. Disease resistance, as determined by fungal inoculation tests, the effect on brewing

quality, or cytological aberrations (Choi et al., 2001) might be analyzed once *pinA*-positive barley is obtained. Some plant breeders think transformed barley, especially with gene gun transformation, disrupts useful and important traits for brewing (personal communication, Xiwen Cai, North Dakota State University, 2003).

It has been 10 years since stable barley transformation was first described. Transformation of elite barley varieties is still difficult to achieve due to the sensitivity of barley tissue culture to the transformation process. However, transformation protocols for important varieties are slowly being established, and this technique may contribute to stable yield, premium price, and reduced chemical use in the field.

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