TRANSFORMATION OF MICROSPORES FOR GENERATING DOUBLED HAPLOID TRANSGENIC WHEAT (*TRITICUM AESTIVUM* L.)

By

WEIGUO LIU

A Dissertation submitted in partial fulfillment of
the requirements for the degree of

DOCTOR OF PHILOSOPHY IN CROP SCIENCE

WASHINGTON STATE UNIVERSITY
Department of Crop and Soil Sciences

DECEMBER 2004

To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of WEIGUO LIU find it satisfactory and recommend that it be accepted.

_____

Chair

_____

_____

_____

ACKNOWLEDGMENT

I would like to thank Dr. Diter von Wettstein for providing the opportunity to work in his laboratory and serving as the chair of my graduate committee. I would also like to thank my other committee members, Dr. Patricia Okubara, Dr. Kimberly Campbell, and Dr. Xianming Chen for valuable advice. My thanks extended to all lab people, Dr. Gamini Kannangara, Ms. Claudia Osorio, Dr. Patrick Schaefer, Ms. Elizabeth Kohl, Ms. Janet Clancy, Mr. Robert Brueggeman, and Dr. Andy Kleinhofs for their help and assistance. My special thanks go to Dr. Calvin Konzak for his encouragement and support on this study, and Dr. Enrique Polle for discussions.

Finally, I would like to thank my dear wife, Hong Liao, for her emotional support and belief in me. This dissertation is dedicated to my parent who has encouraged my professional career.

Transformation of microspores for generating doubled haploid transgenic wheat (*Triticum aestivum* L.)

Abstract

by Weiguo Liu, Ph.D.
Washington State University
December 2004

Chair: Diter von Wettstein

Microspores can form homozygous doubled haploids (DH) in one generation by androgenesis (microspore culture). The goal of this study was to develop a microspore transformation method for the production of transgenic wheat (*Triticum aestivum* L.).

In the first part of this study, optimal conditions for generating DH wheat plants from microspores were identified. First, the chemical inducer formulations effectively triggered microspore embryogenesis. Second, large populations of isolated embryogenic microspores were cultured to form embryoids and green plants at optimal conditions, that required purification of embryogenic microspores, a liquid culture medium with an osmolality around 300 mOsmol Kg^{-1} H_2O, and co-culture with ovaries. Third, conversion of albinos to green plants was obtained by nutrient addition during pretreatment. Fourth, spontaneous chromosome doubling was achieved *in vitro* by use of low toxic chemical caffeine.

In the second part of this study, microspores were transformed by co-cultivation with *Agrobacterium tumefaciens* strain AGL-1. Over 200 putative primary transformants were regenerated and 24 primary (T_0) spontaneously DH transgenic lines were obtained. Polymerase chain reaction (PCR), DNA sequencing of the amplificate, Southern blot analyses and assay of

the recombinant enzyme confirmed the presence of transgenes in T_0 primary transformants and their stable inheritance in homozygous T_1 DH progenies. Several factors for successful transformation were identified: (1) Co-cultivation with *Agrobacterium* for transfer of the plasmid T-DNA into microspores should take place at day 0 for < 24 hours. Volume of the inoculated AGL-1 cells at OD_{600}=1.0~1.5 had to be < 1% of the co-cultivation solution. (2) Killing of AGL-1 cells after co-cultivation was by filtration and addition of timentin at a concentration of 200-400 mg/L. (3) Selection of transformants should be carried out with bialaphos at a concentration of 2-4 mg/L. (4) Identification of transformants by PCR was carried out when regenerating seedlings were at 4-6 leaf stage.

This is the first report on successful transformation of microspores followed by regeneration of homozygous transgenic plants expressing a recombinant protein in wheat grains. The method described and conditions worked out in this study are likely to be applicable to other plant species.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

1.1 Aim of this study

The overall goal of this study was to develop a microspore transformation method for the production of transgenic wheat (*T. aestivum* L.). This required the development of a reliable procedure to generate doubled-haploid wheat plants from cultured microspores via androgenesis (also called pollen embryogenesis). In this process, pseudoembryos (embryoids) were formed from microspores, germinated and developed into mature plants that were homozygous for all genes. Once this goal was achieved, it was investigated if wheat microspores can be transformed by co-cultivation with *Agrobacterium* and the transformed microspores regenerated into doubled-haploid wheat plants.

1.2 Inconveniences with current wheat transformation methods

Wheat is an important crop worldwide. The United States is a major wheat producing country, and the world's leading wheat exporter. Wheat amounts to about 7.5 percent of all U.S. agricultural exports by value (USDA/ARS, 2003).

Production of transgenic wheat with desired traits such as disease and pest resistance is of great importance. Despite the advances in wheat transgenic research and its commercial application, the efficiency in obtaining transgenic wheat plants is still low (Vasil *et al.*, 1992; Cheng *et al.*, 1997). Most of the current methods for genetic transformation in wheat use immature embryos as the target for gene insertion, and plants are regenerated from the transformed cells. One of the major inconveniences in wheat transformation is the lack of an

efficient and reliable regeneration system once the target cells are transformed with cloned genes, thus requiring preparation of large amounts of embryos. Another inconvenience is that it may take several years for genetic analysis, and molecular characterization of the transgenic plants to obtain the desired homozygous plant in the desired genotypic background (Zhou *et al.*, 2003). It then takes several years to obtain genetically true-breeding lines of the transgenic plant suitable for commercial use. Therefore, novel transformation methods are desired to overcome the limitations of current techniques such as low efficiency, labor-intensiveness, and time-consuming breeding procedures.

1.3 Advantages of a microspore regeneration system as applied to wheat transformation

Microspores are haploid cells containing the gametic number of chromosomes. Microspores can be induced to form embryoids (pseudoembryos), which can develop into haploid plants. By spontaneous or chemical induced chromosome doubling with colchicine, herbicides or caffeine, homozygous diploid or amphidiploid plants are generated and the transgenic trait is stably inherited in the following generations. Thus microspores can form homozygous doubled haploids in one generation.

There are several advantages in a functional microspore transformation system over currently available systems:

- It provides a large number of genetically identical and physiologically uniform embryogenic microspores as target cells for transformation. Millions of microspores can be easily isolated with a blender in a few minutes. In contrast, it takes a large effort to prepare similar numbers of immature embryos as targets for genetic transformation. Thus, it would be economic and simpler to use microspores as targets for gene transfer.

- Transgenic microspores are capable of developing into plant embryoids, which are capable of germinating into homozygous doubled haploid transgenic plants in one generation. Traits are "fixed" in a homozygous plant, and recessive genes of interest can be expressed. In contrast, transgenic plants regenerated from transformed somatic cells (immature embryo, callus, protoplast, etc.) have the transgenes generally in a heterozygous state and homozygous transformants will have to be selected in the next generation. Thus, microspore transformation will produce homozygous transgenic plants in large numbers and in shorter time.
- Microspores are cultured at the uni-nucleate stage and are single cells. Foreign DNA may be incorporated into the microspore genome at the beginning of the culture. If the foreign DNA is incorporated into the genome before the first mitosis, the introduced gene(s) are likely to be passed into all daughter cells and chimeras will be avoided.

Despite these advantages, a successful wheat microspore-based transformation system has not been reported. In part this is due to the fact that a highly efficient and reproducible microspore regeneration system has only been worked out recently as a first part of this thesis.

CHAPTER TWO

LITERATURE REVIEW

2.1 Methods for generating doubled haploid wheat plants

Since the first discovery of haploid plants in *Datura stramonium* (Blakeslee *et al*., 1922), haploids were induced in 247 species of angiosperms in 88 genera of 34 families (Maheshwari *et al*., 1983). Haploids of higher plants are sporophytes with gametic chromosomes. Several methods of haploid production have been investigated and reported in the literature, including microspore and/or anther culture (androgenesis), as well as ovule culture (gynogenesis) (Hu, 1997). The importance of *Hordeum bulbosum* or maize (*Zea mays* L.) pollination methods (alien species chromosome elimination), and an alien cytoplasm system has been explored (Kasha and Kao, 1970; Barcley, 1975; Laurie and Bennett, 1988a and b; Dunwell, 1985; Kasha, 1989). Microspore and anther culture methods have the potential to produce more than a thousand haploid plants per cultured anther; all other methods are limited to one haploid plant per floret (Devaux, 1988). Thus androgenesis is a preferred method for generating doubled haploid plants.

In the alien species chromosome elimination method, crosses between *Hordeum vulgare* (female) *or T. aestivum* (female) and *H. bulbosum* were made, and the hybrid embryos were cultured to obtain haploid barley and wheat plants, respectively. The *H. bulbosum* chromosomes were preferentially eliminated during embryo development from a hybrid zygote (Kasha and Kao, 1970; Barcley, 1975). The *bulbosum* technique as originally developed for barley was widely used for wheat haploid production. Similarly, Laurie and Bennett (1988a,b) successfully obtained haploid wheat by carrying out wide hybridization between wheat (female) and maize and/or sorghum (male), and between barley and maize. Fertilization took place, but all maize

chromosomes were eliminated in the initial cell cycle (Wang *et al*., 1991). The maize technique is currently widely used for wheat haploid production. One of the problems in the crosses of wheat with maize and sorghum is that 3% and 10% florets were fertilized and set seeds, indicating the possibility that DNA fragments of maize or sorghum and maize transposon elements may be transferred into the wheat genome (Laurie and Bennett, 1988a).

In the anther or microspore culture method (androgenesis), microspores were induced *in vitro* to develop into mature plants by culturing anthers (anther culture) or isolated microspores (microspore culture). In the case of anther culture, plants may originate from anther somatic tissue other than microspores. Therefore microspore culture is the preferred method for haploid wheat production. Fujii (1970) initially reported the formation of calli by anther culture from *T. aegilopoides* and *T. dicoccoides,* but failed with *T. aestivum.* Pollen plants of wheat (*T. aestivum*) were first obtained by Ouyang and coworkers (1973) by anther culture with low efficiency of green plant regeneration (0.7%). Androgenesis induction in microspores can be limited by various factors and is genotype-dependent (Dunwell, 1985). Most efforts toward improving anther/microspore culture have focused on "stress" treatments to induce androgenesis by redirecting the preprogrammed gametophytic to the sporophytic development pathway (Touraev *et al.*, 1996, 1997; Hu and Kasha, 1999; Zhou and Konzak, 1997; Simonson *et al.*, 1997 and Reynolds, 1997). These early culture systems have been effective only for a narrow range of responsive genotypes, while many genotypes remained recalcitrant.

Low efficiency in doubled-haploid production via androgenesis in wheat has limited exploitation of this potentially powerful method for genetic engineering and crop improvement in this crop plant. Thus, improvement in induction efficiency, culture condition and green plant regeneration was needed to obtain effective protocols for the microspore culture of wheat.

2.2 Gene transfer techniques for wheat transformation

Techniques for incorporating foreign genes into plant cells have rapidly advanced in the past few decades. Genetic transformation of wheat has been obtained by co-cultivation with *A. tumefaciens* (Cheng *et al.*, 1997; Amoah *et al.*, 2001; Weir *et al.*, 2001), particle bombardment (Klein *et al.*, 1987; Vasil *et al.*, 1992 & 1993; Loeb and Reynolds, 1994; Takumi and Shimada, 1996; Mentewab *et al.*, 1999; Pastori *et al.*, 2001; Milligan *et al.*, 2001; Varshney and Altpeter, 2001; Chugh and Khurana, 2003), protoplast electroporation (Zhou *et al.*, 1993; Gustafson *et al.*, 1995; Sorokin *et al.*, 2000) and polyethylene glycol (PEG)-mediated DNA uptake into protoplasts (Marsan *et al.*, 1993).

The *A. tumefaciens* mediated gene transfer has offered promising features such as easy manipulation in vitro, introduction of low gene copy number into the plant genome, and high co-expression of the transgene (Songstad *et al.*, 1995; Cheng *et al.*, 1997; Koncz *et al.*, 1989). These advances have also been exploited in barley (Horvath *et al.*, 2000; Horvath *et al.* 2003). In the case of *Agrobacterium tumefaciens* mediated gene transfer, the bacteria must be killed after co-cultivation of the explants or their growth inhibited to allow formation of callus containing transgenes. Nauerby and others (1997) showed that timentin, a mixture of the penicillin derivative ticarcillin and clavulenic acid, which is a competitive inhibitor of the anti-antibiotic enzyme β-lactamase, was a more favorable bactericide than cefotaxime, and is more cost-effective as well as stable than vancomycin.

Several selectable marker and reporter genes have been successfully used for screening and identifying transgenic plants. The marker genes used in wheat include *uidA* encoding ß-glucuronidase (GUS expression) (Loeb and Reynolds, 1994; Gustafson *et al.*, 1995; Cheng *et al.*,

1997), and anthocyanin biosynthesis stimulating genes (C1 and B-Peru) (Mentewab *et al.*, 1999). The bioluminescent green fluorescent protein (GFP) that can be detected in living cells has been used as visual selection marker in tobacco (Molinier *et al.*, 2000) and wheat (Weir *et al.*, 2001). Widely used reporter genes in wheat include the neomycin phosphotransferase II (NPTII) gene conferring resistance to amino-glycoside antibiotics such as kanamycin (Cheng *et al.*, 1997), the *bar* gene encoding phosphinotricin acetyl transferase (PAT) that inactivates the natural herbicide bialaphos and the chemically synthetic phosphinotricin (PPT) (Altpeter *et al.*, 1996), and the phosphomannose isomerase gene (pmi) (Wright *et al.*, 2001).

Once the gene of interest is successfully introduced to the host plant genome, the transgene must be transcribed and translated. However, this is frequently not the case. Jorgensen and colleagues firstly observed a surprising phenomenon in petunias. They introduced a pigment-producing gene under the control of a powerful promoter in order to deepen the purple color of these flowers. Instead of the expected deep purple color, many of the flowers appeared variegated or even white. They named the observed phenomenon "cosuppression", because the expression of both the introduced gene and the homologous endogenous gene was suppressed (Jorgensen *et al*., 1996; Napoli *et al*., 1990). In addition to petunias, cosuppression has since been found to occur in many species of plants. It has also been observed in fungi, and has been particularly well characterized in *Neurospora crassa*, where it is known as "quelling" (Cogoni and Macino, 2000; Guru, 2000, and Hammond *et al*., 2001).

Two types of gene silencing have been observed. The first type is the transcriptional gene silencing (TGS), which is often associated with heavily methylated and inactive promoter sequences. It can be triggered by short doubled-stranded RNA synthesized in the cell nucleus. It leads to methylation of cytosine bases in the DNA of gene promoters and methylation blocks the

transcription of the gene (Mette *et al*., 1999; Mette *et al*., 2000; Meyer *et al*., 1993; Park *et al*., 1996, Bird, 1992). When a transgene integrates into a chromosomal region that is heavily methylated and or repetitive, it is silenced (Prols and Meyer, 1992). However, a transgene integrated into hypomethylated DNA can also be transcriptionally inactivated (Prols and Meyer, 1992; Meyer and Heidmann, 1994). This selective methylation of foreign DNA may be a cellular defense response against the potential harmful activity of 'foreign' DNA (Doerfler, 1995). Transgenes of T-DNAs that are inserted as a direct repeat or an inverted repeat have a tendency to become inactivated. This is frequently associated with DNA methylation. In general, the more copies a locus contain, the stronger the inactivation (Matzke *et al*., 1994). A transgene locus that is silenced can also silence homologous transgenes at ectopic loci (Matzke *et al*., 1994). Interestingly, methylation of the target genes is erased after crossing out the silencing locus. This does not happen immediately but occurs gradually in successive generations (Matzke and Matzke, 1991). Mette and coworkers (1999 and 2000) showed that introducing plasmids containing inverted repeats of promoter sequences into tobacco and Arabidopsis led to the synthesis of double stranded RNA molecules. These inhibit the transcription of adjacently fused antibiotic resistance genes. Furthermore the insertion and removal of the inverted repeats could be regulated by site-specific recombination employing *lox* DNA elements bracketing one of the repeats. These are recognized by the CRE nuclease, which upon recognition removes the DNA bracketed by the *lox* DNA sequences. Interestingly, gene activation and inactivation can be triggered by homologous pairing of allelic genes in the nuclei of somatic cells (Matzke *et al*., 2001). Jakowitsch and coworkers (1999) found that transgenes driven by the endogenous pararetrovirus (repetitive DNA sequences) enhancer are silenced and methylated but remain unmethylated in *Nicotiana* species that lack the retrovirus sequences in their genome. These

incomplete virus genomes can thus furnish virus resistance through homologous sequence interaction. This finding opens a new area for the basis of viral immunity. Aufsatz and coworkers (2002) studied relationships between RNA signals, DNA methylation and chromatin modifications by performing a genetic screen to recover Arabidopsis mutants defective in RNA-directed transcriptional silencing and methylation of a nopaline synthase promoter–neomycinphosphotransferase II (NOSpro– NPTII) target gene. Studying the methylation sites in the target DNA that have been de-methylated in the *Arabidopsis* mutant reveals that the mutation preferentially addresses the CNG methylation sites. CG methylation in centromeric region was not reduced in this mutant. The identified protein is a putative histone deacetylase indicating the structure of the chromatin to be important for specificity of cytosine methylation (Aufsatz *et al.*, 2002). Using the promoter with inverted repeats as target, mutations identified the gene DRD1. It preferentially causes methylation at CNG sites and target genes in the euchromatin rather than DNA in the centromeric heterochromatin. It is considered to be a protein involved in chromatin reorganization (Kanno *et al.,* 2004).

The second type is the post-transcriptional gene silencing (PTGS), which occurs when the promoter is active but the mRNAs fail to accumulate. Experiments show that the homologous transcript is made, but that it is rapidly degraded in the cytoplasm and does not accumulate (Cogoni and Macino, 2000; Hammond *et al*., 2001; Ingelbrecht *et al*., 1994). It has now become clear that PTGS occurs in both plants and animals and has roles in viral defense and transposon silencing mechanisms. The transgene-induced PTGS mechanism affects expression of the transgenes and of endogenous genes with which they share a considerable degree of sequence identity. Using homologous sense transgenes, the expression of many endogenous genes has been suppressed. PTGS is mediated by a diffusible, trans-acting molecule. Palauqui and

colleagues (1998) induced PTGS in a host plant by grafting a silenced, transgene-containing source plant to an unsilenced tobacco host. From work done in nematodes and flies, it is now clear that the trans-acting factor responsible for PTGS in plants is double-stranded RNA (dsRNA) (Cogoni and Macino, 2000; Guru, 2000; Hammond *et al*., 2001; Guo and Kempheus, 1995; Fire *et al*., 1998). How does dsRNA lead to gene silencing? Hamilton and Baulcombe (1999) discovered the presence of small RNAs of ~25 nucleotides in plants undergoing cosuppression that were absent in non-silenced plants. These RNAs were complementary to both the sense and antisense strands of the gene being silenced. PTGS through RNA interference (RNAi) is initiated by the introduction of double-stranded RNA (dsRNA). Both biochemical and genetic approaches have led to the current models of the RNAi mechanism. In these models, RNAi includes both initiation and effector steps (Hammond *et al*., 2001; Hutvagner and Zamore, 2002; Cerutti, 2003). In the initiation step, perfect and extensive double-stranded RNA (dsRNA) structures, induced by virus or transgene, are processed by Dicer-like proteins (DCLs) into double-stranded short interfering RNAs (siRNAs). In the effector step, siRNAs are recruited into the RNA-induced silencing complex (RISC) that regulates the endonucleolytic cleavage of target mRNAs by the perfect or near perfect base pairing between siRNAs and the targeted sequences. The emerging use of PTGS and, in particular, RNA interference as a functional genomics tool to knock out expression of specific genes in a variety of organisms becomes exciting. This is based on the fact that RNAi has the ability to quickly and easily create loss-of-function phenotypes.

More recently, Song and coworkers (2004) identified the crystal structure of argonaute proteins suggesting a mechanism for siRNA-guided mRNA cleavage, and Liu and coworkers (2004) further identified Argonaute2 being responsible for messenger RNA cleavage activity.

These experimental evidences support a model in which Argonaute contributes "Slicer" activity to the RNA-induced silencing complex (RISC), providing the catalytic engine for RNAi.

Recent work in wheat reveals an overall similarity between the plant and animal RNA-silencing pathways, as well as some intriguing plant-specific aspects (Tang *et al*., 2003; Voinnet, 2003). Tang and coworkers (2003) found that standard wheat germ extract contains Dicer-like enzymes that convert double-stranded RNA (dsRNA) into two classes of small interfering RNAs, as well as an RNA-dependent RNA polymerase activity that can convert exogenous single-stranded RNA into dsRNA. Demeke and coworkers (1999) observed low GUS and NPTII enzyme activities in transgenic wheat seeds, even though Southern blot and PCR analyses indicated the presence of an intact transgene expression cassette. The transgene of these wheat plants was methylated based on Southern blot analysis of genomic DNA restricted with methylation-sensitive enzymes. Northern blot analysis revealed that the plants with the methylated transgene did not accumulate the uidA-nptII fusion gene transcript. Alvarez and coworkers (2000) observed that when wheat HMW glutenin subunit genes 1Ax1 and 1Dx5 were introduced into a commercial wheat cultivar that already expresses five subunits, silencing of all the HMW glutenin subunits was observed in two different events. Interestingly, the silencing of all HMW glutenin subunits was reverted and the revertant T2 seeds expressed the five endogenous subunits plus the 1Ax1 transgene, which was found to be associated with a reduction of transgenes from a high to a low copy number. The expression of 1Ax1 and 1Dx5 transgenes, associated with silencing of all the endogenous high-molecular-weight glutenin subunits, resulted in flours requiring lower mixing time, optimal stretch resistance and sedimentation volumes, and a lower gluten strength (Alvarez et al., 2001).

2.3 Efforts in transformation of microspores in various plant species

Several researchers recognized the great advantage that a functional microspore transformation system would offer, and tried to transform microspores in several species such as barley (Jahne *et al.*, 1994; Yao, *et al.* 1997), tobacco (Stöger *et al.*, 1995), and oil seed rape (Fukuoka *et al.*, 1998; Dormann *et al.* 1995, 1998) by using micro-projectile bombardment and *A. tumefaciens*-mediated methods, demonstrating stable integration of DNA.

Mentewab *et al.* (1999) and Folling and Olesen (2001) used micro-projectile bombardment to transform haploid embryos and isolated microspores in wheat, and observed transient expression of marker genes, but transgenic wheat plants were not obtained, probably because of the inefficient microspore regeneration in the experiments. Less than 100 embryoids were produced from microspores of several spikes in the controls. The efficiency is likely to be further reduced with the introduction of genes by micro-projectile bombardment since microspores are very sensitive to physical damage (Yao, *et al.* 1997).

Fukuoka and others (1998) obtained successful rapeseed microspore transformation demonstrating stable integration of the marker gene (firefly luciferase) in T_0 and T_1 plants by PCR and Southern analyses. A highly efficient microspore culture method was used and modified in which over 10, 000 embryoids were obtained from one million bombarded microspores. Four embryoids were identified to be transgenic from 5 experiments in this study.

Dormann and coworkers (1995, 1998) reported successful *A. tumefaciens*-mediated rapeseed microspore transformation with molecular evidence (PCR and Southern analyses). A single copy of the PAT gene was integrated into the plant genome. An efficient microspore culture method was used, and microspores were transformed by co-cultivation with bacteria for 2 to 3 days. The bacteria were washed from the culture plate and their growth was further inhibited

by 200 mg/L timentin. fourty embryoids were recovered from one million co-cultivated microspores, and upon germination of the embryoids, two plantlets survived a rooting assay for the herbicide glufosinate ammonium (phosphinothricin, PPT) test.

Similarly, the success in microspore transformation in barley (Jahne *et al.*, 1994; Yao, *et al.* 1997) and in tobacco (Stöger *et al.*, 1995) depended upon effective microspore regeneration methods. Thus, a highly efficient microspore culture system in wheat was required as a basis for transformation experiments. Advances in research on androgenesis in many crop species over the past few decades yielded information on critical factors affecting androgenesis. Successful androgenesis required two events:

Step 1: induction of androgenesis. In this process, microspores become embryogenic by changing from the programmed gametophytic pathway (pollen development) to sporophytic pathway (embryo development).

Step 2: embryoid development and plant regeneration. In this process, embryogenic microspores develop into mature embryoids and plants.

For induction of androgenesis, stress treatments (nutrient starvation, high or low temperature) were widely used (Kyo and Harada, 1985; Touraev et al., 1996, 1997; Hu and Kasha, 1999), and responses were dependent on environmental and genetic factors (Anderson *et al*., 1987; Tuvesson *et al*., 1989; Zhou and Konzak, 1997; Simonson et al., 1997 and Reynolds, 1997). A positive relationship between androgenesis and certain chemical treatments was observed (Bennett and Hughes, 1972; Rowell and Miller, 1971; Picard et al., 1987), and there seemed a positive correlation between male sterility and microspore culture response. So it was thus necessary to identify the chemical formulations that could induce a large proportion of microspores to become embryogenic. It was further investigated if the effects of stress and

chemical treatments were additive, to allow the combination of both treatments to achieve high efficiency of androgenesis induction.

It was recognized that after embryogenesis was induced, the induced microspores required an optimal physiological environment to develop further into embryoids able to germinate and develop into green plants. Beneficial effects of ovaries as nurse factors for formation of good quality embryoids were observed (Kohler and Wenzel, 1985; Bruins et al. 1996; Puolimatka et al. 1996; Hu and Kasha 1997). Different media were used for microspore culture in different crops and in the same crop. Most media seemed to contain adequate nutrients to feed developing embryoids. The physical constraints of osmolality therefore became critical for the development of embryoids. Medium osmolality for microspore culture of wheat or other crop species needed optimization.

Plant regeneration and albinism was another problem in anther and microspore culture. Genotypic differences in androgenesis induction did exist (Anderson et al. 1987; Tuvesson et al. 1989). Some genotypes naturally reacted better than others in response to environmental changes, such as stress treatments applied to microspores. To overcome such differences, microspores needed a better environment so that the physiological limitations could be partially compensated for with readily available nutrient resources. It was conceivable that genotypes with reduced plant regeneration and a high frequency of albino plant development were less competent in their efficiency to utilize the nutrients in tissues surrounding the microspores during the pretreatment of whole spikes. If this was the case, then to reduce the number of regenerated albino plants, an adequate supply of nutrients must be readily available to microspores during this pretreatment or "starvation" period. In other words, microspores should

not be “starved” severely. It would be possible to test conditions to improve plant regeneration efficiency and to increase the number of green plants.

It was the goal of this study to determine the conditions for successful microspore transformation and regeneration of transgenic wheat plants from the transformed microspores. Optimal nutrient and chemical inducer formulations were first developed for high androgenic embryoid yields and quality in a number of wheat varieties. Further improvement of androgenesis could be achieved by optimizing the culture conditions, including co-cultivation with ovaries in liquid medium with favorable osmolality. Summary data of these researches have been published in Liu *et al*. 2002a, b and Zheng *et al.* 2001. In the second part of the present work, experiments were carried out to determine the optimal co-cultivation time and concentrations of *A. tumefaciens* and microspores. Furthermore the optimal time of adding the bacteria during embryoid induction culture was worked out.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Generation of doubled haploid wheat plants

3.1.1 Growing wheat plants and selecting microspore-containing tillers

One to three plants per pot (20 by 25 cm) were grown in a greenhouse controlled at $27^{o}C\pm 2^{o}C$(day) and $17\pm2^{0}C$(night), and a light regime of 17 hours light and 7 hours dark. Winter wheat seedlings were artificially vernalized at $6^{o}C$ for two to two and half months before growing in the greenhouse. Further application of fertilizer was achieved by daily watering with nutrient solution containing 200 to 250 ppm of water-soluble fertilizer of nitrogen (N), phosphorus (P_2O_5) and potassium (K_2O), 20% each. Quality donor plants were selected for microspore culture.

Fresh tillers containing microspores enclosed within the anthers in the middle section of a spike at the mid to late-uninucleate stage of development were cut below the second node, counting from the top of the tiller, and immediately placed in a clean container with distilled water. All leaves were removed by cutting at their bases. Morphological features of tillers containing microspores at these stages were established for each cultivar via microscopic examination of microspores in an anther sample with acetocarmine stain.

3.1.2 Treatment of microspores with chemical inducers and chromosome doubling agent

The collected tillers were placed in an autoclaved sterile flask, containing 50 ml of one of the following sterile (autoclaved) solutions:

A. Distilled water (the control treatment for inducer formulations);

B. Inducer formulations (0 to 1 g L^{-1} 2-hydroxynicotinic acid (2-HNA) (Sigma-Aldrich Co, St. Louis, USA), with or without 10^{-6} mol L^{-1} 2,4-dichloro-phenoxyacetic acid

(2,4-D), and 10^{-6} mol L^{-1} 6-benzylaminopurine (BAP) aimed at inducing microspore embryogenesis;

C. Inducer formulation (0.1 g L^{-1} 2-HNA, 10^{-6} mol L^{-1} 2,4-D, and 10^{-6} mol L^{-1} BAP), plus chromosome doubling agent (0 to 1 g L^{-1} caffeine) aimed at conversion of haploid to doubled haploid (DH) *in vitro*;

D. Inducer formulation (0.1 g L^{-1} 2-HNA, 10 mg L^{-1} 2,4-D and 2 mg L^{-1} BAP, 3 mg L^{-1} giberellic acid), with or without 10% nutrient medium aimed at albino-to-green plant conversion *in vitro*. The full-strength nutrient medium contains 232 mg L^{-1} $(NH_4)_2SO_4$, 1415 mg L^{-1} KNO_3, 83 mg L^{-1} $CaCl_2$-$2H_2O$, 200 mg L^{-1} KH_2PO_4, 93 mg L^{-1} $MgSO_4$-$7H_2O$, 5 mg L^{-1} H_3BO_3, 0.0125 mg L^{-1} CoCl-$6H_2O$, 0.0125 mg L^{-1} $CuSO_4$-$5H_2O$, 0.4 mg L^{-1} KI, 5 mg L^{-1} $MnSO_4$-$4H_2O$, 0.0125 mg L^{-1} Na_2MoO_4-$2H_2O$, 5 mg L^{-1} $ZnSO_4$-$7H_2O$, 37.3 mg L^{-1} Na_2EDTA, 27.8 mg L^{-1} $FeSO_4$-$7H_2O$, 50 mg L^{-1} Myo-inositol, 0.5 mg L^{-1} Nicotinic acid, 0.5 mg L^{-1} Pyridoxine-HCl, 5 mg L^{-1} Thiamine-HCl, 500 mg L^{-1} Glutamine, 1 mg L^{-1} Phenylacetic acid (PAA), 90 g L^{-1} Maltose, adjusted to pH7.0 and filter-sterilized.

The open end of a plastic bag was placed over the spikes, wrapped around the neck of the flask and sealed around the flask with masking tape to limit microbial contamination and excessive loss of water. The flask was placed in an incubator at 33°C for a desired period of time, ranging between about 48 hours to 72 hours depending on the genotypes, until microspores enclosed within the anthers from the center section of a spike showed a characteristic embryogenic structure, i.e. the fibrillar-appearing cytoplasm of induced microspores: the embryogenic microspores typically had eight or more small vacuoles immediately adjacent to the cell wall. These vacuoles surrounded the condensed cytoplasm in the center, forming a fibrillar

structure that can be observed under phase contrast in a Zeiss inverted microscope (Axiovert 25) by squashing an anther from the middle section of a treated spike.

Alternatively, a second protocol was used to evaluate chemicals in inducing androgenesis. In this protocol, microspores were isolated right after the spikes were sampled from greenhouse and no pretreatment was conducted. After the isolation of microspores as described in Chapter 3.1.3, these freshly isolated microspores were suspended in a liquid solution A containing chemical formulations, as a pretreatment for inducing androgenesis. Solution A contained in mgL^{-1}: KCl, 1,492; $MgSO_4.7H_2O$, 246; $CaCl_2.2H_2O$, 148; KH_2PO_4, 136; H_3BO_3, 3; KI, 0.5; MnSO4.4H2O, 8.0; $ZnSO_4.7H_2O$, 3.0; $FeSO_4.7H_2O$, 56.0; maltose, 9,000; 2-HNA, 50; Kinetin, 0.2; it was autoclaved without adjusting the pH.

After microspores were treated in solution A for about 48 hours (ranging between 40 and 70 hours or until formation of a characteristic fibrillar structure), microspores were transferred to a new Petri dish containing liquid embryoid induction medium, which contained full-strength nutrient medium, plus 0.2 mg L^{-1} 2,4-D, 0.2 mg L^{-1} Kinetin, adjusted to pH7 and filter-sterilized, and cultured as described in Chapter 3.1.4.

3.1.3 Microspore isolation and purification

After the tillers were pretreated, they were removed from the treatment flask in a laminar flow hood. All foliage beneath the first tiller node was removed, keeping only the boot containing the spike. Isolated boots were then placed on a paper towel and sprayed with 75% ethanol to saturation. The boots were wrapped in the towel and placed in the hood for approximately 45 min, or until the ethanol had fully evaporated. Alternatively, isolated boots were disinfected by immersing them in 20% of a commercial chlorine bleach solution (which contains 6% sodium hypochlorite, 1.2% final concentration) in a cylinder for 20 minutes,

followed by rinsing with distilled water 2 times. The spikes were aseptically removed from each disinfected boot and placed on top of a 125 mL Waring MC II blender cup. Awns (if present), and the upper spikelets were removed, using sterile forceps and scissors. Florets were cut from their bases and allowed to drop into the open blender-cup. Florets obtained from one to three spikes were used for each run of the blending process. Forty mL of a 0.3 mol L^{-1} mannitol solution (autoclaved) was added to the blender-cup, and a sterilized cap was placed on the blender-cup, which was assembled on the blender. The florets were blended for 20 s at 2200 rpm to release most microspores. The blended slurry was poured from the blender-cup into a sterile filter (a container with 100 µm stainless steel mesh at the bottom), and the blender-top was rinsed twice with 5 mL of a 0.3 mol L^{-1} mannitol solution per rinse, and the mannitol solution was poured into the filter. Residue trapped on top of the filter was discarded, and the filtrate was pipetted into 15 mL sterile centrifuge tubes and centrifuged at 100 x g for 3 min. The supernatant was discarded from the tubes, and the pellets were combined and re-suspended in 2 mL of 0.3 mol L^{-1} mannitol solution. The re-suspended pellets were layered over 5 mL of a 0.58 mol L^{-1} maltose solution (sterile) and centrifuged at 100 x g for 3 minutes. A band formed at the interface. Three mL of the upper band (containing microspores) was collected and resuspended in 10 mL of a 0.3 mol L^{-1} mannitol solution in a 15 mL centrifuge tube. The lower band (pellet) was resuspended (for counting purposes) in 12 mL water in a separate 15 mL centrifuge tube. Both centrifuge tubes were centrifuged at 100 x g for 3 min. The supernatant was discarded and the pellet resuspended in 3 mL culture medium for upper band microspores, or 3 mL water for lower band microspores. The number of microspores in each band was counted with a haemocytometer, and after counting the lower band microspores were discarded. The total of microspores isolated was the sum of the microspores from both the upper band and the lower

band. Only the microspores from the upper band were used for culture. The lower band microspores appeared to be those that were too young, or too old and containing starch, thus they had not developed sufficiently or had developed beyond the stage of development useful for DH production. The upper band microspores were resuspended in 10 mL of culture medium in a 15 mL centrifuge tube and centrifuged at 100 x g for 3 min. The supernatant was discarded and the pellet resuspended in induction medium.

3.1.4 Co-cultivation of microspores with live ovaries in liquid medium

Isolated microspores were cultured at a concentration of approximately 1 x 10^4 microspores mL^{-1} as a suspension in liquid embryoid induction medium, which contained full-strength nutrient medium (as described in 3.1.2), plus 0.2 mg L^{-1} 2,4-D, 0.2 mg L^{-1} Kinetin, adjusted to pH7 and filter-sterilized. An aliquot of 2 mL media per 35 mm x 10 mm Petri dish, or 5 mL media per 60 mm x 15 mm Petri dish, at a density of approximately 1 x 10^4 microspores mL^{-1} was used. For optimization of osmotic pressure in the induction media for androgenesis, osmolality was adjusted by changing concentrations of maltose and mannitol in induction medium. Osmolality of each medium was measured by Osmette S Model #4002 (Precision Systems, Inc., 16 Tech Circle, Natick, MA 01760, USA).

Immature ovaries were added to the culture at a density of one mL^{-1} medium, immediately preceding the incubation. Ovaries were aseptically dissected from fresh and disinfected spikes. The ovaries from the cultivar Chris (awnless, spring wheat) were used as convenient sources for supporting embryogenesis of the wheat lines tested. The Petri dish was sealed with Parafilm^{TM}, and incubated in the dark at 27^{o}C.

For testing the effects of ovary source and co-culture methods on androgenesis, ovaries were either freshly isolated right before co-culture from freshly harvested spikes, or extracted

from fresh ovaries of the three genotypes by grinding them in liquid nitrogen and filter-sterilized with 0.22 um filter (Minipore, INC).

3.1.5 Production of microspore-derived embryoids and doubled haploid plants

It took about one month to harvest the first group of mature embryoids. Multi-cellular proembryoids, still enclosed within the microspore wall or exine, were formed in approximately one week after microspores were cultured in liquid embryoid induction medium. In approximately one more week, the exine wall ruptured and immature embryoids emerged, which grew into mature embryoids after another 10 to 14 days. After embryoids had grown to 1 to 2 mm in diameter, they were transferred aseptically to autoclaved solid regeneration/germination 190-2 medium (Zhuang and Xu, 1983) at a density of 25 – 30 embryoids in each 100 x 15 mm Petri dish for germinating into plants. The embryoids were incubated under continuous fluorescent light at room temperature (22 $\pm 3^{o}$C) with 150–180 µmol m^{-2} s^{-1} of illumination. In approximately two weeks, green plants developed and were subsequently transferred to soil and grown to maturity in the greenhouse. To do so, the plantlets in the Petri dish were washed with running water to wash away phytagel and medium. Small 2 x 2 x 2" plastic trays were filled with soil that was premixed with fertilizers (N, P, K). Plantlets were planted in the small plastic trays. The small plastic trays were placed in a 20 x 12 x 2” plastic tray. During the first week after transplanting, a transparent plastic cover tray (propagation dome) was placed over the pots in the tray to maintain the high relative humidity for the plants while light still penetrated the cover. Plants were watered every 2 days or whenever the soil appeared to be dry. The plastic cover tray was lifted gradually so that plants became acclimated to the greenhouse conditions. In about 2 to 3 weeks, the plants grew vigorously. The plastic cover was removed, and the plants were transplanted to larger, 20 x 25 cm pots for doubled haploid production. Green plants were

raised in the greenhouse, much like plants grown from seeds. Stomata size on the 3rd leaf was a relatively good indicator of ploidy. If plants appeared to be haploid, colchicine or caffeine was applied to induce chromosome doubling (Thomas et al., 1997). Briefly, the haploid seedling crowns were immersed in a 3% caffeine solution for 24 hours, followed by rinsing the treated seedlings in running water for 6 hours. Seeds produced on any plants were instantly homozygous.

To avoid bias, the first available 200 embryoids from each Petri dish were transferred in order to evaluate the plant germination rate and doubled haploid percentage. Green and albino plants with well-developed roots and shoots were counted at 14 days after embryoids were transferred to germination culture media. Plant fertility was evaluated on the basis of seed set. More than 20 plants per replication were evaluated for seed fertility.

3.2 Transformation of microspores and regeneration of homozygous transformants

3.2.1 Wheat genotypes

Spring wheat genotypes “Chris”, “Pavon 76”, “WED 202-16-2”, “NPBCT” and “Bobwhite” were used. These genotypes are either highly culture-responsive (Liu *et al.*, 2002) or transformable by particle bombardment (Pellegrineshi *et al.*, 2003).

3.2.2 Plasmids

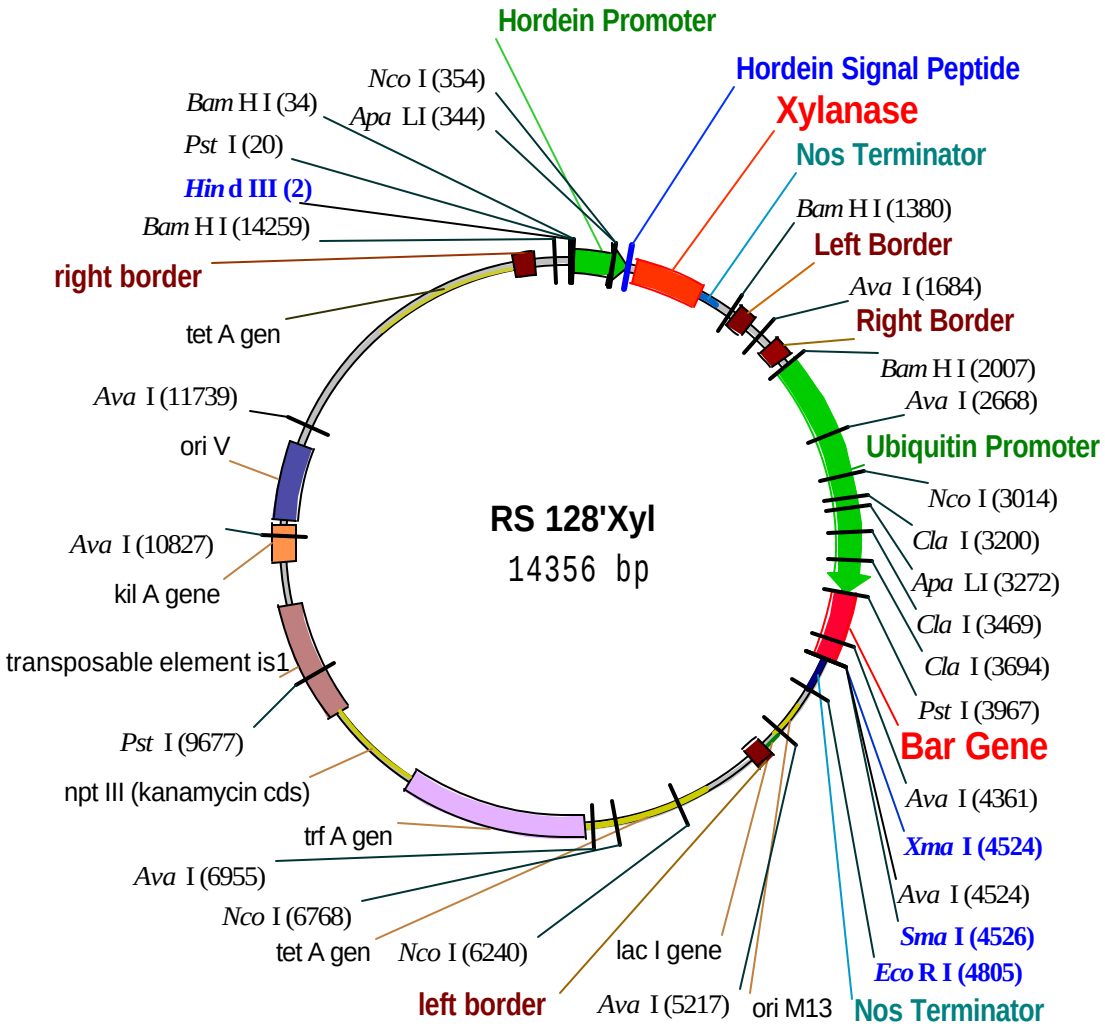
The primary goal of this study was to test the hypothesis that microspores can be transformed. The choices of genes to be used in this study were less important. In order to avoid time-consuming constructions of new plasmids, a plasmid containing transgenes that were expected to express in wheat were selected from the collection of von Wettstein’s lab at WSU. Plasmid RS 128/Xyl (Fig.1) had been used for production of barley transformants (Kohl, 2003) and was used in this study for transformation of wheat microspores.

Plasmid RS 128/Xyl (Fig. 3.2.2) is a double cassette vector containing the *bar* selection marker between one set of T-DNA left and right borders and the codon-optimized target gene for 1,4-ß-xylanase between a second set of T-DNA borders. The target gene was driven by the hordein D gene promoter and was supplied with the code for the signal peptide launching the newly synthesized enzyme precursor into the pathway for endosperm protein storage (Horvath *et al.*, 2000; Horvath *et al.* 2002; Jensen *et al.* 1998; Stahl *et al.*, 2002). The two cassettes were frequently incorporated into different chromosomes or chromosome arms and therefore provided the opportunity for selecting herbicide marker-free transformants (Stahl *et al.*, 2002; Van Fleet, 2001). Wheat transformants expressing the enzyme 1,4-ß-xylanase may be of some practical interest. This enzyme depolymerizes the major endosperm cell wall component of wheat grain, namely the arabinoxylans or pentosans (chains of (1? 4)-ß-D-xylose molecules with a-L-arabinose side chains attached to the xylose by (1→2) and/or (1? 3) linkages). These have been identified as major antinutritive components in mature barley and wheat grains. In a trial with 144 starter pigs (10kg), pigs fed with arabinoxylan-enriched fractions of barley ate less and gained less weight than pigs fed with whole kernel diet. Xylanase addition could counteract the negative effects (Ankrah, 1999). This corroborated the antinutritive effects established for wheat pentosans in broiler chicken, piglets and lactating dairy cows and the possibility for correction by ß-xylanase enzyme treatments (Choct and Annison, 1990; Schingoethe *et al.*, 1999; Rode *et al.*, 1999).

3.2.3 Binary vector construction

Binary vectors were used for microspore transformation experiments. The binary vector consists of a disarmed Ti plasmid with virulent genes for mobilization of the T-DNA, and a plasmid carrying the target transgenes between the left and the right T-DNA borders. *A.*

Figure 3.2.2 Plasmid RS 128/Xyl. It is a double cassette vector containing the *bar* gene (position 3968-4522) driven by the ubiquitin promoter (position 2012-3967) between one set of T-DNA left and right borders, and the codon-optimized target gene for 1,4-ß-xylanase (position 536-1093) driven by the hordein D gene promoter (position 39-472) and supplied with the code for the signal peptide (position 473-535) between a second set of T-DNA borders.



tumefaciens strain AGL-1 carries a disarmed Ti plasmid, which is derived from the hypervirulent, attenuated tumor-inducing plasmid pTiBo542 by precise excision of the T-region (Lazo *et al.*, 1991). It also has an insertion in its *recA* gene that stabilizes the recombinant plasmid and renders the strain resistant to carbenicillin. Plasmid DNA was purified with QiaexII kit from Qiagen, Valencia, CA by following the manufacturer's protocol. AGL-1 cells were transformed with the vector RS 128/Xyl by electroporation as described in a published protocol (Mersereau *et al.*, 1990) and modified as the follows:

(1) Preparation of AGL-1 competent cells: AGL-1 cells were plated out from the glycerol stock on LB plates, and grown at room temperature for 1-2 days or until single colonies appeared. A 5 ml LB culture was inoculated from a single colony on the plate, and grown overnight on shaker with 250 rpm at room temperature. 1 L LB culture was then inoculated with 1 ml of overnight liquid culture, and grown overnight on shaker with 250 rpm at room temperature until A_{600}=1.5~2.0. The cells were harvested by centrifugation at 4500 X g for 5 minutes, and resuspended in 15 ml of cold sterile distilled H_2O. The cells were again harvested by centrifugation at 4500 X g for 5 minutes, and this process was repeated three times. The clean cells were resuspended in 5 ml of cold sterile distilled H_2O with 10% glycerol. The competent cells were stored in 150 µl aliquots in 1.5 ml Eppendorf tubes at –70 ^{0}C after quick-freezing in liquid nitrogen. The cells were used for transformation with the vector.

(2) Transformation of competent AGL-1 cells by electroporation: Cells in glycerol stock in the freezer were thawed on ice, and 40 µl of cells were dispensed into a 0.2 gap cuvette which was pre-chilled on ice. 1 µl of the vector DNA in H_2O (=100 ng) was added into the gap cuvette, and mixed with the cells by tapping so that the cell-DNA mixture was settled down at the bottom of the gap cuvette without any trapped air bubbles. The cold and dry gap cuvette was placed in

the holder of the BIO-RAD Gene Pulser II, and electroporated with the setting of 1.25 kv/cm, 25 µF and 200 µ. 1 ml of LB medium was added into the cuvette immediately after the electroporation. The mixture was then transferred to an Eppendorf tube and incubated at room temperature on a shaker with 200 rpm for 1 hour. 100 µl of the cells were plated out on LB medium containing the antibiotic kanamycin at a concentration of 50 mg/L. The rest of the cells were centrifuged at 13, 000 rpm for 30 seconds. The supernatant was removed, and the remaining cells were plated out on LB medium containing the antibiotics kanamycin. The plates were incubated at room temperature, and colonies appeared in 2 to 3 days, indicating successful transformation of AGL-1 cells with the vector RS 128/Xyl. The plasmid DNA was isolated and purified with a Quantum Prep® Plasmid Miniprep Kit (Catalog number 732-6100, BIO-RAD Laboratories, USA) by following the manufacturer's protocol. The purified plasmid DNA in 100 µl of sterile distilled H_2O was stored at –18 ^{0}C for further use, and was used as a DNA template for PCR analysis to confirm the presence of the vector in the AGL-1 cells. A positive single colony was plated out by stripping on new LB plate with kanamycin (50 mg/L), and incubated at room temperature. It was re-plated out by stripping to new plate every 2 weeks for a period of 2 months. Fresh AGL-1 cells from this plate were used for *Agrobacterium tumefaciens* culture and preparation as described in Chapter 3.2.4 for transformation of microspores.

Alternatively, these freshly transformed AGL-1 cells were used to make glycerol stocks. To do so, the freshly transformed AGL-1 cells were inoculated into 25 ml of LB with kanamycin (50 mg/L) in an autoclaved flask, and incubated on shaker with 200 rpm at room temperature overnight or until media became non-transparent (OD_{600}=1.0 – 1.5). 50% of autoclaved glycerol in H_2O was added to the flask, and mixed well. 250 µl of the medium containing AGL-1 in LB was added to each autoclaved Eppendorf tube (1.5 ml), and stored at –18 ^{0}C.

3.2.4 *Agrobacterium tumefaciens* culture and preparation

The fresh AGL-1 cells containing the binary vector were cultured in 5 ml of LB plus kanamycin (50 mg/L) on a shaker with 200 rpm at 20 ^{0}C for 48 h or until OD_{600}=1.0 – 1.5. For the glycerol stock of AGL-1 cells, each tube with 250 µl of the medium containing AGL-1 in LB was used for inoculation of 5 ml LB without kanamycin on a shaker with 200 rpm at 20 ^{0}C for 48 h or until OD_{600}=1.0 – 1.5.

The AGL-1 cells were centrifuged at 2500 rpm for 2 min and the supernatant was removed and the pellet was resuspended in 2 ml of microspore culture liquid embryoid induction medium, and was then used in co-cultivation experiments.

3.2.5 Microspore preparation and transformation

The method for obtaining microspores as described in Chapter 3.1.1 to 3.1.3 was used. The procedure was modified to be suitable for transformation. After microspores were isolated and suspended in 5 ml embryoid induction medium in a 60 x 15 mm plate, co-cultivation of microspores with AGL-1 cells was performed by adding AGL-1 cells to the plate, which was prepared as described in Chapter 3.1.4. The plate was sealed with Para film and was incubated at 25^{0}C.

Transformation of microspores by co-cultivation of embryoid-forming microspores with *A. tumefaciens* containing the plasmid RS 128/Xyl was carried out for 1 to 48 h with various concentrations of *A. tumefaciens* in the culture media during embryoid induction culture at days 0, 1, 3, 5, 7, 14, 21, and 30.

3.2.6 Methods for elimination of *A. tumefaciens* post co-cultivation

Filtration and rinsing plus the use of antibiotic timentin in the induction medium were tested for elimination or growth inhibition of *A. tumefaciens* after a desired period of time of co-

cultivation with microspores. *A. tumefaciens* cells were smaller than microspores (<5 vs. 40-50 µm). A filter with the desired mesh pore size (38 µm) was constructed. *A. tumefaciens* cells were filtered out by pouring liquid co-cultivation medium through the filter and by rinsing the filter. All microspores, including transgenic microspores, were collected and re-cultured in the embryoid induction medium for embryoid production. Timentin was added to the liquid culture medium to kill the escaped *A. tumefaciens* or inhibit their growth. The strategy was to use filtration and rinsing to eliminate most *A. tumefaciens* cells, followed by the use of antibiotic to kill or inhibit the growth of residual *A. tumefaciens*. Optimal timentin concentration at a range of 0 to 800 mg/L was determined by experimentation.

Five fresh ovaries were then added to the plate. The plate was incubated in dark at 25 ^{0}C for embryoid production.

3.2.7 Plant regeneration from transgenic microspores & selection for transformants

The embryogenic microspores cultured in the liquid embryoid induction medium began to form mature embryoids in 4 to 6 weeks. After embryoids grew to 1 to 2 mm in diameter, they were transferred aseptically to solid bialaphos-containing 190-2 medium in 100 x 15 mm Petri dish for plant regeneration. The embryoids were incubated under continuous fluorescent light at room temperature (22 ^{o}C). In approximately two weeks, green plants developed and were transferred subsequently to soil and grown to maturity in the greenhouse as described in Chapter 3.1.5.

Selection for putative transformants was carried out at the embryoid germinating stage. Bialaphos was used in the regeneration media to inhibit embryoid germination and growth of non-transformants while putative transgenic embryoids could germinate and grow because transformants carried the BAR gene conferring resistance to bialaphos. The bialaphos

concentration from 0 to 5 mg/L was tested, and optimal concentrations were determined. Bialaphos was added to the 190-2 medium after autoclaving the medium.

3.2.8 Identification of transgenic plants with introduced genes

PCR techniques were used for initial screening and identification of putative transformants. Specific primer sets were designed for identification of each transgene (BAR, Xylanase). The amplified single band unique for xylanase was sequenced to determine if this unique band was faithfully amplified from DNA template of the xylanase gene. Southern blot hybridization analysis was performed for T_1 progenies (Kleinhofs *et al*., 1993; Horvath *et al*., 2002) to determine presence of the introduced gene (xylanase) and the gene copy numbers in transgenic plants. The enzymatic assay for xylanase and a rapid method to qualitatively determine the xylanolytic activity were developed previously (Sa-Pereira *et al*., 2002), and were modified to identify transgenic wheat grains expressing (1,4)-β-xylanase.

3.2.8.1 PCR and Reverse Transcription PCR analysis

Polymerase chain reaction (PCR) was performed with plant genomic DNA as a template, which was extracted from young leaves of four week old T_0 and T_1 plants according to the quick extraction protocol of Horvath *et al*. (2002) after modification as described in Appendix A.

The primer sets used for PCR are listed in Table 1. Primer set Bar5' and Bar3' amplified 373 bp of fragments (position 56 from 5' end of bar gene and position 428 at the 3' end of bar gene). Primer set Hor5' and Liuxyldown amplified 837 bp of fragments (position 39 from 5' end of hordein D gene promoter and position 875 at the 3' end of xylanase gene).

For PCR analysis of the xylanase, PCRs were carried out in a total volume of 25 µl reaction mixture, consisting of 1 µl of plant genomic DNA, 1.2 pmol of each primers, 0.2 mM dNTPs, *Pfu* buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.75), 2 mM $MgSO_4$,

0.1% Triton X-100, 100 µg/ml BSA (Bovine Serum Albumin)) and *Pfu* Polymerase (1 unit). DNA was denatured at 97 ^{0}C for 1 minute, followed by annealing at 58 ^{0}C for 20 seconds and extension at 72 ^{0}C for 1 minute. This was followed by 29 amplification cycles of 97 ^{0}C for 30 seconds, 60 ^{0}C for 20 seconds, and 72 ^{0}C for 1 minute. An additional extension at 72 ^{0}C for 5 minutes was followed.

Table 1. Primer sets used for PCR analysis and the expected size of PCR products.

Primer	Sequence	Gene	Band size (bp)
Bar5” Bar3”	5’CGGCGGTCTGCACCATCGTCAACCAC 5’GGCATATCCGAGCGCCTCGTGCATG	Bar	373
Bar-Ubi1-up Bar-Ubi1-down	5’CTTTCCCCAACCTCGTGTT 5’GTACGGAAGTTGACCGTGCT	Bar (with intron)	1212
Bar-Ubi1-up Bar-Ubi1-down	5’CTTTCCCCAACCTCGTGTT 5’GTACGGAAGTTGACCGTGCT	Bar (without intron)	198
Hor5” Liuxyldown	5’AAGCTTCGAGTGCCCGCCGATTTG 5’GTAGCGCGTGGTCGTGTAGATGTCG	Xylanase	837

For PCR analysis of the bar gene, reactions were carried out in a total volume of 25 µl reaction mixture, consisting of 1 µl of plant genomic DNA, 0.8 pmol of each primers, 0.2 mM dNTPs, *Taq* buffer (20 mM Tris-HCl, pH8.4, 50 mM KCl), 10% DMSO (v/v), 5 mM $MgCl_2$ and *Taq* Polymerase (1 unit). DNA was denatured at 94 ^{0}C for 3 minutes, followed by 25 amplification cycles of 94 ^{0}C for 45 seconds, 58 ^{0}C for 30 seconds, and 72 ^{0}C for 1 minute. An additional extension at 72 ^{0}C for 5 minutes followed.

After PCR, the 25 µl PCR mixture was directly used for 1% agarose gel electrophoresis using 1 x TBE buffer (Tris 10.778 g/L, Na_4EDTA 0.93 g/L and Boric acid 5.5g/L, pH 8.3, stored at room temperature). 3 µl of ethidium bromide stock (10 mg/ml, wrap in foil, stored in dark at room temperature.) was added to the 100 ml of 1% agarose gel. 2 µl of Blue Juice (Glycerol 300 ml, Bromophenol Blue 2.5 g, Xylene Cyanol 2.5 g and H_2O 700 ml, store at 4 ^{0}C) was added to

the PCR mixture. All 25 µl of PCR product was loaded to the wells of the gel. 15 µl of 1 kb DNA ladder (50 µl of 1 kb DNA ladder stock, 950 µl of 1x loading buffer (1 ml of Blue Juice, 9 ml of TE buffer pH 7.0 (10 mM Tris, 1 mM EDTA)) was used as marker. The gel was run at 120 V for 1 hour. The DNA band was visualized under UV light and a picture was taken.

To confirm that the DNA template in the PCR reactions for the bar gene was not from the plasmid DNA due to potential *Agrobacterium* contamination on the leaves, primer set Bar-Ubi1-up and Bar-Ubi1-down (Table 1) was designed. This primer set amplified a 1212 bp fragment with plasmid DNA as template (positions 2869 to 4080). However, this same primer set would amplify a 198 bp fragment with the transformant cDNA as template due to the intron removal (positions 2954 to 3967). The cDNA was obtained by Reverse Transcription PCR using total RNA isolated from transformants as described in Appendix C. 1 µl RT-PCR product containing the cDNAs was used in PCR reactions for the bar gene. The reactions were carried out in a total volume of 50 µl reaction mixture, consisting of 1 µl of cDNA from RT-PCR, 1 pmol of each primers (Bar-Ubi1-up and Bar-Ubi1-down), 0.15 mM dNTPs, 1 x Red *Taq* buffer, 0.25 mM $MgCl_2$ and Red *Taq* Polymerase (Sigma D5684). DNA was denatured at 95 ^{0}C for 4 minutes, followed by 35 amplification cycles of 95 ^{0}C for 1 minute, 58 ^{0}C for 1 minute, and 72 ^{0}C for 1 minute. An additional extension at 72 ^{0}C for 5 minutes followed. After PCR, 40µl PCR mixture was directly used for 1% agarose gel electrophoresis using 1 x TAE buffer. 5 µl of ethidium bromide stock was added to the 100 ml of 1 % agarose gel. The gel was run at 100 V for 1.5 hour. The DNA band was visualized under UV light and a picture was taken.

3.2.8.2 Cloning and sequencing of DNA fragment

A unique single band was produced with the designed primer pairs by PCR for identifying the presence of xylanase gene from transgenic plants. This DNA fragment was

purified from agarose gel, cloned into PUC 18 vector, and sequenced. This sequence was compared with the sequence of xylanase gene in the plasmid RS 128/Xyl.

To purify the DNA after PCR, the amplified DNA fragment to be cloned and sequenced was visualized under long UV light with a portable Mineral Light® lamp (Model UVSL-25 with multiband UV of 254/366 nm, Ultra-violet Products, Inc., San Gabriel, CA, USA) with minimal exposure time to avoid DNA mutation, and was carefully cut using a sharp blade. The cut DNA fragment was placed in a 1.5 ml Eppendorf tube, and was purified with a QIAEX II agarose gel extraction kit (QIAGEN Inc., Valencia, CA, USA), by following the manufacturer's protocol. The purified DNA in 20 µl of H_2O was stored at –18 ^{0}C for further use.

To clone the purified DNA fragment into the PUC 18 vector, the DNA was first phosphorylated with T4 polynucleotide kinase (PNK), which was carried out in a total volume of 25 µl reaction mixture, consisting of 17 µl of purified DNA fragment, 1.5 µl of T4 PNK (10 U/µl), 3 µl of 10 X T4 PNK buffer A, 2.4 µl of ATP (10 mM), and H_2O. The reaction was incubated at 37 ^{0}C for 1 hour, and purified with QIAEX II. The concentration of the purified and phosphorylated DNA fragment was examined by running a 0.8% agarose gel, together with the PUC 18 DNA (SmaI, dephosphorylated) and 1 kb DNA ladder. In doing so, 2 µl of the DNA fragment was mixed with 3 µl of TE buffer and 2 µl of Blue Juice. The gel was run at 100 V for 1 hour. The concentrations were determined for adjusting the ratio of concentrations of DNA fragment to PUC 18 in the next ligation step. The purified DNA in 20 µl of H_2O was stored at –18 ^{0}C for further use.

The second step of cloning the purified DNA fragment into PUC 18 vector was to ligate the purified and phosphorylated DNA fragment with PUC 18 vector, which was carried out in a total volume of 20 µl reaction mixture, consisting of 5 to 16 µl of DNA fragment, 1 to 2 µl of

PUC 18 vector, 1 x T4 DNA ligase buffer, 1 µl of T4 DNA ligase, and H_2O. The ratio of amount of DNA fragment to PUC 18 was adjusted to be 5 to 1 in the ligation reaction mixture. The ligation mixture was incubated at room temperature for 30 minutes.

The third step of cloning the purified DNA fragment into the PUC 18 vector was to transform *E. coli* DH5*a* cells with PUC 18 vector containing the DNA fragment. 100 µl of the DH5*a* competent cells from glycerol stock in the freezer in a 1.5 ml Eppendorf tube were thawed on ice. 5 µl of the ligation mixture was added to the tube, and mixed gently. The tube was kept on ice for 30 minutes, and then was heat-shocked at 42 ^{0}C for 45 seconds. It was then kept on ice for 4 minutes to equilibrate the cells. 1 ml of LB medium was added to the tube, and the tube was placed on a shaker with 200 rpm at 37 ^{0}C for 2 hours to generate ampicillin resistance. 150 µl of the cells were plated out on LB medium containing the antibiotics ampicillin at a concentration of 50 mg/L. The rest of the cells were centrifuged at 13, 000 rpm for 2 minutes. The supernatant was removed, and the remaining cells were plated out on LB medium containing the antibiotic ampicillin. The plates were incubated at 37 ^{0}C for about 14 hours or until colonies became visible. As soon as the colonies were visible, 10 single colonies were picked using a sterile tooth stick, and plated out on LB medium containing the antibiotic ampicillin. The position of each of the 10 colonies on the plate was carefully marked. The plate was kept in the incubator at 37 ^{0}C overnight to check the growth of colonies, then placed at 4 ^{0}C for long-term storage. Individual colonies were analyzed for the successful cloning with PCR analysis. To do so, the colony was touched with the tip of a sterile tooth stick, and suspended in 10 µl of sterile distilled H_2O and used directly as a DNA template for PCR. The same single band as in the positive plasmid control would indicate the successful cloning of the DNA fragment into PUC 18 vector present in the bacterium DH5*a* cells.

To prepare the DNA for sequencing, the successful clone as identified by PCR was used for plasmid isolation and purification. To do so, the correctly identified colony was picked using a sterile tooth pick and suspended in 5 ml LB medium containing the antibiotics ampicillin at a concentration of 50 mg/L. The tube was kept on a shaker with 200 rpm at 37 ^{0}C overnight. The plasmid DNA was then purified with a Quantum Prep® Plasmid Miniprep Kit (Catalog number 732-6100, BIO-RAD Laboratories, USA) by following the manufacturer's protocol. The purified DNA in 100 µl of sterile distill H_2O was stored at –18 ^{0}C for further use.

The purified plasmid DNA was analyzed for size and DNA concentration by enzyme digestion, which was carried out in a total volume of 20 µl reaction mixture, consisting of 2 µl of DNA, 2 µl of 10 X reaction buffer (Invitrogen react 2), 0.3 µl of Hind III (Gibco), 0.3 µl of EcoR I (Invitrogen), 15.4 µl of sterile H_2O. The tube containing the reaction mixture was incubated at 37 ^{0}C for 2 hours. The band was visualized by running a 0.8% agarose gel using the thin comb (20 µl capacity). The size and DNA concentration was determined. 100 ng of DNA was needed for the sequencing reaction.

The purified plasmid DNA (PUC 18) containing the cloned DNA fragment was used for DNA sequencing, which was performed with the dideoxynucleotide chain termination method with the BigDye Terminator system on an ABI Prism 377 DNA sequencer (applied Biosystems) by Amplicon Express (1610 NE Eastgate Blvd Suite #880, Pullman, WA, USA). The 837 bp of PCR amplified DNA fragment from wheat transformant carrying the xylanase gene was sequenced in two reactions, i.e. forward and reverse, so that the complete sequence of the 837 bp of PCR amplified DNA fragment was precisely determined. This sequence was compared with the sequence of xylanase gene in the vector RS 128/Xyl.

3.2.8.3 Southern blot analysis

The methods used for DNA isolation, Southern blotting, and hybridization as described by Kleinhofs *et al*. (1993) and Horvath *et al*. (2002) were used and modified as described in Appendix B.

3.2.8.4 Enzymatic assay for xylanase activity

The xylanolytic activity was quantitatively measured by the determination of the amount of reducing sugars liberated from the substrate azo-birchwood xylan (Megazyme). The xylanase standard curve was made with a dilution series of xylanase (from *Thermomyces langinosus* (2500U/g) expressed in *Aspergillus oryzae*, Sigma Cat. X2753) ranging from 0.125 to 0.625 µg in glycine buffer, 0.05 M, pH 6.0 with NaOH. 150 µl of xylanase was mixed with 200 µl of azo-birchwood xylan Brilliant Blue R, which was pre-warmed at 50 ^{0}C for 30 minutes. The mixture was incubated at 50 ^{0}C for 30 minutes. The reaction was terminated by adding 1 ml of precipitant solution (5 x stock contains 1.47 M Na-acetate.3H_2O and 0.11 M Zn-acetate.2H_2O, pH 5.0 with HCl. Work solution contains 200 ml of 5 x stock and 800 ml of methyl cellosolve (2-methoxyethanol)). Unhydrolyzed azo-xylans were removed by centrifugation at 7000 rpm for 3 minutes. The supernatant was transferred to a clean tube, and the optical density of water-soluble products released from the insoluble substrate by xylanase was measured at A_{590}. The xylanase standard curve was made by plotting the data of absorbance A_{590} against amount of xylanase in µg and xylanase activity mini Unit (mU).

To measure amount of xylanase from transgenic wheat grains, 200 mg of ground powder of wheat grains was dissolved in 0.7 ml glycine buffer, 0.05 M, pH 6.0, vortexed for 1 minute, and incubated at room temperature for 5 minutes. The enzyme extraction solution was collected

by centrifugation at 13000 rpm for 10 minutes. 150 µl of the enzyme extraction solution was mixed with 200 µl of azo-birchwood xylan Brilliant Blue R, and incubated at 50 ^{0}C for 30 minutes. The reaction was terminated by adding 1 ml of precipitant solution and centrifuged at 7000 rpm for 3 minutes, supernatant collected, and the optical density was measured at A_{590}. The amount of xylanase contained in the transgenic wheat grains was calculated by the xylanase standard curve.

The xylanolytic activity in transgenic wheat grains was qualitatively determined by a modified quick zymogram method (Sa-Pereira *et al.*, 2002). The wheat seeds were cut into half, and placed onto plates with the cut end facing down. The plates contain 3% (wv^{-1}) oat-spelt xylan (Sigma) in 0.05 M glycine buffer, pH 6.0 and 1% agarose (wv^{-1}), autoclaved. These were incubated overnight (18 to 24 hours) at 50 ^{0}C. The plates were stained in 0.1% Congo Red for 15 minutes, and destained with 1 M NaCl for 15 minutes. Congo Red stains xylan. Xylanase from endosperms of transgenic wheat grains hydrolyzes xylan around the seeds on the plates showing a yellowish ring (unstained area) on the red background. Non-transformed wheat grains or wild type controls do not show this yellowish ring around the grains on the plate.

3.3 Data analysis

3.3.1 Experiments of development of microspore embryogenesis:

All experiments were analyzed as completely randomized designs. There were two to six replications for each treatment. For the 2-HNA dose experiment, similar Chris tillers were assigned to each flask, and each treatment was randomly applied twice to the flasks. Microspores from each of the two flasks with the same treatment were separately isolated and cultured in the same Petri dish (replication), and each Petri dish was separately evaluated. For

the experiments on osmolality or ovary source, microspores from six Pavon 76 or six Chris spikes were first isolated, and equally distributed to each Petri dish, and each treatment was randomly applied twice to the Petri dishes. Each of the two Petri dishes with the same treatment was considered as a replication and was evaluated separately. Liquid embryoid induction media with different osmotic pressures were made by adjusting concentrations of maltose and mannitol. For the genotypic response experiment, the same pretreatment regime with 50 mL of the inducer formulation (0.1g L^{-1} 2-HNA, 10^{-6} mol L^{-1} 2,4-D and 10^{-6} mol L^{-1} BAP) was applied to eight genotypes, and data were pooled means of two to six replications per genotype. The general linear model (Lentner and Bishop, 1993) was used to analyze the data. Analysis of variance was conducted, followed by a 5% least square difference analysis for the three properties, i.e. number of embryoids, green plant percentage, and doubled-haploid percentage.

3.3.2 Experiments with conversion of albino-to-green plants

The experiment was considered as a completely randomized design. There were two treatments, each replicated twice. Two genotypes were used. Each petri dish containing microspores from the three spikes of the same treatment was considered as one replication. The general linear model (Lentner and Bishop 1993) was used to statistically analyze the data. Analysis of variance was conducted, followed by the 5% least square difference analysis, for five traits – i.e. number of embryos, regeneration rate, green versus albino plant percentage and spontaneously DH percentage. At 45 days after microspore plating, embryos visible to naked eyes were counted, while smaller structures were ignored. A small portion of the embryos that matured in the early phase was used for evaluating embryo quality. To avoid bias, the first available 115 and 95 embryos from each petri dish transferred were used to evaluate plant regeneration rate for the genotypes WED 202-16-2 and Svilena and DH percentage for WED

202-16-2, respectively. Embryos were transferred to regeneration media in three groups based on the size of embryos between 30 days and 45 days after microspore plating. Green and albino plants with well-developed roots and shoots were counted at 14 days after embryos had been transferred to regeneration media. Between 13 and 20 plants for each treatment were evaluated for ploidy level based on fertility (10 or more seeds per spike). The experiment was repeated more than three times. The same treatment was also applied to some NPB breeding lines for DH production.

3.3.3 Experiments with microspore transformation:

All experiments were analyzed as completely randomized designs. There were two to six replications for each treatment. Microspores were firstly isolated, mixed thoroughly, and equally distributed to each Petri dish, and each treatment was randomly applied to the Petri dishes. Embryoids of 1 to 2 mm in diameter were randomly chosen and plated out in every Petri dish with different treatment (i.e. bialaphos dose in 190-2 medium). Each Petri dish with the same treatment was considered as a replication and was evaluated separately. The general linear model (Lentner and Bishop, 1993) was used to analyze the data. Analysis of variance was conducted, followed by a 5% least square difference analysis for the four properties, microspore viability, number of embryoids, plant regeneration percentage, and plant surviving percentage.

3.4 Schedule time line for major steps

Day 1	Pretreatment of microspore containing spikes; Growing AGL-1 cells.
Day 3	Isolation & culture of embryogenic microspores in liquid medium.
Day 3 to 30	Co-cultivation of microspores with AGL-1 cells; Eliminating *A. tumefaciens* post co-cultivation.
Day 31	Transferring embryoids to germination medium for plant regeneration; Selection for transformants with bialaphos in the germinating medium.
Day 45	Transferring plants to GH for doubled haploid (DH) seed production.
Day 60	PCR & Southern Blot analysis of leaf DNA samples for identifying positive transformants.
Day 120	Harvesting DH seeds; conducting enzyme assay for xylanase with seeds.

CHAPTER FOUR

RESULTS

4.1 Generation of doubled haploid wheat plants

4.1.1 Microspore embryogenesis triggered by treatment of microspores with chemical inducer formulations

Over 50% of the total microspores in a spike can be routinely induced to become embryogenic by treatment with a formulation, including the chemical 2-HNA at 33^0C, leading to the potential development of thousands of green plants originating from the microspores of a single wheat spike (Table 4.1.1A).

Table 4.1.1A Genotypic response to the developed isolated microspore culture method: the number of green plants obtained from microspores of a single spike of one recalcitrant and seven medium to highly responsive genotypes.

Name	Type†	No. of embryoid‡	Regeneration[a]	Green plant[b]	DH[c]
			%		
Chris	HRS	6294	90	99	50
Pavon76	HWS	4965	50	60	65
WED202-16-2	HWS	4305	61	70	80
Svilena	SWW	2809	90	90	30
Wawawai	SWS	1020	50	48	73
Capo	HRW	2056	50	75	30
Calorwa	SWS/Club	2210	48	8	20
Waldron§	HRS	68	80	99	55

† HRS=Hard Red Spring, HWS=Hard White Spring, SWW=Soft Red Winter, SWS=Soft White Spring, HRW=Hard Red Winter, SWS=Soft White Spring.

‡ Data were based on 200 most advanced mature embryoids and estimation of developing embryoids.

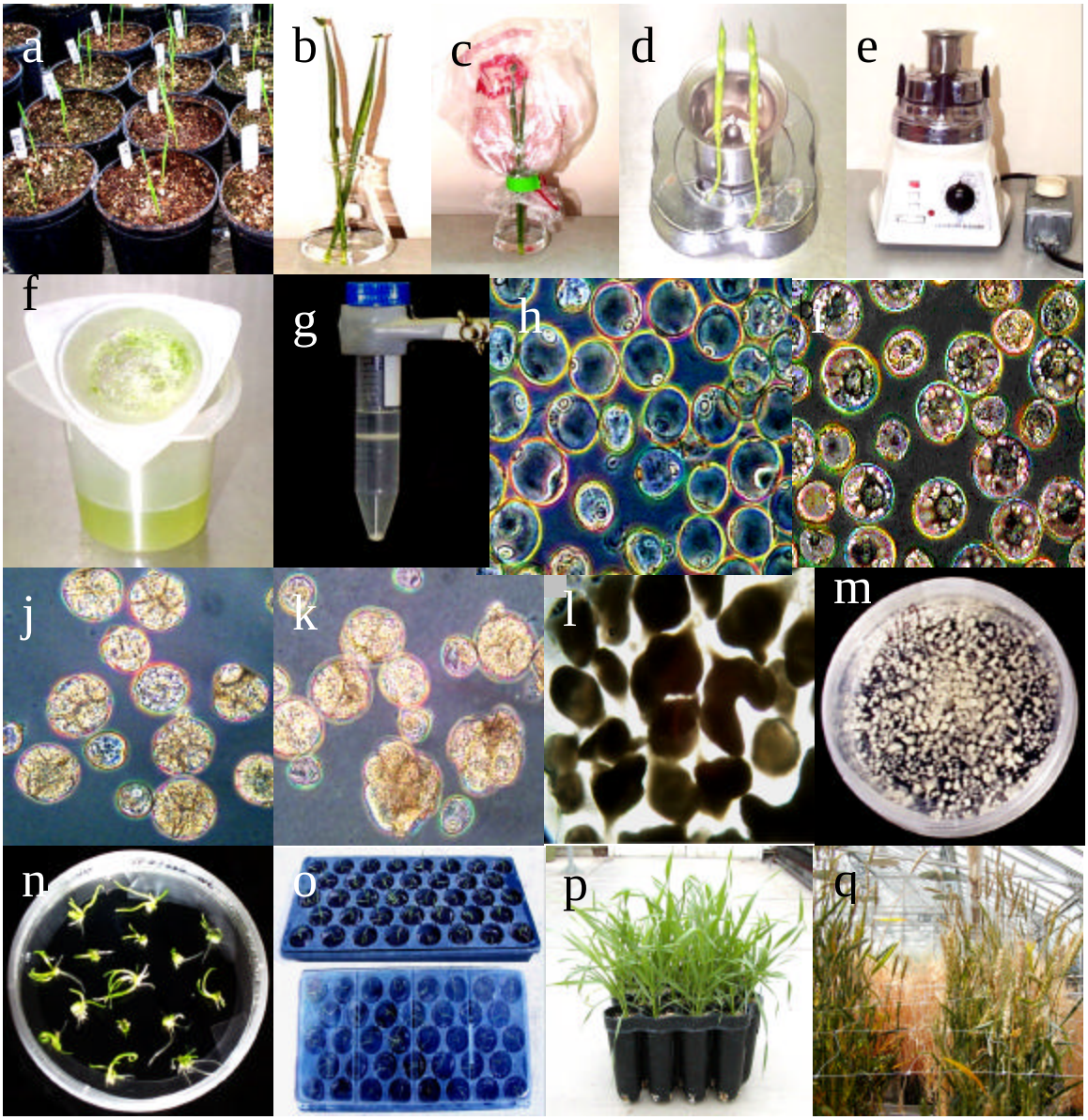
§ Recalcitrant genotype.

a Plant regeneration (%)=100 x (no. of green and albino plants)/no. of embryos plated.

b Green or albino plant regenerants (%)=100 x (no. of green or albino plants)/no. of regenerants.

c DH plants (%)=100 x (no. of fertile plants with 5 or more seeds per spike) /no. of green plants evaluated.

Figure 4.1 The process of generating doubled-haploid wheat plants from microspores. Genotype Chris. **a** Growing explants. **b & c** Sampling and pretreatment of microspore-containing spikes. **d**, **e, f & g** Isolation and purification of embryogenic microspores. **h** Mid- to late-uninucleate microspores from the freshly harvested spikes. **i** Embryogenic microspores with fibrillar cytoplasm induced by treatment of chemical inducer formulations at high temperature for 65 hours. **j, k, l & m** Microspore-derived developing embryoids cultured in liquid embryoid induction medium for 7, 14, 21, and 28 days, respectively. **n** Germination of embryoids on plant regeneration 190-2 medium at day 10. **o** Transplanting microspore-derived seedlings from Petri dish to covered plastic trays in greenhouse. **p** Growing DH plants in greenhouse. **q** Production of DH seeds derived from microspores in 5 months.



After the treatment, the embryogenic microspores typically have eight or more small vacuoles immediately enclosed by the cell wall (Fig 4.1 i). These vacuoles surround the condensed cytoplasm in the center, forming a fibrillar structure. The embryogenic microspores are usually, but not always, of a larger size (about 50 microns) than the average non-treated or non-induced microspores (25-45 microns). The non-treated or non-induced microspores do not divide and die when they are cultured in liquid embryoid induction medium. The embryogenic microspores can form embryoids when they are cultured in liquid embryoid induction medium (Fig 4.1 j-m). These embryoids can germinate and develop into green plants (Fig 4.1 n-q).

The optimal concentration of 2-HNA in the formulation for treating microspores to induce embryogenesis and form mature embryoids was determined to be approximately 100 mg L^{-1} (Table 4.1.1B). The number of induced embryoids increased with increasing concentrations of 2-HNA up to a threshold of 100 mg L^{-1}, while the percentage of germinated green plants (expressed as a percentage of the number of embryoids transferred to germination medium) did not significantly differ between different concentrations of 2-HNA. Spontaneous chromosome doubling percentage reached 65% with 2-HNA treatment at 100 mg L^{-1}. A toxic level of 2-HNA in the pretreatment formulation was observed at the dose 1 g L^{-1}, when the tiller stem tissues deteriorated and microspores died. Microspores, when isolated from tillers pretreated without chemical inducer formulation (in distilled H_2O), appeared to develop toward pollen maturation, and died when cultured in induction medium.

The chemical, 2-HNA, was effectively and conveniently delivered to act on microspores by the described method. The chemical inducer formulation is absorbed by the vascular system of the stem and transported to the anthers and into the microspores, and the 33 ^{0}C temperature

speeds up the efficiency of chemical delivery to microspores. Lower temperatures may be employed, but adjustments must be made for the slower rates of chemical uptake and tiller growth. The optimal period of pretreatment appears to vary somewhat with the genotype and the treatment temperature, ranging between about 48 h and about 72 h at 33 ^{0}C. Tillers can be stored for convenience in a refrigerator at 4^{o}C for up to 1 month before pretreating the microspores with the chemical inducer formulation and temperature, and with nutrient stress. Because the microspore viability falls sharply, tillers should not be stored in a refrigerator at 4^{o}C after the temperature-nutrient stress treatment. The influence of microspore developmental stages on androgenesis was very strong. The mid to late uni-nucleate microspores were the most responsive to chemical induction of androgenesis. Since microspores in wheat spikes are not synchronized in their developmental stage, one can only expect a portion of the microspores from a given spike to be inducible to embryogenesis. The task is to synchronize the maximum number of microspores in a spike at the appropriate developmental stage. Success can be determined by staining microspores of an anther in the middle section of the spike.

Table 4.1.1B Optimization of 2-HNA concentrations for inducing androgenesis. Means of embryoid yields and percentages of green plants and spontaneous doubled-haploids from microspores treated with various concentrations of 2-HNA.

2-HNA (mg L^{-1})	0‡	1	10	100	1000
Number of embryoids†	2^a	118^b	322^b	1238^c	0^a
Green plant (%)	0^a	100^b	97^b	100^b	0^a
Doubled-haploid (%)	0^a	0^a	64^b	65^b	0^a

† No of embryoids with 0.2-2 mm in diameter was counted at day 40 after incubation and smaller structures (developing embryoids) were ignored. Actual figure would be 5 fold higher if all potential embryoids were cultured to maturity. Means followed by the same letter in the same row are not significantly different with ANOVA and 5% LSD analysis.

‡ Microspores developed in their gametophytic pathway during pretreatment, and died upon culture in induction medium.

After identifying the effectiveness of 2-HNA in inducing microspore embryogenesis, other selected chemicals were also tested for their function in inducing androgenesis. The

chemicals were selected based on their structural similarities to 2-HNA, or potential properties for causing male sterility, which were positively correlated to induction of androgenesis. Because it was time-consuming to run experiments with a large number of chemicals, a simpler protocol was developed. It was observed that after the treatment of microspores with chemical inducer formulation including 2-HNA, the embryogenic microspores typically had eight or more small vacuoles immediately enclosed by the cell wall (Fig 4.1 i) and these vacuoles surround the condensed cytoplasm in the center, forming a characteristic star-like fibrillar structure.

Table 4.1.1C The effect of chemical inducers in solution A on formation of embryogenic microspores with fibrillar structures during a 38 – 52 h pretreatment. Microspores were all isolated from the spring wheat WED 202-16-2.

Chemical Inducer†	Concentrations tested (*m*M)	Optimum concentration (*m*M)	Microspore density (no./ml)	Embryogenic Microspores (%)
Solution A	0	0	4,800	45.1
2-HNA	0.06; 0.12; 0.18; 0.30	0.18	6,100	80.6‡
AA	0.09; 0.18	0.09	7,700	67.2‡
BT	0.09; 0.18	0.18	7,700	75.7‡
B-5-CA	0.09; 0.18	0.18	4,600	86.7‡
2,3-BM	0.09; 0.18	0.09	2,700	62.6‡
DL-H	0.09; 0.18	0.09	4,600	72.3‡
2,3-PCA	0.09; 0.18	0.09	2,700	67.4‡
SA	0.045; 0.09; 0.13; 0.16	0.13	1,600	70.0‡
VAM	0.09; 0.18	0.09	5,100	78.4‡

† *AA* Anthranilic acid, *BT* benzotriazole, *B-5-CA* benzotriazole-5-carboxylic acid, *2,3-BM* 2,3-butane-dione monoxime, *DL-H* DL-histidine, *2-HNA* 2-hydroxynicotinic acid, *2,3PCA* 2,3-pyridine carboxylic acid, *SA* sulfanilamide, *VAM* violuric acid monohydrate. Chemicals were added to solution A (control) at described concentrations.

‡ Statistically significant at $P = 0.01$ between each treatment and the control based upon a t-test.

The star-like fibrillar structure can be used as an indicator for successful induction of androgenesis. Based on the results of Kyo and Harada (1985) with tobacco microspores, a

similar procedure was developed in wheat for producing DH from isolated microspores, and was used to evaluate the effect of chemicals for inducing the star-like fibrillar structure in microspores. The results are summarized in Table 4.1.1C.

The results showed that many chemicals had similar function as 2-HNA to significantly increase the number of microspores with a characteristic embryogenic structure. In addition to 2-HNA, two chemicals, benzotriazole-5-carboxylic acid and violuric acid monohydrate were tested for inducing plant regeneration and showed similar results to 2-HNA (data not shown).

4.1.2 Embryoid formation favored by purification of isolated microspores

Once microspores are embryogenic, it is necessary to separate them from the spikes and culture them in liquid nutrient media. Liquid culture of isolated microspores provides many advantages over anther culture. First, the entire process of microspore embryogenesis in the culture plate can be easily monitored under an inverted microscope and the process of embryogenesis followed over time, as desired. Microscopic examination provides an effective way to observe development. Second, all embryoids formed in the culture plate are certain to be microspore-derived, and plants regenerated are either haploid or doubled-haploid, because the only cells placed in the culture plates are microspores (Fig 4.1 h-k).

The isolation process should minimize damage to microspores. Different microspore isolation methods were tested, including use of a vortexer, stirring bars, glass bar grinding and blending. While all methods seemed to work, only isolation by use of a blender produced repeatable results, and high yields of viable and responsive microspores, especially when fixed mechanical conditions such as blend speed and time were monitored. Using a blender is a rapid and efficient means for processing large numbers of samples (Fig. 4.1 d and e). Despite these advantages, It has been observed that using a blender damages over 50% of the embryogenic

microspores, resulting in early abortion of development toward mature embryoids. Future research should aim to improve the isolation methods to reduce damage to the induced embryogenic microspores. Nevertheless, since as many as 50% of the microspores in the spikes can be induced to be embryogenic, the current procedure already can produce large numbers of microspore-derived green plants. In fact, so many embryoids are usually produced that the transfer of the embryoids to germination media can be the factor limiting the number of plants recovered.

Purifying embryogenic microspores is another important step for which results are repeatable. The dead, non-embryogenic microspores or debris may interfere with developing embryoids by releasing phenolics and by changing media composition, such as pH and osmolality. Several purification methods were found to work, but the combination of a simple gradient centrifugation by 0.58 mol L^{-1} maltose and mesh-filter filtration proved to be most effective and efficient (Fig. 4.1 f and g).

4.1.3 Embryoid formation favored by live ovaries in embryoid induction medium

The female part of the wheat reproductive system definitely plays an essential role for the re-programmed sporophytic development. In our studies, even large populations of embryogenic microspores were obtained and cultured in nutrient media, but the majority of the developing embryoids ceased cell division in the process toward forming mature embryoids in media without the presence of live ovaries. Extracts of ovaries were not active, indicating that female nurse substances, only synthesized by live ovaries, were responsible for nursing the majority of embryogenic microspores to become mature embryoids (Table 4.1.3).

Table 4.1.3 Effects of ovary source and co-culture methods on androgenesis.

Ovary source†	Ovary per plate	Number of embryoids	Green plant percentage
Pavon/fresh live	2	225[a‡]	100[a]
Chris/fresh live	2	259[a]	100[a]
WED202/fresh live	2	230[a]	100[a]
Pavon/extract	2	0[b]	0[b]
Chris/extract	2	0[b]	0[b]
Yecora Rojo/extract	2	0[b]	0[b]
Pavon/extract	10	0[b]	0[b]
Chris/extract	10	0[b]	0[b]
Yecora Rojo/extract	10	0[b]	0[b]

† Ovaries were either freshly isolated right before co-culture from freshly harvested spikes, or extracted from fresh ovaries of the three genotypes by grinding them in liquid nitrogen and filter-sterilized with 0.22 um filter (Minipore, INC).

‡ Means followed by the same letter in the same column are not significant by ANOVA and 5% LSD procedure. Actual figure would be 5 fold higher if all potential embryoids were cultured to maturity.

The results also showed that there were no significant differences in the nurse function for androgenesis among the live ovaries of the three different wheat genotypes tested. In fact, it was found that live ovaries from other genotypes, including those of low or non-responsive genotypes and even oat and barley ovaries had similar nurse effects for androgenesis. This finding indicates that a universal mechanism, present in ovaries of any given wheat genotype effectively provides nurse factors for androgenesis. Thus, one can take the advantage of the finding such that Chris ovaries, for example, can be used for androgenesis of all genotypes. This is especially valuable in situations where only a limited number of spikes from a target genotype are available for isolated microspore culture, as often is the case in breeding programs.

4.1.4 Embryoid formation affected by osmolality in the liquid culture media

With a large population of embryogenic microspores isolated, the task is to provide a favorable environment to enable them to develop into mature embryoids. In the described method, embryogenic microspores began their first cell division after approximately 12 h in culture. Multi-cellular proembryoids, still enclosed within the microspore wall or exine, were

formed in approximately one week. After an additional week, the exine wall ruptured and immature embryoids emerged, which grew into mature embryoids within about 10 to 14 days (Fig. 4.1 i-m). This process was affected by a number of factors, of which culture media composition and osmolality were critical (Table 4.1.4).

Table 4.1.4 Optimization of osmotic pressure in the media for androgenesis.

	Media†							
	1	2	3	4	5	6	7	8
Osmolality (mOsmol Kg^{-1} H_2O)‡	152.5	202.5	252.5	299	347	402.5	449.5	497.5
Maltose (g L^{-1})	40	57	76	90	90	90	90	90
Mannitol (g L^{-1})	0	0	0	0	8	17	24.5	34.5
Size of calli on day 14 (mm)	0.05	0.05	0.2	0.2	0.1	0.1	0.1	0.05
Number of embryoids§	5^{a}	22^{ab}	90^{c}	100^{c}	10^{a}	0^{a}	0^{a}	0^{a}

† Osmolality was adjusted by changing concentrations of maltose and mannitol in liquid embryoid induction medium.

‡ Osmolality of each medium was measured by Osmette S Model #4002 (Precision Systems, Inc., 16 Tech Circle, Natick, MA 01760, USA).

§ Actual figure would be 5 fold higher if all potential embryoids were cultured to maturity. Means followed by the same letter in the same row are not significantly different with ANOVA and 5% LSD analysis.

Several media including MS and MN 6 (Murashige and Skoog, 1962; Chu and Hill, 1988) seem to work successfully for embryoid development, and the composition of most media contains adequate nutrients to feed developing embryoids. Thus, the physical constraint of osmolality becomes critical in the development of embryoids. As demonstrated in Table 4.1.4, the number and size of calli/embryoids were influenced by osmotic pressure in the culture media. The number and size of calli/embryoids increased with increasing osmotic pressure up to 300 mOsmol Kg^{-1} H_2O, then decreased with higher osmotic pressure. The results indicated that the optimal osmotic pressure in culture media for embryoid formation is about 300 mOsmol Kg^{-1} H_2O.

4.1.5 Albinism avoided by nutrient addition to pretreatment medium

The presence of additional nutrients in the pretreatment solution during the initiation of microspore embryogenesis did not affect embryo production for the two genotypes tested. All Petri dishes produced a similar number of embryos at 45 days after microspore plating (Table 4.1.5). Embryos eventually completely covered the entire bottom of all Petri dishes. When larger embryos were removed and the culture media and ovaries were refreshed weekly, hundreds more multi-cellular structures or pre-embryos developed into mature embryos. Each Petri dish continuously produced more than 1,000 embryos over a period of several months. This indicates that when microspores are being switched from the programmed gametophytic towards the sporophytic pathway by a chemical inducer formulation under proper physiological conditions, additional nutrients available to microspores are not the essential factors for inducing the formation of embryos. Nutrients present in tissues such as anther walls and stem tissue surrounding microspores may be the nutrient source during the pretreatment. Microspores are not really completely "starved" in this pretreatment regime.

For the genotype WED 202-16-2, the addition of 10% embryo induction medium in the pretreatment solution significantly increased both the plant regeneration rate (15% increase) and the percentage of green plants (27% increase) among regenerants ($P=0.05$) (Table 4.1.5). This result shows that, with respect to a "problematic" genotype, such as WED 202-16-2, the availability of additional nutrients to the microspores during embryo initiation helped enable a large population of microspores to develop into embryos of good quality that were competent for regenerating into green plants, while it did not negatively affect the growth of embryos from a genotype with a "good" genetic background, such as Svilena (Table 4.1.5). The regenerated plants from each treatment looked similar, and no significant difference in the spontaneous DH

frequency was detected for the genotype WED 202-16-2 (Table 4.1.5). Svilena plants were not evaluated for the percentage of spontaneous DHs because there was no difference in green plant regenerants. Similar results for genotypes WED 202-16-2 and Svilena were obtained when the experiments were repeated. When the same nutrient treatment was applied to some breeding lines, there was a significant improvement in green plant frequency among regenerants in those genotypes having a moderate to high frequency of albinos (data not shown).

Table 4.1.5 The effect of nutrients in the pretreatment solution on plant regeneration

	WED 202-16-2†			Svilena†		
	Nutrient	Control	LSD (0.05)	Nutrient	Control	LSD (0.05)
Number of visible embryos at day 45	620^{A}	640^{A}		624^{A}	650^{A}	
Plant regeneration‡ (%)	70^{A}	61^{B}	4	92^{A}	88^{B}	3
Green plant regenerants§ (%)	89^{A}	70^{B}	4	95^{A}	94^{A}	7
Albino plant regenerants§ (%)	11^{A}	30^{B}	4	5^{A}	6^{A}	7
DH plants¶ (%)	63^{A}	64^{A}	52			

† Means followed by the same letter in the same row are not significantly different by ANOVA and 5% LSD analysis

‡ Plant regeneration (%)=100 x (no. of green and albino plants)/no. of embryos plated

§ Green or albino plant regenerants (%)=100 x (no. of green or albino plants)/no. of regenerants

¶ DH plants (%)=100 x (no. of DH plants/no. of green plants evaluated)

4.1.6 Chromosome doubling obtained by treatment of microspores with caffeine in liquid media

Sampling, pretreatment, microspore isolation, culture and plant regeneration were the same as described in Chapter 3.1. Caffeine stock solution was made at a concentration of 20 g/l. It was dissolved in ddH_2O and filter-sterilized with a filter pore size of 0.2 µm. Caffeine treatment was conducted in such a way that caffeine stock solution or its diluted solution was

added to the pretreatment formulation in the flask for the entire period of pretreatment, or/and to the induction medium for a desired period of time. Caffeine was removed from the induction medium either by centrifugation of the microspores or by filtration with mesh-filters. Caffeine was carefully rinsed out twice, each with 5 ml of embryoid induction medium. Microspores then were re-suspended in embryoid induction medium free of caffeine.

The number of embryoids was counted 30 days after the microspores were isolated from spikes and cultured in the embryoid induction medium. Embryoids larger than 0.2 mm in diameter and visible to naked eyes were counted. Embryoids larger than 1 mm in diameter were transferred to 190-2 regeneration medium. Green and albino plants with roots and shoots development were counted. Green plants were transferred to the green house and fertility was evaluated based on seed set.

In the first experiment, the spring wheat Chris was used to evaluate effect of caffeine treatment in the pretreatment solution at different doses, and in the induction media for a short period of time at a high dose on embryogenesis induction, regeneration, and spontaneous chromosome doubling. Data are summarized table 4.1.6.1. The results indicated that 10 g/l of caffeine in the pretreatment solution significantly promoted embryoid induction ($P = 0.02$) for the genotype Chris. The embryoid quality as measured by regeneration and green plant frequency was also affected by caffeine treatment in that 10 g/l caffeine in pretreatment solution increased plant regeneration while caffeine dose at 1 g/l decreased both plant regeneration ($P = 0.002$) and green plant frequency.

Table 4.1.6.1 Effect of caffeine treatment on chromosome doubling of cultivar Chris. Means of each combination of treatment for embryoid number, regeneration percentage, green plant percentage, number of green plants per single spike, doubled-haploid percentage and number of DH plants per spike are given for an experiment with a 3 x 2 factorial design with 2 replications per treatment. Caffeine was added to the pretreatment solution at the concentration of 0, 1 and 10g/l, and in the embryoid induction media for 20 hours at 0 and 0.5 g/L.

Factor A: Caffeine in pretreatment (g/L)	0		1		10		*P*-value		
Factor B: Caffeine in media (g/L)	0	0.5	0	0.5	0	0.5	A	B	A x B
No. of embryoids†	1180	1160	1150	1180	1220	1230	0.016	0.624	0.347
Regeneration percentage‡	36	34	27	19	47	43	0.002	0.179	0.678
Green plant percentage‡	100	78	67	80	98	91	0.010	0.219	0.027
No. of green plants/spike‡	279	204	135	119	369	321	0.000	0.063	0.534
DH percentage§	60	78	75	88	47	73	0.103	0.029	0.705
DH plants/spike¶	168	158	101	104	174	235	0.026	0.420	0.487

† Results were evaluated at 45 days after culturing microspores in induction medium when the first group of embryoids was transferred to regeneration medium. Embryoids larger than 0.2 mm were counted for each plate.

‡ Means were based on 2 replications and 480-transferred embryoids/calli.

§ Chromosome doubling data were based on evaluation of fertility of 142 plants in the greenhouse.

¶ Actual figure would be several folds higher if all embryoids were cultured to maturity.

As a result, the number of green plants per spike was higher when using 10 g/l caffeine in pretreatment ($P < 0.001$). While the high dose caffeine treatment in the induction medium did not show any effect on embryoid induction and plant regeneration, it did promote the chromosome doubling of regenerants ($P = 0.03$). At 1 g/l caffeine resulted in the highest chromosome doubling frequency. Overall, the use of caffeine at a concentration of 10 g/l in the pretreatment medium yielded in the highest number of DH plants per spike used for culture.

This experiment yielded a much higher DH frequency than many previous experiments with the genotype Chris, even though the standard culture procedure was used. I have repeatedly observed that the frequency of DH, and induction response have been affected by the explants sampled. Earlier herbicide experiments showed that controls in 4 groups of experiments, all with the same standard culture procedure, yielded very different DH frequencies ranging from 18 to 49%. Additional studies on the effects of physiological conditions of explants on culture response may be desirable and useful.

In the second experiment, the spring wheat Pavon 76 was used to evaluate the effect of caffeine treatment in the pretreatment solution at different doses, and in the induction media for a short period of time at a high dose on embryogenesis induction, regeneration, and spontaneous chromosome doubling. Data are summarized in Table 4.1.6.2. The results showed clearly that the interactions between factors A and B were significant for all traits ($P < 0.01$), except regeneration percentage ($P = 0.352$). Each combination of caffeine treatment in the pretreatment and in the induction medium needed to be compared in order to choose the best treatment combinations.

For embryoid induction, the best two combinations were 1 or 10 caffeine in the pretreatment followed by 0 or 0.5 g/L caffeine in the induction medium, respectively.

Caffeine treatment at 0.5 g/L in the induction medium decreased the embryoid regeneration frequency ($P = 0.018$) for all three pretreatment regimes.

Green plant and DH frequency showed similar trends. They both were decreased by caffeine treatment merely in the induction medium, or in the pretreatment regimes, suggesting that it was necessary to include caffeine both in the pretreatment and in the induction medium, or

not use it at all. The best treatment for total green plants and DH production per spike was identified as 10 g/L in the pretreatment and 0.5 g/L in the induction medium.

Table 4.1.6.2 Effect of caffeine treatment on chromosome doubling of cultivar Pavon 76. Means for embryoid number, regeneration percentage, green plant percentage, number of green plants per single spike, doubled-haploid percentage and number of DH plants per spike (Factors A and B combinations). In the experiment with a 3 x 2 factorial design with 2 replications per treatment caffeine was added to the pretreatment solution at the concentration of 0, 1 and 10g/l (Factor A), and in the embryoid induction media for 20 hours at 0 and 0.5 g/L (Factor B).

Factor A: Caffeine in pretreatment (g/L)	0		1		10		*P*-value		
Factor B: Caffeine in media (g/L)	0	0.5	0	0.5	0	0.5	A	B	A x B
No. of embryoids†	700	640	740	720	620	730	0.033	0.537	0.009
Regeneration percentage‡	34	31	41	30	39	33	0.312	0.018	0.352
Green plant percentage‡	51	24	43	57	47	66	0.000	0.147	0.000
No. of green plants/spike‡	118	46	127	123	111	158	0.006	0.292	0.004
DH percentage§	45	21	41	50	25	63	0.036	0.050	0.001
DH plants/spike¶	53	10	52	61	27	99	0.009	0.084	0.001

† Results were evaluated at 45 days after culturing microspores in induction medium when the first group of embryoids was transferred to regeneration media. Embryoids larger than 0.2 mm were counted for each plate.

‡ Means were based on 2 replications and 1440-transferred embryoids/calli.

§ Chromosome doubling data were based on evaluation of fertility of 167 plants in the greenhouse.

¶ Actual figure would be several folds higher if all embryoids were cultured to maturity.

It was concluded that caffeine, when used at proper concentrations during the pretreatment regime and in the induction medium, could promote microspore embryogenesis and spontaneous chromosome doubling frequency. For green plant production, caffeine treatment decreased the green plant frequency in a good genotype such as Chris, but increased it in other genotypes, such as Pavon 76, at a combination of 10 and 0.5 g/L in the pretreatment and induction medium, respectively. For chromosome doubling, caffeine treatment generally

increased the DH frequency. In terms of total DH plant production efficiency, a caffeine treatment combination of 10 g/L in the pretreatment for 2-3 days followed by 0.5 g/L in the induction medium for 24 hrs was found to be most effective.

Genotypic differences in response to caffeine treatment were observed. Good genotypes in DH frequency such as WED 202-16-2 did not respond to caffeine treatment, indicating its strong-genetic control over high DH frequency. Upon caffeine treatment, this good genotype response may be damaged, resulting in albinism and reduced DH frequency (data not shown).

DH frequency was also found to vary greatly between experiments without treatment with a chromosome doubling agent for the same genotype such as Chris (18-65%) (Data not shown). The physiological condition of explants is an important factor affecting DH frequency in microspore embryogenesis. It seems that good greenhouse growth conditions are necessary for good explant production and higher DH frequency.

4.2 Transformation of microspores

A procedure was developed by experimentation for successful microspore transformation and plant regeneration from transgenic microspores. Over 200 transgenic wheat plants were produced by transformation of microspores. 24 spontaneously doubled haploid (DH) transgenic lines were obtained. PCR, DNA sequencing, and Southern blot analyses showed that the xylanase gene was present in the primary transformants (T_0), and also in all the T_1 DH progenies, indicating that (1) the xylanase gene was stably integrated into the wheat genome, and (2) the T_1 DH progenies were indeed homozygous.

Various factors affecting microspore transformation were investigated and the results are reported here.

4.2.1 Effect of concentration of AGL-1 cells in the medium on androgenesis

The goal of this study was to transform microspores by co-cultivation with *A. tumefaciens* strain AGL-1 cells containing plasmid RS 128/Xyl. After co-cultivation, the transgenic microspores need to be alive and be capable of developing into embryoids. The androgenesis was affected by introducing *A. tumefaciens* into the microspore culture medium (Table 4.2.1). Both microspore viability and embryoid production decreased with increasing AGL-1 concentration in the co-cultivation medium. To obtain embryoids and regenerate plants, the maximum concentration of AGL-1 cells in the culture medium for 24 hours co-cultivation was identified to be 1%. Higher concentrations of AGL-1 (>1%) caused complete microspore death.

Table 4.2.1 Effect of concentration of *A. tumefaciens* in the culture medium on androgenesis.

AGL-1 dose (%)†	0	0.1	1	5	10	25	50
Viable microspores (%) at Day 7‡	33[a]	11[b]	4[c]	0[d]	0[d]	0[d]	0[d]
No. of embryoids at day 30	860[a]	300[b]	88[c]	0[d]	0[d]	0[d]	0[d]

† *A. tumefaciens* strain AGL-1 cells were co-cultivated with microspores of genotype Chris for 24 hrs before being filtered out. Timentin at the concentration of 200 mg/L was added in the medium post co-cultivation.

‡ Means followed by the same letter in the same row were not significantly different with ANOVA and 5% LSD analysis.

The results indicate that when microspores are inoculated for a long co-cultivation duration (i.e. 24 hours), low concentrations (<1%) of AGL-1 cells should be used.

4.2.2 Effect of co-cultivation duration on androgenesis

As expected, androgenesis was affected not only by introducing *A. tumefaciens* to the microspore culture medium, also by the co-cultivation duration. When 20% of *A. tumefaciens*-containing solution was added to microspore culture plate, both microspore viability and

embryoid production decreased with increasing co-cultivation duration (Table 4.2.2). When 20% of *A. tumefaciens*-containing solution was added to the microspore culture plate, the maximum permissible co-cultivation duration to obtain embryoids and regenerate plants was 45 minutes. Longer co-cultivation duration (>45 min) resulted in complete microspore death.

The results indicate that when high concentrations (i.e. 20%) of AGL-1 cells are used for co-cultivation, the co-cultivation duration should be relatively short (i.e. less than 1 hour).

Table 4.2.2 Effect of co-cultivation duration on androgenesis.

Duration (minute) †	0	15	30	45	60	180	300	420
Viable microspores (%) at Day 7	30	10	8	5	4	2	1	0
Viable microspores (%) at Day 14‡	20[a]	7[b]	5[b]	4[b]	0[c]	0[c]	0[c]	0[c]
No. of embryoids at day 40	940[a]	410[b]	300[b]	282[b]	0[c]	0[c]	0[c]	0[c]

† 20% of *A. tumefaciens*-containing solution was added to microspore culture plate of genotype Chris for 0 to 420 min before *A. tumefaciens* was filtered out. Timentin at the concentration of 200 mg/L was added in the medium post co-cultivation.

‡ Means followed by the same letter in the same row were not significantly different with ANOVA and 5% LSD analysis.

4.2.3 Effect of timing for co-cultivation on transformation

Based on the results of co-cultivation duration and *A. tumefaciens* inoculation concentration, several strategies for microspore transformation were developed:

(1) The first strategy was to use higher initial inoculation concentration of AGL-1 cells (1 to 20%) with shorter co-cultivation duration (<5 hrs); and

(2) The second strategy was to use lower initial inoculation concentrations of AGL-1 cells (0.1 to 1 %) with longer co-cultivation duration (24 hrs).

Both strategies were used to transform microspores. The co-cultivation of AGL-1 cells with microspores was carried out at 0 to 30 days during embryoid induction culture. Over 200

putative transformants were obtained when co-cultivation was carried out at day 0, i.e. co-cultivation was followed immediately after microspores were isolated from donor spikes (Table 4.2.3, Fig. 4.2).

Table 4.2.3 Effect of timing for co-cultivation on androgenesis and transformation.

Timing (day)†	0	1	3	5	7	14	21	30
No. of transformant‡	200	0	0	0	0	0	0	0

† Days during embryoid induction culture when co-cultivation of *A. tumefaciens* and microspores was carried out. Genotypes Chris and WED 202-16-2. *A. tumefaciens*-containing solution was added to the microspore culture plate for 15 min to 24 hrs before *A. tumefaciens* were filtered out. Timentin at the concentrations of 200 or 400 mg/L was added in the medium post co-cultivation.

‡ Transformants were detected by PCR, DNA sequencing, and Southern blot analyses.

4.2.4 Methods for elimination of *A. tumefaciens* post co-cultivation

It is essential to use timentin to kill or inhibit growth of *A. tumefaciens* post co-cultivation. The addition of timentin in the embryoid induction medium may affect androgenesis. Results showed that number of embryoids decreased with increasing concentration of timentin in the embryoid induction medium (Table 4.2.4). The optimal concentration of timentin was determined to be 100 to 400 mg/L. With these doses of timentin present in the medium, a reasonable number of embryoids was produced while quality of embryoids (plant regeneration potential) was maintained.

It was observed that the AGL-1 cells were either completely inactive, presumably dead upon use of timentin post-co-cultivation, or they grew vigorously to eventually kill the microspores. The time of adding timentin in the medium post co-cultivation was critical. If the AGL-1 cells were not killed post-co-cultivation by timentin, AGL-1 cells grew back rather fast, within a couple of days. If this happened, it was difficult to kill AGL-1 cells by re-use of timentin without severely inhibiting embryoid formation. The microspores were very sensitive to

additional manipulation post co-cultivation. Generally, the medium in the culture plate containing transformed microspores should be totally visually transparent and free of active, moving and live AGL-1 cells under microscopical examination. If the medium in the culture plate turns non-transparent by visual inspection post co-cultivation, it indicates the failure to kill AGL-1 cells, and thus results in total failure of obtaining embryoid and plant.

Table 4.2.4 Effect of timentin in the culture medium on androgenesis.

Timentin† (mg/L)	0	100	200	300	400	500	600	700	800
No. of embryoid at day 30‡	312[a]	220[b]	164[b]	156[bc]	144[c]	100[cd]	52[de]	24[e]	4[e]
No. of embryoid transferred	60	60	60	60	60	60	50	20	0
No. of green plant germinated	27	22	20	25	26	21	16	1	0
No. of albino plant germinated	0	0	0	0	0	0	0	0	0
Plant regeneration‡ (%)	45[a]	37[a]	33[a]	42[a]	43[a]	35[a]	32[a]	5[b]	0[b]

† Timentin at the concentrations of 0 to 800 mg/L was added in the embryoid induction medium at the beginning of microspore culture. Embryoids were produced from microspores of wild type genotype Chris.

‡ Means followed by the same letter in the same row are not significantly different with ANOVA and 5% LSD analysis.

4.2.5 Plant regeneration from transgenic microspores and selection of transformants

The use of bialaphos in the regeneration medium serves two purposes: (1) putative transformants that contain the bar gene(s) will survive, and (2) non-transformants will not regenerate. Because no information was available regarding the tolerance level of microspore-derived embryoids that contained the bar gene(s), several selection schemes were tested using the wild type microspore-derived embryoids. The strategy was to determine the minimum concentration of bialaphos in the regeneration medium, which would effectively inhibit plant regeneration from wild type microspore-derived embryoids carrying no bar gene.

Table 4.2.5A Effect of bialaphos used in the regeneration medium on plant regeneration.

Bialaphos (mg/L)†	0	1	2	4	6	8
Morphology of plant at day 7‡	G, green, 5cm	G, green, 3cm	G, pale green, 1cm	G, yellow, 0.5cm	NG	NG
No. of plants at day 28§	43[a]	6[b]	3[b]	2[b]	0[c]	0[c]
Morphology of plants at day 28	Green shoot with root	Green shoot with root	Pale-green shoot without root	Yellow shoot without root	-	-

† Embryoids of 1-2 mm in diameter derived from microspores of wild type genotype Chris were transferred onto 190-2 medium containing bialaphos at concentrations of 0 to 8 mg/L.

‡ G=germinating, NG=non-germinating.

§ Means followed by the same letter in the same row are not significantly different with ANOVA and 5% LSD analysis.

Results indicated that bialaphos at a concentration of 2 mg/L in the regeneration medium would effectively inhibit embryoid regeneration of wild type microspore-derived embryoids (Table 4.2.5A). With bialaphos at a concentration of 2 mg/L or higher in the regeneration medium, only a few embryoids survived and developed shoots but no roots. These, however cannot develop into mature plants with the employed plant regeneration protocol which requires embryoids to develop both shoot and roots in 190-2 medium.

When small germinating embryoids were germinated for 7 days on 190-2 medium containing no bialaphos and transferred to bialaphos-containing media, plant regeneration was completely inhibited on medium containing high dose of bialaphos (4 mg/L) while 38% of plant regeneration was obtained on medium containing a low dose of bialaphos (1 mg/L) (Table 4.2.5B). 70% of these germinating embryoids survived on the low dose of bialaphos (1 mg/L) medium were able to survive when being transferred to media containing high dose of bialaphos (4 mg/L).

Table 4.2.5B Effect of different selection schemes with bialaphos in the regeneration medium on plant regeneration.

Plate Code†	A	B	C
Bialaphos dose (mg/L)	0	1	4
No. of embryoid transferred	80	80	80
No. of green plants germinated at day 7	51	0	0
No. of green plants germinated at day 21	0	2¶	0
Plant regeneration (%)‡	64[a]	3[b]	0[c]
Plants transferred from A to B & C§			
No. of green plants transferred from A to B & C at day 7		26	25
No. of green plants survived at day 21		10	0
Plant surviving (%)		38[a]	0[b]
Plants transferred from B to C§			
No. of green plants transferred from B to C at day 21			10
No. of green plant survived at day 35			7
Plant surviving (%)			70

† Embryoids of 1-2 mm in diameter derived from microspores of wild type genotype NPBCT were transferred onto 190-2 medium containing bialaphos concentrations of 0(Plate A), 1(Plate B), and 4 mg/L (Plate C).

‡ Means followed by the same letter in the same row are not significantly different with ANOVA and 5% LSD analysis.

§ The 7-day-old germinated green plants on Plate A were transferred to Plate B Plate C. Green plants survived on Plate B were again transferred to Plate C.

¶ Plants died 14 days after transferring to Plate C.

To avoid escapes (false positive transformants containing no introduced genes which survive the selection process), the best selection strategies is to germinate putative transgenic embryoids either on media containing 2 mg/L or higher bialaphos; or to germinate putative transgenic embryoids on media containing none or a low dose (1%) bialaphos followed by transfer of the germinated embryoids to media containing a high dose of bialaphos (4 mg/L). These selection schemes can completely prevent false positive transformants from germinating (escaped) on the regeneration medium.

Based on these selection strategies, over 200 transgenic wheat plants were generated. During summer of 2003 to early spring of 2004, the greenhouse was heavily infected with mites,

and many of the transgenic wheat plants were lost. However, some 24 primary (T_0) spontaneously doubled haploid (DH) transgenic lines were obtained.

4.2.6 Identification of transgenic plants with introduced genes

Polymerase chain reaction (PCR) was performed with plant genomic DNA as a template obtained from four-month-old young leaves of the primary transformants (T_0). Optimal PCR conditions were developed for reliable identification of the bar and xylanase genes in the samples. Various parameters were tested. Among all the PCR parameters, primer sequence was found to be critical. 16 and 9 different combinations of primer sets were tested for the xylanase and bar genes, respectively. The primer sets BAR5' and BAR3', and 5'Hor5 and 3'Liuxyldown were found to be robust for generating a single band for the bar and the xylanase genes, respectively (Table 3.8.1, Figure 4.2.6A). An expected DNA fragment of 373 bp for the bar gene was amplified by PCR with wheat transformants. An expected DNA fragment of 837 bp for the xylanase gene was amplified by PCR with different DNA concentrations of the transformant B4. It is concluded that with the standard quick DNA extraction methods as described in Chapter 3.8.1, by using the described PCR conditions and 0.2 to 2 µl of template DNA, a single band can be reliably generated from positive transformants containing the introduced transgenes bar and xylanase.

In order to confirm that the single band PCR product was truthfully amplified from the bar gene from the transgenic plants other than potential contaminant *Agrobacterium* on plant leaves, total RNA was isolated from transformants, and cDNA was obtained by reverse transcription from the total RNA. The cDNA was used as the DNA template for PCR reactions. The primer set Bar-Ubi1-up and Bar-Ubi1-down was designed to amplify a 1212 bp DNA fragment with the plasmid DNA (Figure 4.2.6B), which contained an intron (1014 bp). PCR

reactions with the same primer set produced an expected 198 bp band with the cDNAs from the transformants (Figure 4.2.6B). The results showed that the intron was successfully removed after the introduced bar gene was transcribed into mRNA. This demonstrates that the wheat transformants carry the bar gene and the PCR products are not due to *Agrobacterium* contamination.

Figure 4.2.6A PCR analysis of primary transformants for identification of the bar and xylanase genes. **a).** Sample code from left to right: 1 kb DNA ladder; 9 transformants; and plasmid DNA. A 373 bp band was produced for the bar gene. **b).** Sample code: 1 = H_2O, 2 = wild type wheat DNA, 3 = 1 kb DNA ladder; 4 = plasmid DNA; 5 to 8 = DNA sample of the primary transformant (T_0) B4 at four concentrations 2, 1, 0.5, and 0.2 µl with the standard DNA extraction methods as described in Chapter 3.8.1. A 837 bp band was produced for the xylanase gene.

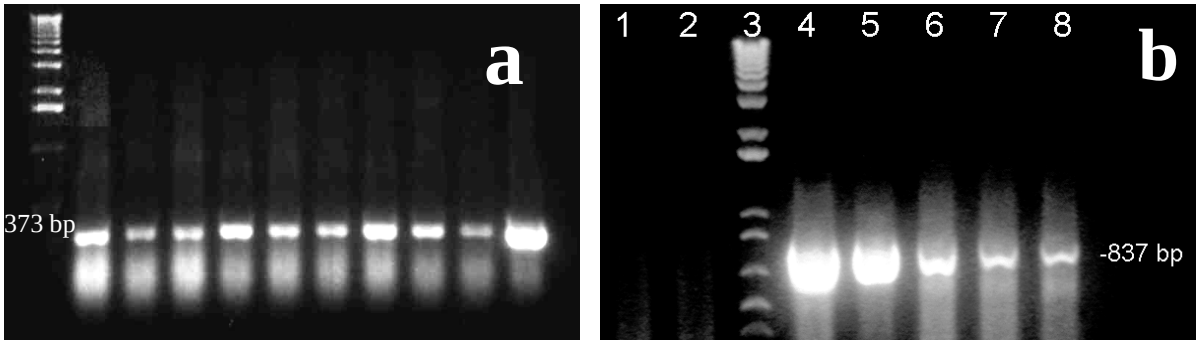
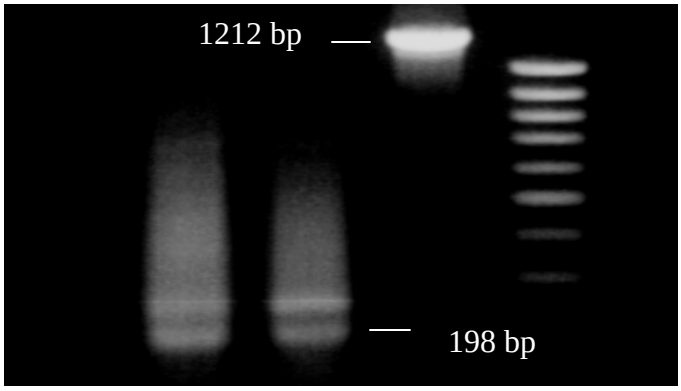
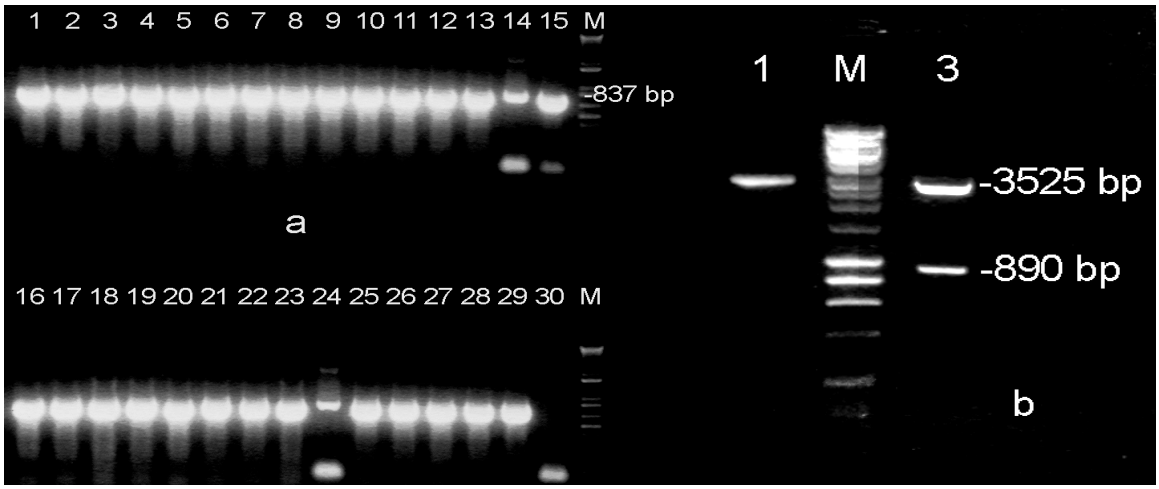


Figure 4.2.6B Reverse Transcription PCR analysis for the bar gene. Sample code from left to right: cDNAs of two wheat transformants; plasmid DNA; 100 bp DNA ladder. A DNA fragment of 198 bp was amplified by PCR from cDNAs of transformants, while a 1212 bp band was produced with plasmid DNA. The gel shift was due to intron removal.



To confirm that the 837 bp DNA fragment amplified by PCR was truly from the transformed xylanase gene, it was purified and cloned into PUC18 vectors and sequenced (Figure 4.2.6C). The sequence was compared to the sequence of xylanase gene used for construction of plasmid RS128/Xyl. Both sequences showed exact match (100% identity). The results indicate that xylanase gene is present in the primary transformants (T_0), and the quick DNA extraction procedure and PCR conditions used in this study works reliably for identification of the xylanase gene in the positive transformant samples.

Figure 4.2.6C Cloning and sequencing of a DNA fragment of 837 bp amplified by PCR from the primary transformant (T_0) B4. **a).** Successful cloning of the DNA fragment into PUC 18 vector that was transformed into the *E. coli* strain DH5*a* cells. An expected single DNA fragment of 837 bp was amplified by PCR in DH5*a* clones (1 to 13, 16 to 23, and 25 to 29). Sample code: 15 = plasmid DNA (positive control). 30 = H2O (negative control). M = 1 kb DNA ladder. **b).** Successful purification of plasmid DNA (PUC 18 containing 837 bp of DNA fragment) and estimation of DNA concentration by enzyme digestion. Sample code: 1 = uncut PUC 18 containing the 837 bp DNA fragment, M = DNA ladder I, 3 = PUC 18 containing the 837 bp DNA fragment digested with enzyme *Hind* III and *EcoR* I. An expected band of 890 bp was cut by enzymes, and DNA concentration was estimated at 80 ng/µl. plasmid DNA purified from Clone #1 was used for sequencing.

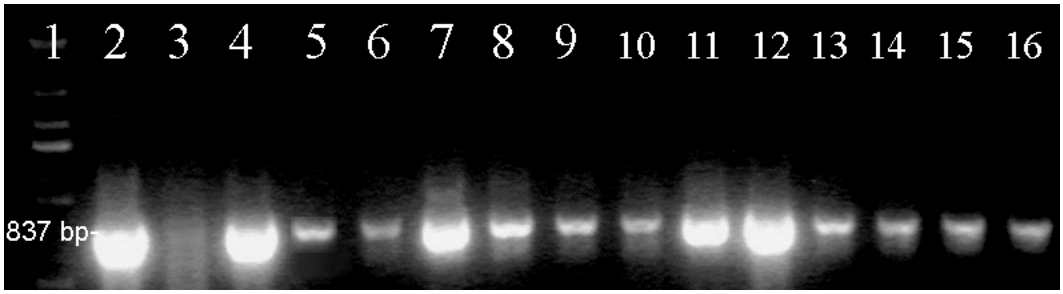


To confirm that xylanase gene was stably integrated into the wheat genome and the integrated gene in T_0 was stably inherited to next generation (T_1), 13 randomly chosen DH seeds (T_1 progenies) from the primary transformant (T_0) B4 were planted and their leaf DNA samples were individually extracted and used for PCR analysis. The primer set (5'Hor5 and 3'Liuxyldown) was used. All 13 T_1 progenies had the 837 bp DNA fragment (Figure 4.2.6D). The results indicate that

(1) The xylanase gene is stably integrated into the wheat genome, and inherited into T_1 generation, and

(2) The primary transformant (T_0) B4 is homozygous for the introduced transgene (Xylanase), and no gene segregations occurred in the T_1 DH progenies.

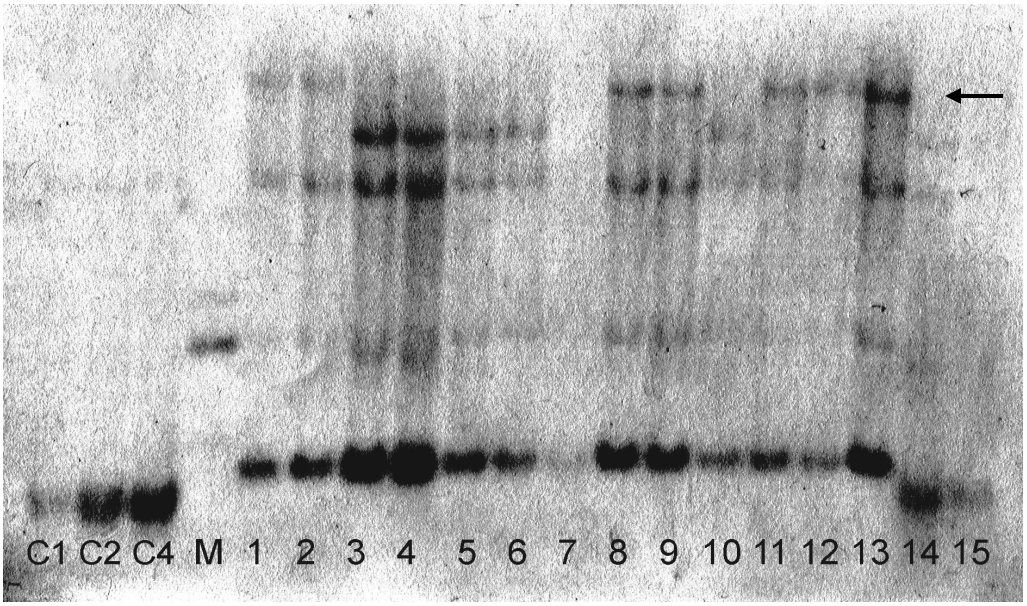
Figure 4.2.6D PCR analysis of 13 randomly chosen DH seeds (T_1 progenies) from the primary transformant (T_0) B4. Sample code: 1 = 1 kb DNA ladder, 2 = plasmid DNA, 3 = wild type wheat DNA; 4 to 16 = 1 to 13 T_1 progenies.



Southern blot analyses were performed with 10 T_1 progenies each from different T_0 transformants, and 3 T_1 progenies originated from the same T_0 transformant (B4). The results showed that DNA samples 8, 9, 11, 12, and 13 had unique hybridization band (high molecular weight, on the very top indicated by an arrow), which the wild type samples (negative controls, lanes 14 and 15) lacked (Fig. 4.2.6E). So it can be concluded that transformants 8 and 9 contain the introduced xylanase gene. Transformants 11, 12, and 13 are three different T_1 progenies from the same T_0 primary transformant B4 as identified by PCR and sequencing analysis (Fig. 4.2.6

A, C & D), showing homozygous status of the introduced xylanase gene (no segregation). Southern analysis also showed one T-DNA insertion site (one copy of xylanase gene) per haploid genome for 5 T_1 seedlings analyzed in this study. The Southern blotting results were in agreement with the PCR and sequence results.

Figure 4.2.6E Southern blot analysis of homozygous T_1 seedlings for identification of transformants containing xylanase gene. Putative transformants were obtained by co-cultivation of microspores with *A. tumefaciens* strain AGL-1 cells containing plasmid RS 128/Xyl carrying xylanase gene driven by *Hor3* promoter and D hordein signal peptide. The genomic DNA was isolated and purified. 10 µl of genomic DNA was digested with Hind III, separated on 1% agarose gel, blotted onto positive charged nylon membrane and probed with the digoxogenin (DIG)-labeled coding region and promoter as well as signal peptide sequences of the transgene xylanase (837 bp). Picture ID: C1, C2, C3 = 2, 4, and 8 copies of the 837 bp probe DNA; M = molecular marker; lanes 1-10 = T_1 doubled-haploid seedlings of 10 different primary (T_0) transformants; lanes 11-13 = T_1 doubled-haploid seedlings of the same primary (T_0) transformant B4; lanes 14-15 = wild type wheat DNA (negative controls).



A fast zymogram method was successfully developed and used for identifying transgenic wheat grains expressing the transgene xylanase. Unstained areas around the transgenic seeds on plate were observed (Fig. 4.2.7), indicating the presence of active xylanase activity. The wild

type wheat grain lacked the xylanase activity, as xylans around the seed on the plate were not hydrolyzed. This method enables fast identification of transgenic grains from large number of seeds.

The standard curve for the amount of xylanase activity at A_{590} was established (Fig. 4.2.8). At the range from 0.125 to 0.625 µg of xylanase, it is a good fit (R^2= 0.999). It was used to quantitatively measure the xylanase activity in transgenic wheat grains.

Transgenic wheat grains (T_2) from six different T_0 transformants, and two barley transgenic grains (T_1) were tested for xylanase activity. All six transgenic wheat samples had significantly higher xylanase activity compared with the non-transformant wild type (Table 4.2.6). The amount of xylanase in the six transgenic wheat samples were estimated to be 0.19 to 0.23 µg per 60 mg of grain or 9.5 to 11.5 µg per gram of wheat grains. The xylanase activity in these transgenic wheat grains was estimated to be 0.49 to 0.59 mini U per 60 mg ground grains or 8.2 to 10 mini U per gram grains. Two transgenic barley samples also had higher xylanase activity compared with the wild type variety Golden Promise (Table 4.2.6).

All results in this study suggest that wheat microspores can be transformed with *Agrobacterium*-mediated techniques, and the transgenic doubled-haploid wheat plants can be generated from the transformed microspores via androgenesis. Moreover, the introduced gene xylanase is successfully expressed in the target tissue i.e. wheat grains.

Figure 4.2.7 Zymogram for identifying transgenic wheat grains containing the active recombinant enzyme 1,4-β-xylanase. Wild type wheat grains (arrow) lack the yellowish ring around the seeds, while transgenic wheat grains have unstained areas around the seeds against the Congo Red stained background.

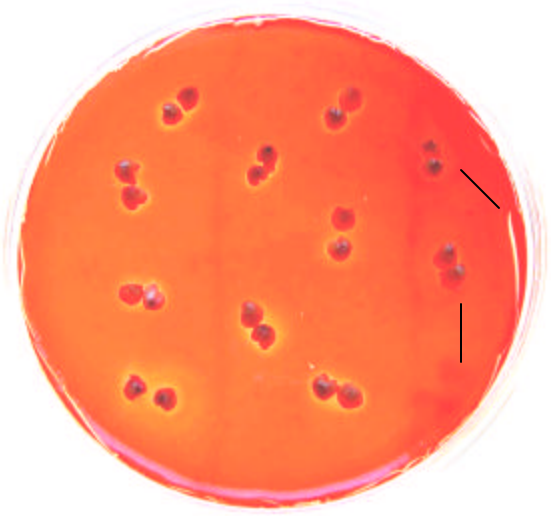


Figure 4.2.8 The standard curve for the amount of xylanase activity plotted against A_{590}. Xylanase was from *Thermomyces langinosus* with 2500U/g expressed in *Aspergillus oryzae* (Sigma Cat X2753).

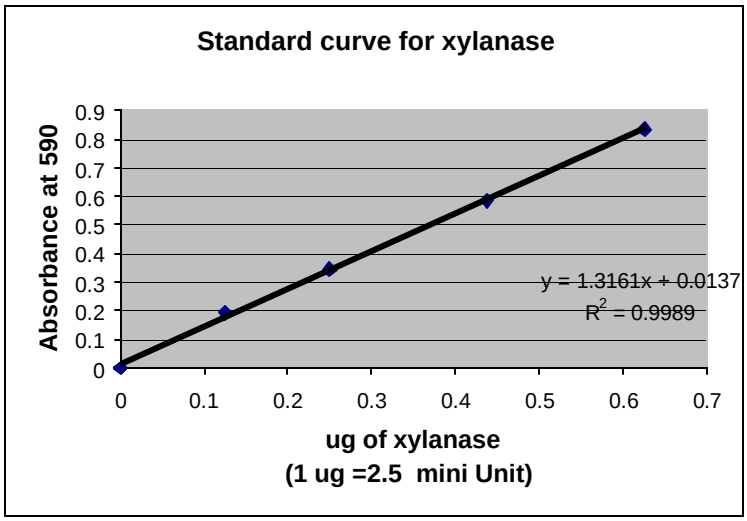


Table 4.2.6 Xylanase activity measured form transgenic wheat and barley grains. The amount of xylanase in seed grains was measured from the standard curve for xylanase and 1 μg xylanase has 2.5 mini U according to product information from Sigma. 150 μl of the 500 μl enzyme extraction solution from 200 mg ground grains was used for the assay.

Sample Name	Genotype	A_{590}	Xylanase amount (μg per 60 mg ground grains)	Xylanase activity (mU per 60 mg ground grains)
WED202-16-2	Wheat wild type	0.1437	0.0988	0.2469
Chris	Wheat wild type	0.1648	0.1148	0.2869
B4	Transgenic WED	0.2989**	0.2167**	0.5418**
RS128/xyl 1	Transgenic WED	0.3036**	0.2203**	0.5507**
RS128/xyl 6	Transgenic Chris	0.2714*	0.1958*	0.4894*
RS128/xyl 8	Transgenic Chris	0.2789*	0.2015*	0.5037*
RS128/xyl 10	Transgenic WED	0.2981**	0.2161**	0.5402**
RS128/xyl 11	Transgenic WED	0.3241**	0.2358**	0.5896**
Golden Promise	Barley wild type	0.3667	0.2682	0.6704
2c	Transgenic GP	0.4270	0.3140	0.7851
2b	Transgenic GP	0.4260	0.3132	0.7831
P-value		0.0108	0.0078	0.0000
LSD (0.01)		0.1464	0.1069	0.2673
LSD (0.05)		0.1038	0.0758	0.1894

** Value is significantly different from the same wild type control with ANOVA and 1% LSD analysis.

* Value is significantly different from the same wild type control with ANOVA and 5% LSD analysis.

CHAPTER FIVE

DISCUSSION

5.1 High efficiency of generating doubled haploid wheat plants by optimizing several conditions

Doubled haploid wheat plants were obtained with high efficiency in this study. The culture system established represents a major advance for DH production in wheat. The overall efficiency in terms of number of estimated green plants/single spike ranges from 50 to 5500, indicating that the procedure would be effective for use in genetic transformation, breeding and research programs. Winter wheat genotypes responded as well as spring wheats when they were fully vernalized. Several factors are important. First, the procedure provides an effective and efficient means to obtain large populations of embryogenic microspores, as demonstrated for the eight genotypes tested. The chemicals 2-HNA and others can effectively trigger microspore embryogenesis. Second, large populations of isolated embryogenic microspores are cultured at optimal conditions to form embryoids and green plants. The optimal conditions include purification of embryogenic microspores from dead microspores and tissue debris, a liquid culture medium with an osmolality around 300 mOsmol Kg^{-1} H_2O, and co-culture with ovaries for providing the nurse factors, which the embryogenic microspores apparently cannot efficiently synthesize. Third, conversion of albinos to green plants can be obtained by adjustment of pretreatment condition, i.e. nutrient addition. Fourth, spontaneous chromosome doubling can be achieved *in vitro* by use of low toxicity chemical caffeine. When this procedure was applied for production of doubled-haploids for over 50 wheat genotypes, microspores of a single spike from

moderately responsive genotypes and crosses have yielded over 100 embryoids and over 50 green plants.

Albinism was one of the major problems in microspore culture. Other reports indicate that the ratio of green to albino plants could be affected by the genetic and environmental factors, including the temperature during pretreatment for inducing microspore embryogenesis (Zhou and Konzak 1997; Hu and Kasha 1999). This study demonstrates that an apparently genetic influence on androgenesis can be overcome, to some extent, by providing improved conditions to microspores for induced androgenesis. While stresses including reduced nutrient availability may be beneficial for the induction of androgenesis, some nutrients are needed for normal green plant formation at the very beginning of androgenesis induction. There are reports that albino plants in wheat and barley have altered plastids in which the DNA has been changed or partially deleted (Day and Ellis 1984, 1985; Chen et al. 1986). The question still remains as to how the lack of nutrients readily available to microspores relates to alterations or deletions in plastid DNA. Further study should be directed towards determining if the observed responses are due to major or minor nutrients, or maltose, and towards determining the effects of nutrient concentration and treatment duration.

If plants appear to be haploid as determined by examining the stomata size and plant morphology, colchicine is usually applied to induce chromosome doubling. However, colchicine has high toxicity, especially to humans. Additionally, it takes extra time and labor to convert haploid to doubled haploid plants by treating haploid plants. It is therefore highly desirable to obtain doubled haploid plants when the chromosome doubling events happen spontaneously in the process of embryoid development. Previous reports indicated that treating plant cells with caffeine causes nuclear fusion in *Vicia faba* root meristem cells (Roper, 1976; Davidson and

Armstrong, 1979), and formation of binucleate 2n-2n cells in onion bulb root-tip cells (Gimenez-Martin, *et al*., 1968). Caffeine was reported to restore fertility of wheat haploid plants (Thomas, *et al.*, 1997). This study demonstrates that an increased spontaneous DH production can be accomplished by the adjustment of culture conditions, with addition of inexpensive chromosome-doubling agents of low toxicity (caffeine) to the induction medium.

Because of the genetically fixed status of the DH lines, the time needed to produce homozygous transgenic wheat plants can be reduced by many months. These make isolated microspore culture an ideal tool for genetic transformation. The developed microspore culture method provided a basis for transformation experiments.

5.2 Successful microspore transformation

In the second part of this study, the developed microspore culture method was used for transforming microspores to generate transgenic wheat plants successfully. Molecular and biochemical evidences (PCR, DNA sequencing of the amplificate, Southern blot analyses and assay of the recombinant enzyme) confirmed the hypothesis that microspores can be transformed and transgenic microspores can be selected and regenerated to produce homozygous DH wheat plants with stably inherited transgene expressing a recombinant protein.

Several factors for successful transformation were identified. First, co-cultivation with *Agrobacterium* for transfer of the plasmid T-DNA into microspores had to take place at day 0 for < 24 hours. The volume of the inoculated AGL-1 cells at OD_{600}=1.0~1.5 had to be < 1% of the co-cultivation solution. It can be presumed that the T-DNA can enter microspores through germinating pores, which are only closed by the plasma membrane, the plasmalemma (Mizelle *et al*., 1989). It is also possible that damages or wounding of microspore cell walls by the blender

may serve as channels for T-DNA invasion into microspores, and enhance transformation (Weber *et al*., 2002). Dormann and co-workers (1995, 1998) transformed rapeseed microspores by co-cultivation with *Agrobacterium* for 2-3 days. However, it was observed in this study that co-cultivation of wheat microspores with *Agrobacterium* could not go beyond 24 hours, or embryogenesis was generally inhibited. Second, killing of AGL-1 cells after co-cultivation was achieved by filtration and addition of timentin at a concentration of 200-400 mg/L. Careful handling of microspores is required to obtain viable microspores after co-cultivation. Third, selection of transformants should be carried out with bialaphos at a concentration of 2-4 mg/L. A high level of escapes has been reported (up to 95%) with the *bar* selection scheme (Vasil et al., 1992, Altpeter et al., 1996, Witrzen *et al*., 1998). In those studies, callus have gone through many days exposure to phosphinothricin (PPT) and glufosinate ammonium, the active ingredient in the herbicide Basta (Hoechst AG) and Liberty (AgrEvo). Callus without the transgenes under these conditions may become tolerant to the herbicide, resulting in escapes. The low level or lack of escapes observed in this study is likely due to the different plant regeneration system used. In the process of androgenesis, individual microspores generally develop into embryoids (no callus stage) (Liu *et al.*, 2002), and the selection scheme starts at the plant regeneration stage. Microspore-derived embryoids without the *bar* gene are sensitive to bialaphos, and are inhibited for germination and plant development. Fourth, identification of transformants by PCR can be carried out when regenerating seedlings were at 4-6 leaf stage, and the transgenic wheat grains expressing the target enzyme (1,4)-β-xylanase can be quickly identified by zymogram.

This is the first report on successful production of transgenic wheat grains expressing a recombinant protein by microspore transformation.

5.3 Outlook

The method described and conditions worked out for microspore transformation in this study are likely applicable to other plant species, especially monocots which have lower transformation efficiency as compared to dicots.

Still, the described method in this study is considered to be imperfect, and there is room for further improvements. One of the areas to be modified is the selection marker design for easy identification of transgenic microspores at the embryoid stage. Visual screening markers such as GFP may be used to aid the identification/selection of transgenic embryoids under florescence microscope. The transgenic embryoid containing the GFP gene will emit florescence, so only transgenic embryoids need to be picked up from large population of embryoids for plant regeneration. Thus, using visual screening markers can save time and labor. This is especially economic in the case of microspore transformation because a large number of embryoids can be produced from a large population of microspores. The drawback is the need of microscope equipped with florescence generator with correct filter sets in order to identify transgenic embryoids containing GFP gene. It is expected that the labor- and time-saving will compensate for the expense of the equipment.

Another area of future research is the early counter-selection of non-transgenic microspores and/or non-transgenic developing-embryoids. This requires application procedures for antibiotics or herbicides in the embryoid induction medium that inhibit non-transgenic microspores to develop into mature embryoids. Such a strategy is expected to work but modification of the embryoid induction medium from liquid to solid or semi-solid is needed when bialaphos is to be used. If this is successful, only transgenic microspores and/or developing-embryoids will regenerate into mature embryoids. Again this will save time and

labor, which are needed for transferring large amount of embryoids for selection at plant regeneration stage as required with the present procedure.

It is also interesting to apply other successful gene-transfer techniques to embryogenic microspores. One promising technique that is expected to work with microspores is the liposome-mediated gene delivery method. The germination pore of microspores may provide a good channel for a liposome-encapsulated T-DNA complex to get into microspores. This method is expected to be more efficient, simpler, easier, and more economic than the *Agrobacterium*-mediated and particle-bombardment techniques. Liposomes are spherical vesicles, with particle sizes ranging from 30 nm to several micrometers, consisting of one or more lipid bilayers surrounding aqueous spaces, and were first described by Bangham and co-workers (1965). The liposome-vesicles can encapsulate aqueous solutions, and have been introduced as drug delivery vehicles due to their structural flexibility in size, composition and bilayer fluidity as well as their ability to incorporate a large variety of both hydrophilic and hydrophobic compounds (Vemuri and Rhodes, 1995; Voinea and Simionescu, 2002). Cationic liposomes form complexes with DNA and can be used in gene delivery (Felgner *et al*., 1987; Farhood *et al*., 1995; Meyer *et al*., 1998; Nakanishi, 2003). The advances in liposomes-mediated gene delivery combined with the elegance of T-DNA offer a very promising future for a simple transformation method. Extensive researches on *Agrobacterium tumefaciens*-mediated plant transformation have yielded detailed information on the T-DNA processing and integration into the host plant genome (Gelvin, 2000; Ziemienowicz *et al*., 1999, 2000, 2001; Ziemienowicz, 2001). Two bacterial virulence proteins, VirD2 and VirE2, play key roles in the nuclear uptake and genomic integration of T-DNA in plants. VirD2 plays a vital role in T-DNA synthesis by recognizing and cleaving the pTi plasmid within the conserved border sequences. VirD2 then is

covalently bound to the 5' end of the ssT-DNA via a phosphotyrosine bond resembling an eukaryotic nuclear localization signal (NLS), which is essential for the nuclear import of the complex. *Agrobacterium* strains deficient in VirD2 are unable to produce viable T-DNA, and mutations in VirD2 motifs result in reduced integration efficiency and deletion of the T-DNA 5' ends. VirE2 covers the entire length of T-DNA thus protecting it from degradation and maintaining its conformation for nuclear import. *Agrobacterium* strains deficient in VirE2 result in extensive deletion of the T-DNA 3' ends and drastically lower transformation efficiency. VirD2 and VirE2 proteins are both essential and sufficient to mediate nuclear import of *in vitro* synthesized T-DNA complexes into mammalian and plant cell nuclei (Ziemienowicz *et al.*, 1999, 2001; Pelczar *et al.*, 2004). This strategy coupled with high efficiency in a microspore regeneration system may yield promising transformation methods for quick production of transgenic plants.

The established method for generating homozygous transgenic wheat plants in one generation via microspore transformation may provide useful means in wheat research and trait development. It allows researchers to routinely introduce cloned genes into wheat plants and express the introduced genes in target tissue in a relatively short period of time. It is expected that the transgenic wheat research will likely play a more important role in wheat improvement in several aspects, such as improving tolerance to biotic and abiotic stress, and nutritional enhancements. Advances in transgenic rice and barley research provide an impetus for wheat researchers. Transgenic rice with agronomically useful genes has been produced, as reviewed by Giri and Laxmi (2000). More recently, rice researchers have tried to manipulate dwarfism, a high value trait. The LAX Panicle gene *LAX* was overexpressed in rice resulting dwarfism accompanied by severe sterility (Komatsu *et al*., 2003). When the gene *OsGA2ox1* coding the

gibberellin catabolic enzyme GA 2-oxidase was overexpressed in rice shoots, normal fertile semi-dwarf rice plants were obtained (Sakamoto *et al.*, 2003). Increase tillering in transgenic rice plants was achieved by overexpressing *MOC1* gene, which encodes a GRAS family nuclear proteins found mainly in axilliary buds (Li *et al.*, 2003). Wang and co-workers (2003) identified the pollen-development specific protein RAFTIN by RNAi method in rice. Katiyar-Agarwal and co-workers (2003) obtained transgenic rice plants with enhanced heat tolerance by overexpression of Hsp101, a heat shock protein from *Arabidopsis thaliana*. Transgenic rice carrying an Na^+/H^+ antiporter gene *OsNHX1* had enhanced salt tolerance (Fukuda *et al*., 2004). Expression of the barley trypsin inhibitor CMe in transgenic rice plants improved their insect resistance (Alfonso-Rubi *et al.*, 2003). Transgenic rice and barley plants have increased disease tolerance (Itoh *et al.*, 2003; Liu *et al.*, 2004; Horvath *et al.*, 2003). Ye and co-workers (2000) introduced the entire provitamin A (β-carotene) biosynthetic pathway into rice endosperm and produced yellow colored rice grains containing provitamin A (Golden Rice). Human milk proteins were expressed in rice and the recombinant protein lactoferrin has similar properties to the native protein (Lonnerdal *et al.*, 2002; Suzuki *et al.*, 2003). Horvath and co-workers (2000) produced transgenic barley grains expressing a recombinant protein (1,3-1,4)-β-glucanase, which depolymerizes the β-glucans of the endosperm cell wall and converts barley into a high-metabolic feed grain.

In this study, transgenic wheat grains were produced that expressed a recombinant enzyme, (1,4)-β-xylanase, which depolymerizes the major wheat endosperm cell wall component (arabinoxylans) therefore increases the feed value of grains (Ankrah, 1999). Researchers used transgenic approaches to combat Fusarium head blight (scab) in wheat and barley (Dahleen *et*

al., 2001; Okubara *et al.*, 2002), and to improve wheat flour quality (Masci *et al.*, 2003; Blechl *et al.*, 2004).

In conclusion, this study demonstrates that it is possible to transform microspores and generate transgenic wheat plants expressing recombinant proteins in the target tissue, i.e. wheat grains. Additionally, this method can generate homozygous DH transgenic grains in less than 6 months for spring wheat. It is anticipated that advances in many areas of biotechnology will lead to more efficient transformation methods and their utilization in wheat improvement.

BIBLIOGRAPHY

Alfonso-Rubi, J., F. Ortego, P. Castanera, P. Carbonero, and I. Diaz. 2003. Transgenic expression of trypsin inhibitor Cme from barley in *Indica* and *Japonica* rice, confers resistance to the rice weevil *Sitophilus oryzae.* Transgenic Res. 12:23-31.

Altpeter, F., V. Vasil, V. Srivastava, E. Stöger, and I.K. Vasil. 1996. Accelerated production of transgenic wheat (*Triticum aestivum* L.) plants. Plant Cell Reports. 16:12-17.

Alvarez, M.L., M. Gomez, J.M. Carrillo, and R.H. Vallejos, 2001. Analysis of dough functionality of flours from transgenic wheat. Molecular Breeding: New Strategies In Plant Improvement. 8(1):103-108.

Alvarez, M.L., S. Guelman, N.G. Halford, S. Lustig, M.I. Reggiardo, N. Ryabushkina, P. Shewry, J. Stein, and R.H. Vallejos. 2000. Silencing of HMW glutenins in transgenic wheat expressing extra HMW subunits. Theoretical and Applied Genetics. 100(2):319-327.

Amoah, B.K., H. Wu, C. Sparks, and H.D. Jones. 2001. Factors influencing Agrobacterium-mediated transient expression of uidA in wheat inflorescence tissue. Journal of Experimental Botany. 52 (358):1135-1142.

Anderson, S.B., I.K.Due, and A. Olesen. 1987. The response of anther culture in a genetically wide material of winter wheat *(Triticum aestivum* L.). Plant Breeding. 99:181–186

Ankrah,N. 1999. Morphology and chemical composition of pearlings from kernels of hulless barley and other cereal grains and the nutritional significance of arabinoxylans from hulless barley in the diets of young pigs. Ph.D. thesis. Washington State University. Pullman, WA.

Aufsatz, W., M.F. Mette, J. van der Winden, M. Matzke, and A. J.M. Matzke. 2002 HDA6, a putative histone deacetylase needed to enhance DNA methylation induced by double-stranded RNA. EMBO J. 21(24):6832-684.

Bangham, A.D., M. M. Standish, and J. C. Watkins. 1965. Diffusion of univalent ions across the la-mellae of swollen phospholipids. J. Mol. Biol. 13:238–252.24.

Barclay, I.R. 1975. High frequencies of haploid production in wheat (*Triticum aestivum*) by chromosome elimination. Nature. 256:410-411.

Bennett, M.D., and W.G. Hughes. 1972. Additional mitosis in wheat pollen induced by Ethrel. Nature. 240:566.

Bird, A. 1992. The essentials of DNA methylation. Cell. 70:5-8.

Blakeslee, A.F., J. Belling, M.E. Farnham, and A.D. Bergner. 1922. A haploid mutant in the jimson weed *Datura stramonium*. Science. 55:1433.

Blechl, A.E., P.P. Bregitzer, K. O'Brien, J.W. Lin, S.B. Nguyen, and O.D. Anderson. 2004. Agronomic, biochemical and quality characteristics of wheats containing hmw-glutenin transgenes. Proceedings - 8th Gluten Workshop. P:9.

Bruins, M.B.M., M. Rakoczy-Trojanowska, and C.H.A. Snijders. 1996. Isolated microspore culture in wheat *(Triticum aestivum* L.): the effect of co-culture of wheat or barley ovaries on embryo-genesis. Cereal Res. Commun. 24:401–408.

Cerutti, H. 2003. RNA interference:traveling in the cell and gaining functions? Trends Genet. 19:39 –46.

Chen, Y.R., Y.F. Huang, T.L. Lin. 1986. Variations of plastids in pollen-derived albino plants of rice. In: Bailey GW (ed) Proc 4th Annu Meet Electron Microsc Soc Am, vol 44: 196–197. San Francisco Press, San Francisco.

Cheng, M., J.E. Fry, S. Pang, H. Zhou, C.M. Hironaka, D.R. Dunkan, T.W.Conner, and Y. Wan. 1997. Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiology. 115:971-980.

Choct, M., and G. Annison. 1990. Anti-nutritive activity of wheat pentosans in broiler diets. British. Poultry Science. 31(4)**:**811-821.

Chu, C.C., and R.D. Hill. 1988. An improved anther culture method for obtaining higher frequency of pollen embryoids in *Triticum aestivum* L. Plant Sci. 55:175-181.

Chugh, A., and P. Khurana. 2003. Regeneration via somatic embryogenesis from leaf basal segments and genetic transformation of bread and emmer wheat by particle bombardment. Plant Cell, Tissue and Organ Culture. 74:151-161.

Cogoni, C., and Macino G. 2000. Post-transcriptional gene silencing across kingdoms. Genes. Dev. 10: 638-643.

Dahleen, L.S., P.A. Okubara, and Blechl. 2001. Transgenic approaches to combat Fusarium head blight in wheat and barley. Crop Sci 41:628-637

Davidson, D., and S.W. Armsytrong. 1979. Cell cycle duration and time of DNA synthesis in binucleate cells induced in V. faba meristems by caffeine or isobutyl-methylxanthine. Protoplasma. 102(3-4):281-293.

Day, A., and T.H.N. Ellis. 1984. Chloroplast DNA deletions associated with wheat plants regenerated from pollen: possible basis for maternal inheritance of chloroplasts. Cell. 39:359–368.

Day, A., and T.H.N. Ellis. 1985. Deleted forms of plastid DNA in albino plants from cereal anther culture. Curr. Genet. 9:671–678.

Demeke, T., P. Hucl, M. Baga, K. Caswell, N. Leung, R.N. Chibbar, 1999. Transgene inheritance and silencing in hexaploid spring wheat. Theoretical and Applied Genetics. 99(6):947-953.

Devaux, P. 1988. Comparison of anther culture and the *Hordeum bulbosum* method for the production of doubled haploids in winter barley. Plant Breeding. 100:181-187.

Doerfler, W. 1995. Uptake of foreign DNA by mammalian cells via the gastrointestinal tract in mice: methylation of foreign DNA- A cellular defense mechanism. In: Meyer P, ed. Gene silencing in higher plants and related phenomena in other eukaryotes. Berlin: Springer-Verlag, 209-224.

Dormann, M., N. Datla, A. Hayden, D. Puttick, and J. Quandt. 1998. Non-destructive screening of haploid embryos for glufosinate ammonium resistance four weeks after microspore transformation in *Brassica*. Acta Horticulturae. 459:191-197.

Dormann, M., H.M. Wang, N. Datla, A.M.R. Ferrie, W.A. Keller, M.M. Oelck. 1995. Transformation of freshly isolated *Brassica* microspores and regeneration to fertile homozygous plants. (Murphy D) Ninth Intern. Rapeseed Congress, (3):816-818.

Dunwell, J.M. 1985. Anther and ovary culture. In: S.W.J. Bright and M.G.K. Jones (ed.) Cereal Tissue and Cell Culture. Matinus Nijhoff/Dr. W. Junk Publishers, Dordrecht/Boston/Lancaster. Netherlands. pp.1-44.

Farhood, H., N. Serbina, and L. Huang. 1995. The role of DOPE in cationic liposomes mediated gene transfer. Biochim Biophys Acta. 1235:289-295.

Felgner, P.L., T. Gadek, M. Holm, R. Roman, H. Chan, M. Wenz, J. Northrop, G.M. Ringold, and R.M. Danielsen. 1987. Lipofection, A highly efficient, lipid-mediated DNA transfection procedure. Proc Natl Acad Sci. 84:7413-7417.

Fire, A., S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, and C.C. Mello. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. 391:806-811.

Folling, L., and A. Olesen. 2001. Transformation of wheat (Triticum aestivum L.) microspore-derived callus and microspores by particle bombardment. Plant Cell Reports. 20(7): 629-636.

Fujii, T. 1970. Callus formation in wheat anther. Wheat Inf. Serv. 31:1-2.

Fukuda, A., A. Nakamura, A. Tagiri, H. Tanaka, A. Miyao, H. Hirochika, and Y. Tanaka. 2004. Function, intracellular localization and the importance in salt tolerance of a vascular Na^+/H^+ antiporter from rice. Plant Cell Physiol. 45:146-159.

Fukuoka, H., T. Ogawa, M. Matsuoka, Y. Ohkawa, and H. Yano. 1998. Direct gene delivery into isolated microspores of rapeseed (*Brassica napus* L) and the production of fertile transgenic plants. Plant Cell Report, 17(5):323-328.

Gelvin, S.B. 2000. *Agrobacterium* and plant genes involved in T-DNA transfer and integration. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 51:223–256.

Gimenez-Martin, G., J.F. Lopez-Saez, P. Moreno, and A.Gonzalez-Fernandez. 1968. On the triggering of mitosis and the division cycle of polynucleate cells. Chromosoma. 25: 282-296.

Giri, C.C., and G.V. Laxmi. 2000. Production of transgenic rice with agronomically useful genes: an assessment. Biotechnol. Adv. 18:15898-15903.

Guo S, and K.J. Kempheus. 1995. *Par-1*, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell. 81:611-620.

Guru, T. 2000. A silence that speaks volumes. Nature. 404:804-808.

Gustafson, V.D., P.S. Baezinger, A. Mitra, H.F. Kaeppler, C.M. Papa, and S.M. Kaeppler. 1995. Electroporation of wheat anther culture derived embryoids. Cereal Research Communications. 23:207-213.

Hammond, S.M., A.A. Caudy, G.J. Hannon. 2001. Post-transcriptional Gene Silencing by Double-stranded RNA. Nature Rev. Gen. 2:110-119.

Hamilton, A.J., D.C. Baulcombe. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science. 286:950-952.

Horvath, H., J. Huang, O.T. Wong, E. Kohl, T. Okita, C.G. Kannangara, and D. von Wettstein. 2000. The production of recombinant proteins in transgenic barley grains. Proc. Natl. Acad. Sci. USA 97:1914-1919.

Horvath, H., J.Huang, O.T. Wong, and D.von Wettstein. 2002. Experiences with genetic transformation of barley and characteristics of transgenic plants. In: Barley Science. G.A. Slafer, J.L. Molina-Cano, R. Savin, J.L. Araus & J. Romagosa (eds.) The Harworth Press, New York. pp. 143-176.

Horvath, H., N. Rostoks, R. Brueggeman, B. Steffenson, D. von Wettstein, and A. Kleinhofs. 2003. Genetically engineered stem rust resistance in barley using the *Rpg 1* gene. Proc. Natl. Acad. Sci. USA. 100:364-369.

Hu, H. 1997. In vitro induced haploids in wheat. *In* M.S. Jain et al. (ed.) In Vitro Haploid Production in Higher Plants. Kluwer Academic Publishers, Boston/Dordrecht.

Hu, T, and K.J. Kasha. 1997. Improvement of isolated microspore culture of wheat (*Triticum aestivum* L.) through ovary co-culture. Plant Cell Rep. 16:520–525.

Hu, T, and K. Kasha. 1999. A cytological study of pretreatments used to improve isolated microspore cultures of wheat (Triticum aestivum L.) cv. Chris. Genome. 42(3):432-441.

Hutvagner, G, and P.D. Zamore. 2002. RNAi: nature abhors a double-strand. Curr Opin. Genetics & Development. 12:225-232.

Ingelbrecht, I., H. Van Houdt, M. Van Montagu, and A. Depicker. 1994. Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. Proc. Natl. Acad. Sci. USA. 91:10502-10506.

Itoh, Y., K. Takahashi, H. Takizawa, N. Nikaidou, H. Tanaka, H. Nishihashi, T. Watanabe, and Y. Nishizawa. 2003. Family 19 chitinase of *Streptomyces griseus* HUT6037 increases plant resistance to the fungal disease. Biosci. Biotechnol. Biochem. 64:847-855.

Jahne, A., D. Becker, R. Brettschneider, and H. Lorz. 1994. Regeneration of transgenic, microspore-derived, fertile barley. Theor. Appli. Genet. 89(4):525-533.

Jakowitsch, J., M.F. Mette, J. van der Winden, M.A. Matzke, and A.J.M. Matzke. 1999. Integrated pararetroviral sequences define a unique class of dispersed repetitive DNA in plants. Proc. Natl. Acad. Sci. *USA* 96:13241-13246.

Jensen, L.G., O. Politz, O. Olsen, K. K.Thomsen, and D.von Wettstein. 1998. Inheritance of a codon optimized transgene expressing heat stable (1,3-1,4)-β-glucanase in scutellum and aleurone of germinating barley. Hereditas 129:215-225.

Jorgensen, R.A., P.D. Cluster, J. English, Q. Que, and C.A. Napoli. 1996. Chalcone synthase cosuppression phenotypes in petunia flowers: comparison of sense vs. antisense constructs and single-copy vs. complex T-DNA sequences. Plant Mol. Biol. 31:957-973.

Kanno, T., M.F. Mette, D. Kreil, W. Ausatz, M. Matzke, and A. Matzke. 2004. Involvement of putative SNF2 chromatin remodeling protein DRD1 in RNA-directed DNA methylation. Current Biology. 14:801-805.

Kasha, K.J. 1989. Production of haploids in cereals. In: M. Maluszynski (ed.) Current Options for Cereal Improvement. Kluwer Academic Publishers, London. pp. 71-80.

Kasha, K.J., and K.N. Kao. 1970. High frequency of haploid production in barley (*Hordeum vulgare* L.). Nature. 225:874-875.

Katiyar-Agarwal, S., M. Agarwal, and A. Grover. 2003. Heat-telerant basmati rice engineered by over-expression of *hsp 101*. Plant Molec. Biol. 51:677-686.

Klein, T.M., E.D. Wolf, R. Wu, and J.C. Sanford. 1987. High-velocity microprojectiles for delivering nucleic acids into living cell. Nature. 327:70-73.

Kleinhofs, A., A. Kilian, M.A.S. Maroof,, R.M. Biyashev, P. Hayes, F.O. Chen, N. Lapitan, A. Fenwick, T.K. Blake, and V. Kanazin. 1993. A molecular, isozyme and morphological map of the barley (Hordeum vulgare) genome. Theor. Appl. Genet. 86(6):705-712

Kohl, E.A. 2003. Development of transgenic barley expressing (1,4)-β-xylanase. M.Sc. thesis. Washington State University. Pullman, WA.

Kohler, F., and G. Wenzel. 1985. Regeneration of isolated barley microspores in conditioned media and trials to characterize the responsible factors. J. Plant Physiol. 121:181-191.

Komatsu, K., M. Masahiko, S. Ujiie, Y. Satake, F. Ikuo, H. Okamoto, K. Shimamoto, and J. Kyozuka. 2003. *LAX* and *SPA*: Major regulators of shoot branching in rice. Proc. Natl. Acad. Sci. USA. 100:11765-11770.

Koncz, C., N. Martini, R. Mayerhofer, Z. Koncz-Kalman, H. Korber, G.P. Redei, and J. Schell. 1989. High-frequency T-DNA-mediated gene tagging in plants. Proc. Natl. Acad. Sci. USA. 86:8467-8471.

Kyo, M., and H. Harada. 1985. Studies on conditions for cell division and embryogenesis in isolated pollen culture of *Nicotiana rustica*. Plant Physiol. 79:90-94.

Lazo, G.R., P.A. Stein, and R.A. Ludwig. 1991. A DNA transformation-competent *Arbidopsis* genomic library in *Agrobacterium*. Bio/Tech. 9:963-967.

Laurie, D.A., and M.D. Bennet. 1988a. The production of haploid wheat plants from wheat x maize crosses. Theor. Appl. Genet. 76:393-397.

Laurie, D.A., and M.D. Bennet. 1988b. Cytological evidence for fertilization in haxaploid wheat x sorghum crosses. Plant Breeding. 100:73-82.

Lentner, M., and T. Bishop. 1993. Experimental design and analysis. Valley Book Company. P.O. Box 884, Blacksburg, VA 24063, USA.

Li, X., Q. Qian, Z. Fu, Y. Wang, G. Xiong, D. Zeng, X. Wang, X. Liu, S. Teng, F. Hiroshi, M. Yuan, D. Luo, B. Han, and J. Li. 2003. Control of tillering in rice. Nature. 422:618-621.

Liu, J., M.A. Carmell, F.V. Rivas, C.G. Marsden, J.M. Thomson, J.J. Song, S.M. Hammond, L. Joshua-Tor, and G.J. Hannon. 2004. Argonaute2 Is the Catalytic Engine of Mammalian RNAi. Science. 305:1437-1441.

Liu, M., Z. Sun, J. Zhu, T. Xu, G.E. Harman, and M. Lorito. 2004. Enhancing rice resistance to fungal pathogens by transformation with cell wall degrading enzyme genes from *Trichoderma atroviride*. J. Zhejiang Univ. Sci. 5:133-136.

Liu, W., M. Zheng, and C.F. Konzak. 2002. Improving green plant production via isolated microspore culture in bread wheat (*Triticum aestivum* L.). Plant Cell Reports. 20:821-824.

Liu, W., M. Zheng, E. Polle, and C.F. Konzak. 2002. Highly efficient doubled-haploid production in wheat (T*riticum aestivum* L.) via induced microspore embryogenesis. Crop Science. 42:686-692.

Loeb, T.A., and T.L. Reynolds. 1994. Transient expression of the *uid*A gene in the pollen embryoids of wheat following microprojectile bombardment. Plant Sci. 104:81-91.

Lonnerdal, B., 2002. Expression of human milk proteins in plants. J. Am. Coll. Nutr. 21:218S-221S.

Maheshwari, S.C., A. Raishid, and A.K. Tyagi. 1983. Anther pollen culture for production of haploids and their utility. IAPTC Newsl. 41:2-7.

Marsan, P.A., E. Lupotto, F. Locatelli, Y.M. Qiao, and M. Cattaneo. 1993. Analysis of stable events of transformation in wheat via PEG-mediated DNA uptake into protoplasts. Plant Science. 93:85-94.

Masci, S., R. D'Ovidio, F. Scossa, C. Patacchini, D. Lafiandra, O.D. Anderson, and Blechl. 003. Production and characterization of a transgenic bread wheat line over-expressing a low-molecular weight glutenin subunit gene. Molecular Breeding. 12:209-222.

Matzke, M.A., and A.J.M. Matzke. 1991. Differential inactivation and methylation of a transgene in plants by 2 suppressor loci containing homologous sequences. Plant Molecular Biology. 16:821-830.

Matzke, M.A, M.F. Mette, J. Jakowitsch, T. Kanno, E. A. Moscone, J. van der Winden, and A. J. M. Matzke. 2001. A Test for Transvection in Plants: DNA Pairing May Lead to *trans*-Activation or Silencing of Complex Heteroalleles in Tobacco. Genetics. 158: 51-461.

Matzke, A.J.M., F. Neuhuber, Y.D. Park, P.F. Ambros, and M.A. Matzke. 1994. Homology-dependent gene silencing in transgenic plants: epistatic silencing loci contain multiple copies of methylated transgenes. Molecular & General Genetics. 244:219-229.

Mentewab, A., V. Letellier, C. Marque, and A. Sarrafi. 1999. Use of anthocyanin biosynthesis stimulatory genes as markers for the genetic transformation of haploid embryos and isolated microspores in wheat. Cereal Research Communications. 27(1-2):17-24.

Mersereau, M., G.J. Pazour, and A. Das. 1990. Efficient transformation of *Agrobacterium tumefaciens* by electroporation. Gene. 90:149-151.

Mette, M.F., J. van der Winden, M.A. Matzke, and A.J.M. Matzke. 1999. Production of aberrant promoter transcripts contributes to methylation and silencing of unlinked homologous promoters *in trans*. EMBO J. 18:241-248.

Mette, M.F., W. Aufsatz, J.van der Winden, M.A. Matzke, and A.J.M. Matzke. 2000. Transcriptional gene silencing and promoter methylation triggered by double stranded RNA. EMBO J. 19:5194-5201.

Meyer, O., D. Kirpotin, K. Hong, B. Sternberg, J.W. Park, M.C. Woodle, and D. Papahadjopoulos. 1998. Cationic liposomes coated with polyethylene glycol as carriers for oligonucleotides, J Biol Chem. 273:15621-15627.

Meyer, P., and I. Heidmann. 1994. Epigenetic variants of a transgenic petunia line show hypermethylation in transgene DNA: an indication for specific recognition of foreign DNA in transgenic plants. Molecular & General Genetics. 243:390-399.

Meyer, P., I. Heidmann, and I. Niedenhof. 1993. Differences in DNA-methylation are associated with a paramutation phenomenon in transgenic petunia. Plant Journal. 4:89-100.

Milligan, A.S., A. Daly, M.A.J. Parry, P.A. Lazzeri, and I. Jepson. 2001. The expression of a maize glutathione S-transferase gene in transgenic wheat confers herbicide tolerance, both in planta and in vitro. Molecular breeding: new strategies in plant improvement. 7(4):301-315.

Mizelle, M.B., R. Sethi, M.E. Ashton, and W.A. Jensen. 1989. Development of the pollen grain and tapetum of wheat (Triticum aestivum) in untreated plants and plants treated with chemical hybridizing agent RH0007. Sexual Plant Reproduction. 2(4):231-252.

Molinier, J., C. Himber, and G. Hahne. 2000. Use of green fluorescent protein for detection of transformed shoots and homozygous offspring. Plant Cell Reports. 19:219-223.

Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Nakanishi, M. 2003. New strategy in gene transfection by cationic transfection lipids with a cationic cholesterol. Curr Med Chem. 10(14):289-96.

Napoli, C., C. Lemieux, and R. Jorgensen. 1990. Introduction of a chalcone synthase gene into *Petunia* results in reversible co-suppression of homologous genes in trans. Plant Cell. 2:279-289.

Nauerby, B., K. Billing, and R. Wyndaele. 1997. Influence of the antibiotic timentin on plant regeneration compared to carbenicillin and cefotaxime in concentrations suitable for elimination of *Agrobacterium tumefaciens*. Plant Science. 123:169-177.

Okubara, P.A., A.E. Blechl, S.P. McCormick, N.A. Alexander, R. Dill-Macky, and T.M. Hohn. 2002. Engineering deoxynivalenol metabolism in wheat through the expression of a fungal trichothecene acetyltransferase gene. Theor. Appl. Genet. 106:74-83.

Ouyang, T.W., H. Hu, C.C. Chuang, and C.C. Tseng. 1973. Induction of pollen plants from anther of *Triticum aestivum* L. cultured *in vitro*. Sci. Sin. 16(1):79-95.

Palauqui, J.C., T. Elmayan, J.M. Pollien, and H. Vaucheret. 1998. Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. Embo. J. 16:4738-4745.

Park,Y.D., I. Papp, E.A. Moscone, V.A. Iglesias, H. Vaucheret, A.J.M. Matzke, and M.A. Matzke. 1996. Gene silencing mediated by promoter homology occurs at the level of transcription and results in meiotically heritable alterations in methylation and gene activity. Plant Journal. 9:183-194.

Pastori, G.M., M.D. Wilkinson, S.H. Steele, C.A. Sparks, H.D. Jones, and M.A.J. Parry. 2001. Age-dependent transformation frequency in elite wheat varieties. Journal of Experimental Botany. 52(357):857-863.

Pelczar, P., V. Kalck, D. Gomez, and B. Hohn. 2004. Agrobacterium proteins VirD2 and VirE2 mediate precise integration of synthetic T-DNA complexes in mammalian cells. EMBO. 5(6):632-637.

Pellegrineshi, A., L.M. Noguera, B. Skovmand, R.M. Brito, L. Velazquez, M.M. Salgado, R. Hernandez, M. Warburton, and D. Hoisington. 2002. Identification of highly transformable wheat genotypes for mass production of fertile transgenic plants. Genome. 45:421-430.

Picard, E., C. Hours, S. Gregoire, T.H. Phan, and J.P. Meunier. 1987. Significant improvement of androgenetic haploid and doubled-haploid induction from wheat plants treated with a chemical hybridization agent. Theor. Appl. Genet. 74:289-297.

Prols, F., and P. Meyer. 1992. The methylation patterns of chromosomal integration regions influence gene activity of transferred DNA in *Petunia hybrid*a. Plant Journal. 2:465-475.

Puolimatka, M., S. Laine, and J. Pauk. 1996. Effect of ovary co-cultivation and culture medium on embryogenesis of directly isolated microspores of wheat. Cereal Res. Commun. 24:393–400.

Reynolds, T.L. 1997. Pollen embryogenesis. Plant Molecular Biology. 33(1):1-10.

Rode, L.M., W.Z. Yang, and K.A. Beauchemin. 1999. Fibrolytic enzyme supplements for dairy cows in early lactation. J. Dairy Sci. 82 (10):2121-2126.

Roper, W. 1976. Nuclear fusion and irregular cytokinesis in binucleate and tetraploid cells of Vicia faba after caffeine treatment. Experientia. 32(10):1260-1262.

Rowell, P.L., and D.G. Miller. 1971. Induction of male sterility in wheat with 2-Chloroethylphosphonic Acid (Ethrel). Crop Sci. 11:629-631.

Sakamoto, T., Y. Morinaka, K.Ishiyama, M. Kobayashi, Y. Itoh, T. Kayano, S. Iwahori, M. Matsuoka, and H. Tanaka. 2003. Genetic manipulation of gebberellin metabolism in transgenic rice. Nature Biotechnol. 21:909-913.

Sa-Pereira, P., M. Costa-Ferreira, and M.R. Aires-Barros. 2002. Enzymatic properties of a neutral *endo*-1,3(4)-β-xylanase Xyl II from *Bacillus subtilis*. J. Biotechnology. 94:265-275.

Schingoethe, D.J., G.A. Stegeman, and R.J. Treacher. 1999. Response of lactating dairy cows to a cellulase and xylanase enzyme mixture applied to forages at the time of feeding. J. Dairy Sci. 82 (5):996-1003.

Simonson, R.L., P.S. Baenziger, and V.D. Gustafson. 1997. Wheat anther culture as affected by various cultural changes and supplements. J. of Appl. Genet. 38(4):381-392.

Song, J.J., S.K. Smith, G.J. Hannon, and L. Joshua-Tor. 2004. Crystal structure of argonaute and its implications for RISC slicer activity. Science. 305(5689):1434-1437.

Songstad, D.D., D.A. Somers, and R.J. Griesbach. 1995. Advances in alternative DNA delivery techniques. Plant Cell Tiss. Org. Culture. 40:1-15.

Sorokin, A.P., X. Ke, D. Chen, and M.C. Elliott. 2000. Production of fertile transgenic wheat plants via tissue electroporation. Plant Sci. 156:227-233.

Stahl, R., H. Horvath, J. Van Fleet, M. Voetz, D. von Wettstein, and N. Wolf. 2002. T-DNA integration into the barley genome from single and double cassette vectors. Proc. Natl. Acad. Sci. USA. 99:2146-2151.

Stoger, E., C. Fink, M. Pfosser, and E. Heberle-Bors. 1995. Plant transformation by particle bombardment of embryogenic pollen. Plant Cell Rep. 14(5):273-278.

Suzuki, Y.A., S.L. Kelleher, D. Yalda, L. Wu, J. Huang, N. Huang, and B. Lonnerdal. 2003. Expression, characterization, and biologic activity of recombinant human lactoferrin in rice. J. Pediatr. Gastroenterol. Nutr. 2:190-199.

Takumi, S., and T. Shimada. 1996. Production of transgenic wheat through particle bombardment of scutellar tissues: frequency is influenced by culture duration. J. Plant Physiol. 49:418-423.

Tang, G., B.J. Reinhart, D.P. Bartel, and P. D. Zamore. 2003. A biochemical framework for RNA silencing in plants. Genes Dev. 17:49–63.

Thomas, J., Q. Chen, and N. Howes. 1997. Chromosome doubling of haploids of common wheat with caffeine. Genome 40:552-558.

Touraev, A., A. Indrianto, I. Wratschko, O. Vicente, and E. Heberle-Bors. 1996. Efficient microspore embryogenesis in wheat (*Triticum aestivum* L.) induced by starvation at high temperature. Sexual Plant Reproduction. 9(4):209-215.

Touraev, A., O. Vicente, and E. Herberle-Bors. 1997. Initiation of microspore embryogenesis by stress. Trends in Plant Science Reviews. 2(8):297-302.

Tuvesson, I.K.D., S. Perdersen, S.B. Andersen. 1989. Nuclear genes affecting albinism in wheat *(Triticum aestivum* L.). Theor. Appl. Genet. 78:879–883

USDA/ARS, Briefing Room: wheat, U.S. Department of Agriculture, available at http://www.ers.usda.gov/Briefing/Wheat/ (last visited Dec. 16, 2003).

Van Fleet, J. 2001. Production of recombinant human lysozyme and lactoferrin in transgenic barley grains. M.Sc. thesis. Washington State University. pp. 1-75.

Varshney, A., and F. Altpeter. 2001. Stable transformation and tissue culture response in current European winter wheats (Triticum aestivum L.). Molecular breeding: new strategies in plant improvement. 8(4):295-309.

Vasil, V., A.M. Castillo, M.E. Fromm, and I.K. Vasil. 1992. Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Bio/Technology. 10:667-674.

Vasil, V., V. Srivastava, A.M. Castillo, M.E. Fromm, and I.K. Vasil. 1993. Rapid production of transgenic wheat plants by direct bombardment of cultured immature embryos. Bio/Technology. 11:1553-1559.

Vemuri, S., and C.T. Rhodes. 1995. Preparation and characterization of liposomes as therapeutic delivery systems: a review. *Pharmacautica Acta Helvitae.* 70:95–111.

Voinea, M., and M. Simionescu. 2002. Designing of 'intelligent' liposomes for efficient delivery of drugs. J Cell Mol Med. 6(4):465-74.

Voinnet, O. 2003. RNA silencing bridging the gaps in wheat extracts. Trends in Plant Science. 8(7):307-309.

Wan, Y., and Lemaux, P.G. .1994. Generation of large numbers of independently transformed fertile barley plants. Plant Physiology. 104:37-48.

Wang, A., Q. Xia, W. Xie, R. Datla, and G. Selvaraj. 2003. The classic Ubisch bodies carry a sporophytically produced structural protein (RAFTIN) that is essential for pollen development. Pro. Natl. Acad. Sci. USA. 100:14487-14492.

Wang, J.L., C.S. Sun, T.G. Lu, A. Fang, H.R. Cui, S.Z. Cheng, and C. Xiang. 1991. Fertilization and embryological development in wheat x maize crosses. Acta. Bot. Sin. 33(9):674-679.

Weber, S., W. Friedt, N. Landes, J. Molinier, C. Himber, P. Rousselin, G. Hahne, and R. Horn. 2002. Improved *Agrobacterium* -mediated transformation of sunflower (*Helianthus annuus* L.): assessment of macerating enzymes and sonication. Plant Cell Reports. 10.1007/s00299-002-0548-7.

Weir, B., X. Gu, M.B. Wang, N. Upadhyaya, A.R. Elliot, and R.I.S. Brettell. 2001. Agrobacterium tumefaciens-mediated transformation of wheat using suspension cells as a model system and green fluorescent protein as a visual marker. Australian Journal of Plant Physiology. 28(8):807-818.

Witrzens, B., R.I.S. Brettell, F.R. Murray, D. McElroy, Z. Li, and E.S. Dennis. 1998. Comparison of three selectable marker genes for transformation of wheat by microprojectile bombardment. Australian J. Plant Physiol. 25:39-44.

Wright, M., J. Dawson, E. Dunder, J. Suttie, J. Reed, C. Kramer, Y. Chang, R. Novitzky, H. Wang, and L. Artim-Moore. 2001. Efficient biolistic transformation of maize (Zea mays L.) and wheat (Triticum aestivum L.) using the phosphomannose isomerase gene, pmi, as the selectable marker. Plant Cell Reports. 20(5):429-436.

Yao, Q., E. Simion, M. William, J. Krochko, and K.J. Kasha. 1997. Biolistic transformation of haploid isolated microspores of barley (*Hordeum vulgare* L.). Genome. 40:570-581.

Ye, X. S. Al-Babili, A. Kloti, J. Zhang, P. Lucca, P. Beyer, and I. Potrykus. 2000. Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotene-free) rice endosperm. Science. 287:303-305.

Ziemienowicz A. 2001. Odyssey of agrobacterium T-DNA. Acta Biochim Pol. 48(3):623-35.

Ziemienowicz A, B. Tinland, J. Bryant, V. Gloeckler, and B. Hohn. 2000. Plant enzymes but not Agrobacterium VirD2 mediate T-DNA ligation in vitro. Mol Cell Biol. 20(17):6317-22.

Ziemienowicz A, D. Gorlich, E. Lanka, B. Hohn, and L. Rossi. 1999. Import of DNA into mammalian nuclei by proteins originating from a plant pathogenic bacterium. Proc Natl Acad Sci U S A. 30;96(7):3729-33.

Ziemienowicz A, T. Merkle, F. Schoumacher, B. Hohn, and L. Rossi. 2001. Import of Agrobacterium T-DNA into plant nuclei: two distinct functions of VirD2 and VirE2 proteins. Plant Cell. 13(2):369-83.

Zheng, M.Y., W. Liu, Y. Weng, E. Polle, and C. F. Konzak. 2001. Culture of freshly isolated wheat (*Triticum aestivum* L.) microspores treated with inducer chemicals. Plant Cell Rep. 20:685–690.

Zheng, M.Y., Y. Weng, W. Liu, and C. F. Konzak. 2001.The effect of ovary-conditioned medium on microspore embryogenesis in common wheat (*Triticum aestivum* L.). Plant Cell Rep. 20:802–807.

Zhou, H., J. D. Berg, S. E. Blank, C. A. Chay, G. Chen, S. R. Eskelsen, J. E. Fry, S. Hoi, T. Hu, P. J. Isakson, M. B. Lawton, S. G. Metz, C. B. Rempel, D. K. Ryerson, A. P. Sansone, A. L. Shook, R. J. Starke, J. M. Tichota, and S. A. Valenti. 2003. Field Efficacy Assessment of Transgenic Roundup Ready Wheat. Crop Sci. 43:1072–1075.

Zhou, H., and C.F. Konzak. 1997. Influence of genetic and environmental factors on anther culture response of wheat. J. of Appl. Genet. 38(4):393-406.

Zhou, H., C.M. Stiff, and C.F. Konzak. 1993. Stably transformed callus of wheat by electroporation-induced direct gene transfer. Plant Cell Reports, 12:612-616.

Zhuang, J. J., and J. Xu. 1983. Increasing differentiation frequencies in wheat pollen callus. In: Hu, H. and M. R. Vega (ed.) Cell and Tissue Culture Techniques for Cereal Crop Improvement. Science Press, Beijing, P. R. China. pp. 431.

APPENDIX

A. Plant genomic DNA isolation procedure

(1) From approximately 4 week old plants 1-2 cm of young leaf is punched into a 1.5 ml Eppendorf tube using the lid of the tube. Avoid touching the cut end. Put leaf into the tube, close lid and tear leaf off. Make sure the leaf is not hanging out of the tube. Close the lid and place it on ice.

(2) Store the tube containing leaf samples in a freezer at –18 ^{0}C for future use.

(3) Take tubes out of freezer. Add 400 µl of extraction buffer and immerse the leaf completely in the buffer by using the pipette tip. Place the tubes in a rack at room temperature. Grind leaf samples to a fine powder using a grinder for about 1 minute. Place a clean pestle attached to a drill and grind directly in the Eppendorf tube, the blue plastic reusable pestles fit down into the 1.5 ml tubes. Be careful to place the pestle tip so that the leaf is under the tip or to the side, and move the tube for efficient grinding. Avoid spilling by adjusting the grind speed (speed controller position 3-4).

DNA extraction buffer (10 ml):

Stock solution		Final concentration
1M Tris-HCl pH 8.0	2 ml	20 mM
5M NaCl	0.5 ml	250 mM
0.5 M EDTA pH 8.0	0.5 ml	25 mM
20% SDS	0.25 ml	0.5%
dH_2O	6.75 ml	

(4) In hood add 400 µl of chloroform: isoamyl alcohol (24:1 v/v), vortex well and centrifuge at 13, 000 rpm for 10 minutes.

(5) Transfer 300 µl of the clear supernatant to a fresh tube. Use yellow tips and avoid touching with pipette tip any debris or chloroform. Dump the chloroform into a waste bottle and spent tube to a waste container in the hood.

(6) Add 300 µl of isopropanol and mix well by inverting the tube a couple of times. Allow standing at room temperature for 1 to 2 hours and centrifuge at 13, 000 rpm for 10 minutes. A small pellet should be visible.

(7) Decant supernatant and wash the pellet 3 times with 75% ethanol using a squirt bottle. Take care not to squirt at the pellet and dislodge the pellet. After last wash Suck off any ethanol visible in the tube without touching the pellet.

(8) Leave the tube on the counter for about 45 minute so that the tubes are completely dry.

(9) Add 50 µl of sterile H_2O and use the pipette tip to suspend the pellet. Leave at 4 ^{0}C overnight for the entire DNA to dissolve.

(10) Centrifuge for 3 minutes at 13, 000 rpm and collect the clear supernatant with DNA. Store at 4 ^{0}C or –18 ^{0}C for long term storage. Use 1 µl as a template for PCR analysis.

B. Southern blot procedure

Isolation of plant genomic DNA

(1) Harvest 1.5-2 g (about 3-4 full leaf blades) fresh leaf material of 3-4 week old young seedlings or very young inflorescence by placing cut pieces (1 in.) into a 50 mL centrifuge tube to be closed with a cap or into a ziplock bag, and place into liquid nitrogen after sealing and cutting small vent into bag. Minimize the time (1-2 min per plant).

(2) Lyophilize to total dryness and store samples in sealed plastic bag (with desiccant) at -20 ^{0}C until used. Samples prepared and stored this way will be good for extraction for many years. Or store fresh samples at -80 ^{0}C.

(3) Put 0.1 g dry or 1.0-1.5 g fresh materials in a 50 ml mortar and pestle, add some liquid nitrogen and carefully grind to a very fine powder. This takes about 30 seconds. Put the powdered sample into a15 ml polypropylene conical tube.

(4) Add 7 ml of extraction buffer (110 mM Tris pH8.0, 55 mM EDTA pH8.0, 1.54 M NaCl, 1.1 % CTAB; Tris is from a 1 M stock pH8.0, EDTA is from a 0.5M stock pH8.0, NaCl is from a 5 M stock, CTAB is from a 10% stock kept in 3 7 ^{0}C incubator.), which has been pre-warmed to 65 ^{0}C. Do one tube and then do step 5 & 6 to that tube before doing another tube.

(5) Immediately vortex tube thoroughly so there are no chunks.

(6) Immediately add 0.7 ml of 20% SDS. Mix tube gently without making bubbles by end over end rotation and incubate in 65 ^{0}C water bath for 2 hr with occasional inversion. (The final composition is now 100 mM Tris, 50 mM EDTA, 1.4 M NaCl, 1 % CTAB, and 2% SDS).

(7) Remove from water bath and let cool 5 minutes on lab bench.

(8) Add chloroform: (24 parts chloroform: 1 part isoamylalcohol) to 14ml mark on tube.

(9) Mix thoroughly for at least 15 minutes by inversion (not too fast) to form an emulsion (two phases not visible).

(10) Centrifuge 20 minutes in clinical centrifuge at 2000 rpm to separate the phases.

(11) Remove top phase slowly with disposable 10 ml pipette and transfer to fresh 15 ml polypropylene conical tube. Do all the tubes.

(12) Do this step one tube at a time. Add 0.6 volume of isopropanol or 2-propanol (e.g. if 8ml top phase add 4.8ml isopropanol). Mix carefully by holding tube flat (sideways) and rocking slowly so that solution rocks back and forth: one end of tube and then the other (2 seconds between ends). When strands of DNA form at the interface of the two phases rock for about 1-2 minutes more. If bubbles attach to DNA strands gently flick sides of tube to dislodge them.

(13) Centrifuge 4 minutes in clinical centrifuge at 2000 rpm to collect DNA at tube bottom.

(14) Carefully pour off liquid and make sure that DNA is not poured off by using a blue pipette tip to block the DNA pellet.

(15) Add 4.0 ml 1x TE buffer. Invert tube to dislodge DNA then let tube stand at 4^0C overnight or may proceed directly to next step.

(16) Next morning, place DNA tubes in 45 ^{0}C water bath for 20 minutes and mix gently. Do this until DNA is dissolved.

(17) Add RNase A (Ribonuclease A, R-4875, Sigma) to final concentration of l0 µg/ml mix gently and let digest at 37^0C. If DNA is not completely dissolved then continue to incubate at 45 ^{0}C up to 2 hrs. Be sure that the RNase A has had at least 1 hr to digest at 37 ^{0}C.

(18) Add about 8 ml of chloroform and rotate tubes end over end for 10 min and centrifuge for15 min at 2000 rpm to separate phases.

(19) Remove top phase slowly with disposable 10 ml pipette and transfer to fresh 15 ml polypropylene conical tube.

(20) Add 1/10th volume (about 0.4ml) 3 M sodium acetate solution (pH 5.2). Mix gently.

(21) Add two volumes (about 9 ml) of 95% ice-cold ETOH.

(22) Mix carefully by holding tube flat and rocking slowly so that solution rocks back and forth: one end of tube and then the other. When mixed well you should be able to see the blob of DNA.

(23) Centrifuge for 20 minutes at 2000 rpm. Pour off liquid. Using a sterile tip pull the DNA pellet up the side of the tube about 3 cm. Using a sterile blue pipette tip to flatten the pellet onto the wall of the tube.

(24) Add about 2 ml of 70% ETOH, and mix so that the pellet comes loose and is washed thoroughly for 30 seconds and then pour off liquid carefully so as not to lose the DNA pellet.

(25) Using a sterile blue pipette tip, squeeze each DNA pellet and drag the DNA up about l cm. Using a pipette to remove the liquid, continue to squeeze the DNA until it has reduced its size about 10 fold removing all liquid.

(26) Place tube containing DNA into dry heat at 37 ^{0}C for 20 minutes.

(27) Add 100-150 µl of TE to dissolve DNA and cap tube. Let tube stand at 4 ^{0}C overnight. Next day place in 45 ^{0}C water bath for 15 min to dissolve DNA.

(28) Transfer the DNA solution to 1.5 ml bullet tube.

(29) Concentration of working solution should be quantified by visual appearance (brightness of band) of uncut band (1:10 dilution) in 1% agarose gel after electrophoresis with TAE buffer at 45 V for 3 hours and staining with ethidium bromide. ? DNA at concentrations of

5-500 ng/µl are used as markers. Adjust sample DNA to 0.5 to 1.0 µg/µl and store at 4 ^{0}C. Pay attention to sterile technique in all steps.

Southern blotting and hybridization

(1) Use 10 µg DNA per sample.

(2) Digest DNA using 30 units of the proper enzyme (Hind III 3µl (10 unit/µl, Invitrogene), 10 x React 2 buffer 3 µl, H_2O 4 µl, DNA 20 µl (0.5 µg/µl)) in 30 µl reaction mix in a PCR tube at 37 ^{0}C for at least 10 hours (overnight).

(3) Electrophoresis is performed in agarose (0.8 - 1.0%) submarine gels using lx TAE buffer without ethidium bromide at 20-25 V overnight (15-18 hours). 1 kb DNA ladder is used as size marker, and DNA digest plus 3 µl of 10 x loading dye is used. After the blue dye has run about 10 - 11 cm, trim gel at sample slots and then measure the length and cut at 10 cm. The gels are 23 cm wide using a variety of combs. Cut upper right corner of the gel. Stain with ethidium bromide for 20 min and photograph the gel if necessary. 10-15 µg DNA/sample are used; this allows for quick turnaround time on the blots and routinely gets 20+ hybridizations per filter. To determine the copy numbers of the introduced gene in T_1 transformants, the DNA fragment of 837 bp containing the xylanase gene sequence was used. The concentration of the DNA fragment of 837 bp was determined to be 40 ng/µl by running 1% agarose gel electrophoresis at 60 V for 1 hour with TAE buffer and ethidium bromide stain with 5µl/lane DNA ladder I as marker. The DNA fragment of 837 bp was obtained by PCR band purification. Wheat haploid genome has about 1.6×10^{10} bp. Amount of DNA of the 837bp needed for 2 copies equals to 10 µg (Sample DNA for gel) x (2 x 837) / (3.2×10^{10}), i.e. 0.52 pg. For 4 copies, it is 1.04 pg, and 2.08 pg for 8 copies. 10 ug of wild type WED 2020-16-2 DNA was used for enzyme digestion, and then mixed as carrier DNA with the copy number marker DNA (837 bp) at three concentrations.

(4) Using a large dish, wash each gel in about 500 ml of 0.25 N HCl (10ml concentrated HCl/500 ml water) for 15 min. This depurinates the DNA and allows for transfer of high molecular weight DNA fragments.

(5) Rinse gels with deionized water. Using a large dish, wash each gel in about 1 L of Rinse Solution (0.4 M NaOH containing 0.6 M NaCl; for 1L: 16 g NaOH, 35 g NaCl) on shaking platform for 15 min.

(6) Cut three 25x30 cm 3M filter papers for wicks. In a 12x9 inch tray place 1 L Rinse Solution and wet the wicks in this solution. Place a 6.5xl2.5x125 inches glass plate support over tray and drape wicks over glass plate.

(7) Take gel stab from step 2 by using two glass plates with the gel sandwiched between them, and place it face down on the wicks (add a bit of solution to wick before putting on gel). Smooth out any air bubbles.

(8) Place Para film strips between tray edge and right up to the gel; do all four edges.

(9) In a smaller tray filled with 200 ml deionized water wet a prelabeled l0x22 cm sheet of genescreen plus (NEN LifeSci: NEF-976). Label the upper right corner of the DRY membrane. This corresponds to the upper right corner of the gel. Genescreen plus and HyBond N+ work very well since one can load lots of DNA onto them and you can hybridize 20+ times.

(10) To the gel surface add a small amount of deionized water and carefully place the genescreen sheet (label side down) on the smooth gel surface so as not to trap any air bubbles. Use a plastic/Teflon roller to remove bubbles and make smooth.

(11) Cut three 3M filter papers to l0x22 cm and wet these in deionized water. Place them on the genescreen membrane; smooth out any air bubbles. Place a 3 inch wad of paper towels

(James Rivers 755 single fold, 13x23 cm) on top of the filter papers followed by another glass plate (same size) and then three 250 ml E-flasks filled with deionized water.

(12) Leave overnight to transfer DNA to membrane. Disassemble transfer apparatus and air-dry membrane on 3M papers for about 1-2h at 37 ^{0}C.

(13) Soak membrane in 2x SSC (20x SSC for 5 L: 877 g NaCl, 44l g Na_3 citrate.$2H_2O$) for 5 min, and then blot off the excess moisture. Go to pre-hybridization (this is best) or wrap with saran wrap and freeze.

After DNA was separated by agarose gel electrophoresis and blotted onto nylon membranes, they were hybridized to the 837bp DNA probe of xylanase gene. The hybridization probe was obtained by labeling the 837 bp DNA fragment of plasmid RS 138/xyl with [α-^{32}P]dCTP using the All-in-One random labeling system (Sigma R7522, R9647). 25 ng DNA was used. The DNA was added to 1 x TE buffer or H_2O to a volume of 45 µl, then boiled for 5 min to denature double strand. The tube was hold for 10 seconds under cold water and spun down for 10 seconds. The 45 µl DNA was transferred into ALL-in-One reaction tube and 2.5µl [α-^{32}P]dCTP was added. The mix was incubated at 37 ^{0}C for 30 min on a counter top protected by plastic to prevent radiation to human. The [α-^{32}P]dCTP was stored in a special container in a refrigerator. A piece of plastic was placed between human and the container or reaction tube containing [α-^{32}P]dCTP or radioactive labeled DNA probe.

Once the DNA was blotted to the membrane filter, and the probe DNA was radioactively labeled, the next step was to do prehybridization and hybridization.

(1) Make prehybridization buffer:

18 ml ddH_2O (DNase free)

6 ml 5 x HSB

3 ml Denhardts III

Mix together and heat to 65 °C and add 3 ml of freshly boiled Carrier DNA.

5 x HSB

30.3 g PIPES in 300 ml dd H_2O and adjusted to pH 6.8, add 600 ml 5 M NaCl and 40 ml 0.5 M EDTA Na_2 $2H_2O$, recheck pH and adjust volume to 1 L, and autoclave.

Denhardts III

150 ml ddH_2O

4 g BSA (fraction V)

20 g SDS

4 g Ficoll-400

4 g PVP-360

10 g $Na_4P_2O_7$ x 10 H_2O

Dissolve each ingredient, bring volume to 200 ml, and store at RT

Carrier DNA

5 g salmon testis DNA (Sigma D-1626) in one liter of ddH_2O

Heat to boiling, turn off heat and stir until DNA is dissolved

Autoclave for 30 min and aliquot to 50 ml reaction tubes

Store at -20 °C

(2) Before adding prehybridization buffer to the membrane, boil the buffer for 7 min (avoids to get black spots on membrane);

(3) Soak membrane in 2 x SSC so that there is no air bubble hampering the wetting. Transfer membrane from 250 ml 2 x SSC to another tray containing 250 ml 2 x SSC. Remove abundant

SSC sandwiching the membrane between to filter papers and press with your hands on it (will be not harmful to membrane);

(4) Transfer membrane to prehybridizatio n buffer and slightly shake it with hands so there are no air bubbles disturb contact of membrane to buffer, cover membrane with saran wrap just above membrane to prevent loss of prehybridization buffer, close lid of the plastic tray, incubate at 65 ^{0}C overnight (at lease 8 hours for new membrane);

(5) Make hybridization buffer: for 1-3 membranes

10 ml ddH_2O

4 ml 5 x HSB

2 ml Denhardts III

(6) Prewarm hybridization buffer to 65 °C

(7) Add 1 ml of freshly boiled Carrier DNA

(8) Add labeled DNA probe

(9) Boil buffer 7 min before transfer to membrane (should prevent the occurrence of black spots)

(10) Pour hybridization buffer in a new tray, transfer the whole membrane into the tray and make sure the membrane is moistened by hybridization buffer

(11) Get rid off all air bubbles between membrane and hybridization tray

(12) Hybridize over night (15-20 hours) at 65 °C

Once the membrane is hybridized overnight, the next step is to wash membrane:

(1) Make washing solutions:

Solution A: 2 x SSC/2 % SDS

Solution B: 1 x SSC/1 % SDS

(2) Store hybridization buffer with labeled probe in a 50 ml reaction tube

(3) Add 100 ml of solution A and shake 1 min; discard the solution in waste container protected by organic glass plate

(4) Add 300 ml of solution A and let shake at 100 rpm at 65 °C for 30 min

(5) Discard 300 ml of solution A in waste container and add 300 ml of solution B and let shake at 100 rpm at 65 °C for 30 min

(6) Discard solution B in waste container (if radioactivity > 1000 cpm) or sink with running tap water, now membrane is not so hot that you do not have to work behind the glass screen

(7) Dry membranes with two filter papers and cover it completely with saran wrap

(8) Check the membrane with a monitor (Geiger counter, Model TBM-3S, Technical Associates, Canoga Park, California) and related to the activity deduce the days of exposure on a film: 10 days for 300 cpm, 5 days for 300 - 500 cpm, 2 days for 500 - 1000 cpm, overnight for 1000 – 2000 cpm, 4 hours for >2000 cpm

(9) Put the membrane into a Kodak film cassette, front side up, and use tape on edges to hold filter in place

(10) In the dark room with only the safelight on, take a piece of film (Kodak, X-OMAT AR, 20 x 25 cm Cat. 1651454) and clip or bend its corner, which will mark the right corner of the filter. Place the film over the filter

(11) Place intensifying screen (Dupont, Cronex Lighting Plus) face down onto film and close cassette and fix the cassette in a press

(12) Store the membrane during the exposure at - 80 °C for 4 hours to 15 days

(13) Thaw cassette, develop film in a dark room (1-4 min in developer, then rinse in water, agitate in fixer for 2 min, rinse well in water, air dry).

Membrane can be re-used for hybridization by stripping the filter:

(1) Remove filters from saran wrap and wash in 2x SSC for 5 min

(2) Strip filter by pouring about 500 ml 80 ^{0}C strip solution (0.2x SSC + 0.2% SDS) over filter and agitating for 5 min in shaker at 100 rpm at 65 ^{0}C

(3) Strip filter by pouring about 500 ml 80 ^{0}C strip solution (0.1x SSC + 0.1% SDS) over filter and agitating for 5 min in shaker at 100 rpm at 65 ^{0}C

(4) Blot excess moisture from membranes and check radioactivity with counter. If they need more stripping continue to step 5

(5) Strip filter by pouring about 500 ml 85 ^{0}C strip solution (0.1x SSC + 0.1% SDS) over filter and agitating for 5 min in shaker at 100 rpm at 65 ^{0}C

(6) Blot excess moisture from membranes and check radioactivity with counter. If they need more stripping continue to step 7

(7) Strip filter by pouring about 500 ml 90 ^{0}C strip solution (0.1x SSC + 0.1% SDS) over filter and agitating for 3-4 min in shaker at 100 rpm at 65 ^{0}C

(8) After final strip, quickly blot off excess moisture from membrane and wrap in saran wrap. Store at –20 ^{0}C, flat or do directly to prehybridization.

C. RNA isolation and Reverse Transcription PCR procedure

(1) Grind 1-gram tissue with a mortar and pestle in liquid nitrogen.

(2) Add 10 ml TRIzol (pre-heated to 60°C) to tissue in a falcon tube. Swirl to mix. Work in the hood. Wear safety glasses when working with TRIzol and chloroform.

TRIzol Reagent

		Final Concentration
Phenol, pH. 4.3	380 ml	38%
Guanidine thiocyanate	118.2 g	0.8 M
Afnmonium thiocyanate	76.12 g	0.4 M
3M Sodium Acetate, pH 5.0	33.4 g	0.1 M
Glycerol	50 ml	5%
H_2O to 1 liter		
Total	1 liter	

(3) Incubate samples at 60°C for 5 minutes.

(4) Centrifuge @3000 x rpm @ 4°C for 10 minutes. Transfer supernatant to 50 ml pp (SS34) centrifuge tube that has been dipped in chloroform to rinse out any residual RNases.

(5) Add 2 ml of chloroform. Vortex the sample until color shade is uniform at least 5 seconds, and incubate at room temperature for 5 minutes.

(6) Centrifuge @ 3000 x rpm for 15 minutes @ 4°C.

(7) Collect the upper aqueous layer and transfer to a new 50 ml pp (SS34) centrifuge tube dipped in chloroform.

(8) Add 0.5 volume of isopropanol & 0.8 M Na-citrate/1.2 M NaCl solution per 1 ml of aqueous solution. Mix gently. Incubate at room temperature for 20 minutes.

(9) Centrifuge @ 3000 rpm for 10 minutes @ 4°C.

(10) Wash the clear RNA pellet with 10 ml of cold 75% ethanol. Swirl & centrifuge at 9000 rpm for 5 minutes @ 4°C.

(11) Discard supernatant & air-dry the pellet for 10 minutes, inverted on a kimwipe. Dissolve pellet in 300 ml of DEPC-treated H_2O. Resuspend by pipeting up & down a few times.

(12) Add 2 µl RNasin. Incubate at 55-60°C for 10 minutes to resuspend.

(13) Transfer to 1.5 ml microcentrifuge tube and rinse the 50 ml pp (SS34) centrifuge tube with 100 µl DEPC-treated H_2O. Check concentration and run a gel of 2 µl of RNA samples (see qualitative/quantitative analysis).

(14) Freezing at this step (and storing at -80)..

Qualitative/Quantitative Analysis

(1) To determine concentration, dilute 2 µl RNA to 78 µl H_2O. Measure the absorbancy 260/280 and calculate concentration.

(2) Prepare and run a 1% 0.5X TBE-agarose gel (1 µl of RNA, 9 µl H_2O and 2 µl of tracking dye) at 100 volts for 1 hr. 12 µl 100 DNA ladder is used and the 500 bp band containa 170 ng DNA.

The Reverse Transcription PCR reaction

(1) Mix 2 µl RNA, 1 µl each of 10 pmol primers (Bar-Ubi1-up and Bar-Ubi1-down), 1 µl PCR H_2O, and 1 drop oil, quickly spin down and incubate at 72 ^{0}C for 2 min, put on ice for 2 min.

(2) Add 5 µl master mix (2 µl M-MLV RT 5x buffer (Promega M531A), 1 µl DDT (Clontech S 2665), 1 µl 10 mM dNTP (Clontech S2666), M-MLV Reverse Transcriptase (Promega M170A, 200 U/µl, Promega, Madison, WI)).

(3) Incubate at 42 ^{0}C for 1 hr, 75 ^{0}C for 10 min, and cool to room temperature.

(4) Add 1 µl RNase H (New England Biolabs # M0297S, 5000U/ml) and incubate at 37 ^{0}C for 20 min.

(5) Use 1 µl of this RT-PCR product for regular PCR.