The Potential for Green Fluorescent Protein as a Screening Tool in the Production of Haploid Potato Plants

Rose Palumbo

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science in Horticulture

Richard Veilleux, Chair

Vladimir Shulaev

Gregory Welbaum

December 17, 2003

Blacksburg, Virginia

Keywords: *Solanum tuberosum, Solanum phureja*, haploid-inducing pollinator, GFP, genetic transformation, transgenic plants

The Potential for Green Fluorescent Protein as a Screening Tool in the Production of Haploid Potato Plants

Rose Palumbo

(Abstract)

A hybrid between a highly regenerative diploid clone (BARD 1-3) of *Solanum phureja* and haploid inducer IVP 101 was transformed with *Agrobacterium tumefaciens* strain 4404 containing plasmid pHB2892 with genes for green florescent protein (GFP) and kanamycin resistance. Hemizygous primary transformants (T_o) were produced from three leaf discs: 17 diploid plants from one leaf disc, three and nine tetraploids from the other two leaf discs. GFP expression was observed qualitatively under fluorescence microscopes and quantitatively with a GFP meter. Anther culture of tetraploids produced 29 plants, none with high levels of GFP. Segregation ratios for tetraploid T_1 seedlings fit models for single duplex insertions (35 transgenic: 1 non) or double simplex insertions (15 transgenic: 1 non). Diploid T_1 seedlings segregated for deleterious traits: dwarfed size and curled leaves, as well as the GFP transgene. Similar segregation patterns in diploid families implied that all diploids may have been from the same transformation event. The cumulative segregation showed the dwarfed and curled plants fit a single recessive gene ratio (3 normal: 1 mutant), and GFP fit a double-copy insertion ratio (15 transgenic: 1 non). There was substantial GFP silencing evidenced by the loss of expression in plants that had originally been selected for high GFP. However, six selections were found to be free of deleterious traits, consistently high expressers of GFP, and producers of stainable pollen with less 2n than IVP 101.

Acknowledgements

I would like to thank some of the people whose assistance has made this project possible:

- My advisor, Dr. Veilleux, for all the encouragement and advice regarding this thesis and my education, and for his patience with all the problems that have come up
- The rest of my committee, Dr. Shulaev and Dr. Welbaum, for their help with this project
- Suzanne Piovano for instruction and advice with lab techniques and procedures
- Dr. Williams for introducing me to this program and encouraging me to succeed
- Neal Stewart for the loan of the prototype GFP
- The horticulture department for funding my education through teaching assistantships
- My fellow students who have assisted with the generation of transformants and anther regenerants (Melissa Huhn, Tom Martin, Brian Ikenberry) , and with flow cytometery and field harvests (Gordon Lightbourn, Jeff Skonecka, Leslie Blischak, Aaron Baxter, Phil Wadl, Vishal Arora, Rahul Gupta)
- Bennett Deddens for continuing this research

Table of Contents

List of Tables and Figures

Chapter 1: Introduction

Haploids

Haploids always have half the chromosomes of the plant from which they were produced. So, a haploid from a tetraploid (4x) would be a diploid (2x) and a haploid from a diploid would be a monoploid (1x). Most potatoes are tetraploid: however, diploid and even monoploid selections have been used extensively in breeding research, not only for potato but also for many crop plants. The main benefits of plants with reduced ploidy are that segregation of phenotypes is more distinct and there are fewer alleles per locus simplifying genetic studies (Ross, 1986).

In self-pollinating crops, such as wheat or rice, doubling the chromosome number of haploids can result in the direct release of new cultivated varieties whereas in cross-pollinating crops, such as potato or maize, doubled haploids are just the starting material for breeding hybrid cultivars. An example of a way that haploids can be used is found in *Solanum phureja* Juz. & Buk. monoploids. Since monoploids only have one allele at each loci, and therefore cannot be recessive, selection of the strongest monoploids should cause deleterious traits to be removed from the population. Surviving monoploid plants can then be used for *in vitro* shoot regeneration, which can produce doubled monoploids (Paz and Veilleux, 1999).

Haploid Inducing Pollinators

A haploid potato can be produced by pollination as the product of a haploid inducing pollinator. These pollinators either initiate the formation of seed without fertilization, or facilitate preferential elimination of the haploid inducer's chromosomes during development of the zygotic embryo following fertilization. Evidence of transfer of fragments of the paternal genome to the haploid derivatives has been used to support the latter possibility (Allainguillaume

et al., 1997; Clulow and Rousselle-Bourgeois, 1997). In the case IVP 101, which we used as our haploid inducing pollinator, Straadt and Rasmussen (2003) found no evidence of IVP 101 DNA fragments in the haploids produced.

In *Pseudogamic Production of Dihaploids and Monoploids in* Solanum tuberosum *and some related species* (1981) van Breukelen described the effects of the pollinator and seed parent on the number of haploid seeds produced, which he identified through the embryo spot method described later. As reported by van Breukelen (1981), haploid induction is a heritable trait, linked to at least five loci, and affected by the environment. Specifically, temperatures of approximately 18^{o}C were low enough to increase haploid induction without decreasing flowering. While studying the mechanism of haploid induction, van Breukelen found that 1n pollen was more likely to induce haploids than 2n pollen, provided that the pollen was from a plant with the necessary haploid inducing genes.

Unfortunately, haploid inducing pollinators used to pollinate diploids generally produce a few monoploids and many diploid hybrids, so seeds must be painstakingly screened under a dissecting microscope to separate the haploids from the other seeds. The current method of screening is through the use of a gene that produces a tiny purple spot of anthocyanin pigmentation on the embryo of non-haploid hybrid seed. Seeds without a spot are likely to be haploids. By developing haploid inducing pollinators that are homozygous for this dominant trait, Hermsen and Verdenius (1973) were able to screen seeds, which resulted from crosses between the haploid inducing pollinator and whatever potato germplasm (without the embryo spot marker) had been selected for haploidization, for the embryo spot phenotype that can be observed through the seed coat. However, there are often cases where the presence/absence of the spot is ambiguous (van Breukelen, 1981).

Transformations

Agrobacteria tumefaciens Smith and Townsend is a bacterial pathogen that actively transforms wounded plant tissue with genes for tumor growth as well as production of opines. When transformations are successful, all the tumor tissue produced will also be producing opines, which the *Agrobacteria* can use as food. The transformations require that a region of the DNA, called T-DNA, be transferred to the plant. T-DNA is located on the Ti plasmid in between a left and right border with specific sequences. Eliminating these pathogenic genes from the T-DNA provides the opportunity to use *Agrobacteria* to transfer other genes into plant tissue by replacing the pathogenic genes with the desired transgenes (Gelvin, 2003 - review). Once *Agrobacteria* has inserted the T-DNA into the plant tissue, whole plants containing a hemizygous copy of the transgene (but otherwise genetically the same as the parent plant) can be regenerated. Inbreeding can be used to develop homozygous carriers of the transgene.

Green Fluorescent Protein

Green Fluorescent Protein (GFP) has been studied for many research applications (Stewart, 2001). It is the product of a gene that was first identified in *Aequorea victoria* Murbach and Shearer (jellyfish) and, through genetic engineering, can be expressed in many different species of plants and animals. For example, it has been shown that GFP can be used as an accurate tracer of transgenes in plants (Niedz, 1995). In addition, Olivares-Fuster et al. (2002) found that GFP could be used to monitor the success of protoplast fusion, and as a selectable marker for somatic hybrids. The sequence of DNA that encodes GFP is known and has even been altered to improve its expression in plants (Rouwendal, 1997).

GFP is useful as a reporter gene because it allows the screening of live plants, in contrast to other reporter genes such as GUS, for which the screening process results in dead tissue. Screening for GFP requires only that an ultraviolet (or blue) light be shined on the tissue in question to see the green glow produced by GFP; it requires no other preparation or chemical reaction. GFP is excited by wavelengths between 380 and 420 nm, and less so near 480 nm, and the emitted fluorescence is most intense at just above 500 nm (Chalfie et al., 1994). The emission wavelengths can be altered to produce fluorescence in colors other than green (Hawley et al., 2001). These modified GFP can be used separately to avoid interference from autofluorescence, or in combinations to trace multiple transgenes simultaneously. According to Chalfie et al. (1994), the modifications to the GFP gene used in our transformations (obtained from Molinier et al., 2000) did not alter either the excitation (strongest at ~400nm) or emission (strongest just above 500nm) wavelengths from that of the jellyfish GFP.

Purpose

Because haploids have importance in plant breeding, developing more efficient means to derive them has become a plant breeding objective in itself. The goal of this project is to produce a haploid inducing pollinator homozygous for the GFP transgene to use in crosses to produce haploids. Seeds resulting from these crosses can be screened for the production of GFP in order to determine which seeds are haploid. Any seeds that inherit the GFP from the haploid inducing pollinator are expected to be sexual hybrids and therefore cannot be haploid, so they would be discarded. Transgenic crop plants have been quite controversial over the past few years; however this use of transgenics would have no direct connection to the food supply. In this scheme, the plants expressing a transgene are undesirable and will be discarded; however,

the obvious transgene expression of the undesirable hybrids will help distinguish the rare haploid non-transgenics. We would effectively be substituting the GFP marker for the embryo spot in an attempt to screen seed lots more easily. Of course, GFP fluorescence visible through the seed coats from hemizygous embryos bearing the transgene is a prerequisite to the success of this scheme.

In the process of producing the GFP expressing haploid inducing pollinator, we will examine the stability of the GFP expression in this application. Evaluations of GFP fluorescence will be both qualitative by using fluorescence microscopy, and quantitative by using a GFP meter. GFP segregation ratios will be used to estimate copy number, since the most stabile expression is expected from a single insertion. In addition, an attempt will be made to distinguish homozygous from hemizygous GFP fluorescence by the results of the GFP meter. Rather than calibrating the GFP meter to exclude autofluorescence, an approximate maximum autofluorescence detectable by the GFP meter will be determined.

Chapter 2: Materials and Methods

Plant Material:

IVP 101: A diploid (2n=2x=24) descendent from the *Solanum phureja* plants van Breukelen (1981) studied was originally selected as the haploid inducing pollinator for the transformations in this project. The plant material van Breukelen (1981) started with was developed by Hermsen and Verdenius (1973) to be effective at inducing haploids and also homozygous for embryo spot. In an effort to increase haploid inducing ability, van Breukelen (1981) used full-sib F_3 and selfed progeny of the IVP clones. IVP 101 was obtained from the USDA/ARS Inter-Regional Potato Introduction Station, Sturgeon Bay, Wis. However, this plant did not respond well to leaf disc regeneration, an important step in the *Agrobacterium* mediated transformation.

BARD 1-3: This is a diploid (2n=2x=24) clonal selection of Haynes' (1972) adapted *Solanum phureja* population; the clone derived from PI 225669 was initially selected for high tuber yield in the field and subsequently for its regenerability in anther culture (Boluarte et al., 2001). Its derivatives have also been found to be regenerable in protoplast culture (Lightbourn, data not published).

SMP-C: A cross between BARD 1-3 and IVP 101 was used in an attempt to combine the haploid inducing abilities of IVP 101 and the regenerative abilities of BARD 1-3. The seeds that inherited the embryo spot, verifying hybridity with IVP 101, were planted and a vigorous, easily regenerable diploid (2n=2x=24) seedling was chosen for use in the transformations.

Transformation:

Agrobacterium tumefaciens containing a construct with the genes for GFP expression and kanamycin resistance was obtained from Molinier et al. (2000): Strain LBA 4404 and plasmid pHB2892. The plasmid uses neomycin phosphotransferase (kanamycin resistance) as a selectable marker and it has two Cauliflower Mosaic Virus promoters in front of the S-GFP gene. The S-GFP gene Molinier et al. (2000) used had already been studied by Chalfie et al. (1994). Chalfie et al. (1994) reports that the sequence they used for expression in *Escherichia coli* Migula has a change in the 80th codon that replaces glutamine with arginine, and that the first methionine for GFP has been replaced with an alanine preceded by a new methionine. According to Chalfie et al. (1994), these changes did not affect the excitation or emission wavelengths of the GFP produced.

Transformation of potato with *Agrobacterium* is a relatively simple procedure that has been used to develop plants with transgenic resistance to insects and diseases (Douches et al., 1988). The process involves incubation of callused leaf discs (pieces of leaves without stem tissue and all edges cut to encourage maximum callus production) in an *Agrobacterium* solution, and subsequent placement of the discs on callus induction medium including kanamycin (50 mg l^{-1}) and plant growth regulators (0.9 mg l^{-1} thiamine HCl, 0.8 mg l^{-1} zeatin riboside, 2.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D)) that promote the formation of callus (disorganized plant tissue). After 2 weeks the transformation is complete; only transformed cells that are resistant to kanamycin will be able to grow. The remaining *Agrobacterium* is removed from the callus with cefotaxime (250 mg l^{-1}), and leaf disc regeneration is encouraged by transferring the callused leaf discs to Murashige & Skoog (MS) medium (Murashige and Skoog, 1962) with kanamycin and

growth regulators (0.9 mg l^{-1} thiamine HCl, 0.8 mg l^{-1} zeatin riboside, 2.0 mg l^{-1} gibberellic acid (GA_3)) to recover plants from the transformed callus.

Agrobacterium preparation: A small scraping (2-3 sq. mm) of the frozen A. *tumefaciens* obtained from Molinier (2000) was added to 5 ml of Ty media with 50 mg l^{-1} kanamycin, in order to maintain an *Agrobacterium* population containing the plasmid. This mixture was incubated at 30^{o}C on a shaker for 36 h. All 5 ml of the mixture was transferred into 50 ml of Ty media (with 50 mg l^{-1} kanamycin) and incubated at 30^{o}C on a shaker for 12h, to obtain bacteria in a log phase of growth for the transformation.

Agrobacteria transformation: Leaf discs that had been on callus induction media for 2 days were soaked in 25 ml of the *Agrobacterium* culture for 10 min. The inoculated leaf discs were placed on callus induction media with 50 mg l^{-1} kanamycin, to select for transformed calli.

Agrobacterium elimination: After 1 week, the inoculated the leaf discs were washed for 10 min in 25 ml of liquid regeneration media (no phyto-agar) with 250 mg l^{-1} cefotaxime, to discourage growth of the *Agrobacterium.* Cleaned leaf discs were transferred to leaf disc regeneration media with 50 mg l^{-1} kanamycin to continue selection for transformed shoots. Leaf discs were transferred onto new media every 2 weeks until shoots were harvested at approximately 2 cm in height. The shoots were maintained in tissue culture on MS basal medium, with kanamycin that was phased out at the next subculture.

Media: Ty: 5.0 g l^{-1} tryptone, 3.0 g l^{-1} yeast extract, 500 mg l^{-1} $CaCl_2 \cdot 2H_2O$, 50 mg l^{-1} kanamycin

Callus Induction: MS basal salts, 0.9 mg l^{-1} thiamine HCl, 3% sucrose, 0.8 mg l^{-1} zeatin riboside, 2.0 mg l^{-1} 2,4-D, 0.7% agarose

Liquid Regeneration: MS (Murashige and Skoog, 1962) basal salts, 0.9 mg l^{-1} thiamine HCl, 3% sucrose, 0.8 mg l^{-1} zeatin riboside, 2.0 mg l^{-1} GA_3, 250 mg l^{-1} cefotaxime, 50 mg l^{-1} kanamycin

Leaf Disc Regeneration: MS basal salts, 0.9 mg l^{-1} thiamine HCl, 3% sucrose, 0.8 mg l^{-1} zeatin riboside, 2.0 mg l^{-1} GA_3, 50 mg l^{-1} kanamycin, 0.7% agarose

Flow Cytometry:

Plants that regenerated from transformed shoots of SMP-C were expected to be diploid and hemizygous for GFP (Figure 1). However, it is possible for the chromosome number to spontaneously double during leaf disc regeneration, so the ploidy level of regenerated plants must be tested. Flow cytometry provides a quick estimate of ploidy (Owen et al., 1988). Flow cytometry was used to determine the ploidy level of the original transformants, as well as the progeny from crosses to test haploid inducing ability.

Approximately 0.5 g of in vitro plant tissue was chopped over ice in a Petri dish that contained 1.5 ml chopping buffer (3.528 g L^{-1} sodium citrate, 1.676 g L^{-1} MOPS, 3.66 g L^{-1} $MgCl_2$, 0.4 ml L^{-1} Triton X-100). Once the tissue was thoroughly chopped, the mixture was filtered through 250 μm and 63 μm filters. From filtrate containing intact nuclei 0.5 ml was transferred to a microcentrifuge tube and kept on ice until all samples were ready. Samples were then incubated at room temperature after 0.25 ml ribonuclease A (800 mg L^{-1} ribonuclease A in chopping buffer) was added to each sample. After 30 min, 0.125 ml propidium iodide (400 mg

L^{-1} PI in chopping buffer) was added to each sample, and incubation was continued on ice for 30 min to 3 h.

Fluorescence levels from individual nuclei were measured by flow cytometry using a Coulter Epics XL Flow cytometer (Coulter International Corp., Miami, FL). Results were displayed as graphs of 1000 counts along with the total number of nuclei that fell within predetermined ranges, corresponding to 1x, 2x, and 4x DNA peaks for potato by comparison to a known monoploid (2n=1x=12) control. Each sample always has multiple peaks, but samples should not have peaks corresponding to ploidy levels lower than the ploidy level of the plant. Therefore, the peak for the lowest ploidy identifies the plant's ploidy level.

Primary Transformant GFP Testing:

After visual confirmation of GFP using a fluorescence microscope, we transferred all of the transformed plants to the greenhouse and tested them with a prototype GFP meter kindly provided by Neal Stewart (University of Tennessee). The instrument uses fiber optic cable to emit and detect specific wavelengths of light, (emit: 395nm/495nm, detect: 530nm) and record GFP expression in relative units called tics. One end of the cable has a clip to attach a leaf, while the other end displays and stores the results (Figure 2). Three leaves on each plant were tested, excluding the midvein, and the results were averaged for each plant. Since several plants had been clonally propagated, plant means were averaged to give clonal means whenever possible. Results were also collected from IVP 101 as a control to quantify autofluorescence.

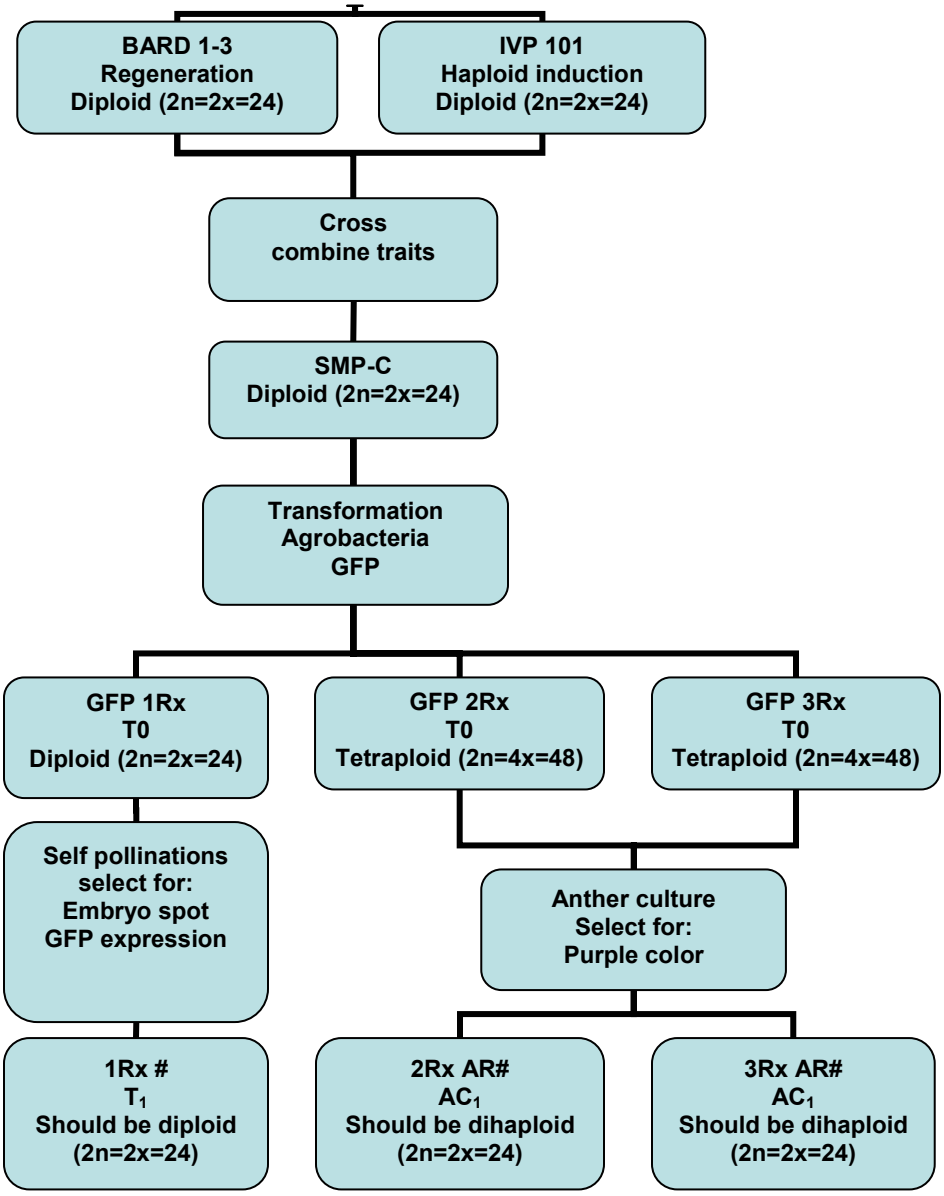


Figure 1: Flow chart from germplasm development by transformation through selection by sexual reproduction and anther culture

Figure 2: A potato leaf clipped into the GFP Meter (top) and the control panel where results are displayed (bottom).

Self pollinations:

All 29 of the original transformants were self-pollinated with the expectation that one in 16 progeny from single insert transgenics would be homozygous for both GFP expression and haploid induction. Nearly all plants (all but 1R14 and 1R16) set fruit on selfing. Selections from the progeny were made based on the results of the GFP tests on the original transformants (Table 1). The seed from selfs of the selected T_0 diploids (1R1, 1R5, 1R7, 1R11, 1R12, 1R15 and 1R21) were sorted while still hydrated under a fluorescence microscope, and only those seed that inherited both brightly fluorescing embryos and the embryo spot were planted *in vitro* (Figure 3). GFP categories of high, medium and none were based on how brightly the embryo of each seed glowed through the moist seed coat of seeds that were freshly extracted from the berries.

The in vitro selfed seedlings that developed from the high GFP seeds (with the embryo spot) had a low germination percentage (22.5%). Of 41 seedlings, only 15 were vigorous enough in vitro for subculturing into multiple copies, and only six survived transfer to the greenhouse. Therefore we abandoned our efforts to preselect the population for embryo spot and GFP fluorescence and planted seeds from all remaining fruit from selfs of the diploid primary transformants. This yielded 879 diploid seedlings from which to select homozygous GFP and haploid inducing (embryo spot) plants.

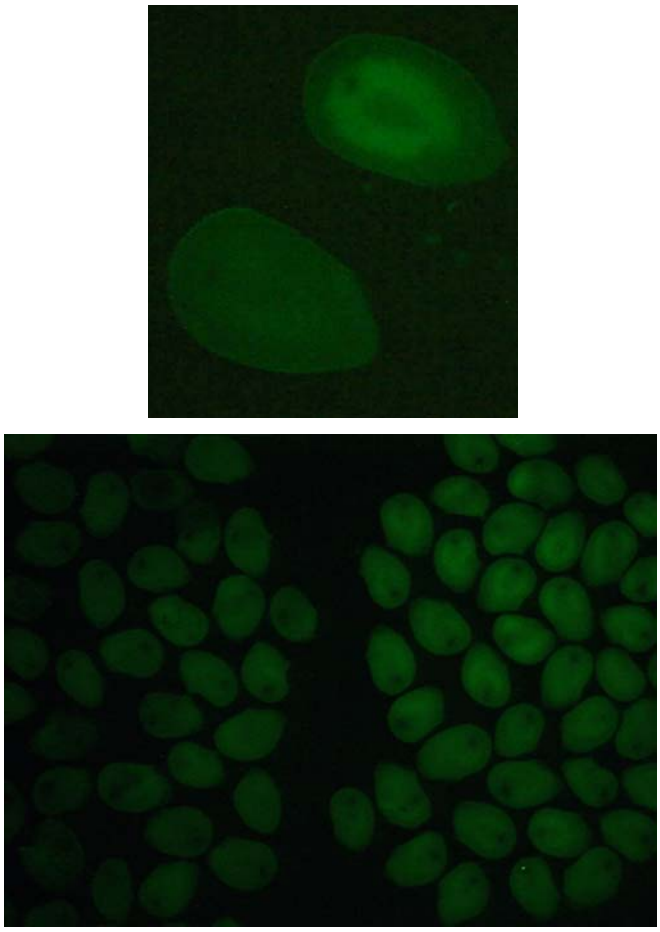


Figure 3: Seeds from 3R2⊗ have been sorted into glowing and not glowing, but some obviously glow more brightly. (GFP filter on a dissecting microscope)

Anther culture:

The tetraploid 2Rx and 3Rx selections (2R1, 2R2, 2R3, 3R4, 3R5 and 3R7) were anther cultured to derive dihaploid regenerants with the expectation that, if the transformation occurred before chromosome doubling produced the tetraploids, one sixth of the anther derived dihaploid regenerants from single insert plants would be homozygous for GFP. Since one sixth of those plants would also be homozygous for haploid induction, one out of 36 should be homozygous for both traits (Figure 1).

Procedure: Flowers from 3R4, 3R6, 3R7, 3R9 and 2R3 were sterilized in 80% ethanol for 1 min, followed by 100% bleach with Tween 20 for 5 min and two rinses in sterile water. One anther from each flower (25 flowers per rep) was put into each of five treatments. Anther culture was performed using either solid or liquid anther culture media. The liquid anther culture media had either sucrose or maltose as the sugar, and cultures were incubated in the dark on a shaker at either 20^{o}C all day or 30^{o}C during the day (12 h)and 20^{o}C at night (12 h). The solid media was incubated at 20^{o}C continuously without a shaker. After incubating for 6 weeks the embryos were harvested onto embryo media solidified with either phytagel or agarose, and transferred to fresh media every 2 weeks. Embryos were examined twice for visual confirmation of GFP, and showed absolutely no consistency in results.

Anther culture was repeated with selections based on GFP fluorescence in the T_0 plants (2R1, 2R2, 2R3, 3R4, 3R5 and 3R6). In this case the sucrose liquid media was for all anthers, and each anther was cultured in an individual well of 24 well plates. All plates were incubated on a shaker at 30^{o}C during the day and 20^{o}C at night. After

incubating for 6 weeks, the embryos were harvested, placed on embryo media solidified with phytagel, and transferred to fresh media every 3 weeks. As the embryos developed into plantlets, they were transferred to MS media and maintained in tissue culture.

Media: Solid Anther Culture Media: 2.165 g L^{-1} MS basal salts, 0.4 mg L^{-1} thiamine HCl, 0.1 mg L^{-1} indoleacetic acid (IAA), 2.5 mg L^{-1} benzyladenine (BA), 6% sucrose, 0.25% activated charcoal, 0.6% phytagel

Liquid Sucrose AC Media: 2.165 g L^{-1} MS basal salts, 0.4 mg L^{-1} thiamine HCl, 0.1 mg L^{-1} IAA, 2.5 mg L^{-1} BA, 6% sucrose, 0.25% activated charcoal

Liquid Maltose AC Media: 2.165 g L^{-1} MS basal salts, 0.4 mg L-1 thiamine HCl, 0.1 mg L-1 IAA, 2.5 mg L-1 BA, 6% maltose, 0.25% activated charcoal

Embryo Medium: 3.2 g l^{-1} Gamborg B5 Media (Gamborg et al., 1968), 34 ml l^{-1} MS II stock solution (Murashige and Skoog 1962), 1% sucrose, 0.05 g l^{-1} $CaHPO_4$, 0.25 g l^{-1} NH_4NO_3, 5 mg l^{-1} GA_3, 0.6% phytagel (or agarose), 50 mg l^{-1} kanamycin

Phenotypic selections:

In addition to the poor germination found in the first selection of selfs, the families that provided the 879 diploid selfs also segregated for curled leaves, dwarfed size and purple color. Of those three traits only purple color is desirable, since it should be linked to embryo spot and haploid inducing ability. Curled leaves are a problem because they obstruct clipping the GFP meter on the plant reliably, and dwarfism produces small plants with few, if any, flowers. Also, the curled and dwarfed plants both tended to be less robust than their normal phenotype siblings. After elimination of the plants with deleterious traits at the seedling stage, we selected the

remaining plants for purple color, and also eliminated any plants with late signs of abnormal phenotypes.

Seedling GFP Testing:

Initial screening: All 879 diploid progeny, and all available anther culture regenerants, were screened with the commercially available GFP meter (Opti-sciences, Tyngsboro, MA). Three leaves were again tested on each plant, but this time the midvein was intentionally sampled in order to include some of the tiny dwarfed plants in the testing. Since there was only one copy of each plant the initial results were based on the average of three leaves for each plant. The top 57 GFP expressers (with normal phenotypes and purple color) were maintained in the greenhouse, and retested as they matured.

High, Medium, Low Replicated Study: Based on the initial screening, the three plants with GFP fluorescence closest to 0, 400, 800, 1200, 1600 tics and the three highest expressing plants were selected, and multiple cuttings were taken from each. Once three clones of each selection became available they were arranged in three separate replications with different randomization patterns, and given several weeks to mature. The GFP meter was used to evaluate fluorescence from three leaf samples on each plant, with the vein included.

Low, None Replicated Study: Based on the initial screening the plants surrounding an apparent drop in GFP fluorescence, between a little GFP and none, were selected for use in a second replicated study to determine the cut-off point for an absence of GFP. Three

clones of each were obtained though cuttings and arranged in three separate replications with different randomization patterns. After the plants matured for several weeks, the GFP meter was used to evaluate expression from three leaf samples on each plant, with the vein included.

Pollen screening:

Pollen counts were used to eliminate plants that released low levels of pollen, or had high 2n pollen levels. Pollen from at least three flowers of each plant was collected and mixed. The mixed sample was stained with acetocarmine (Darlington and LaCour, 1942) and individual pollen grains were counted based on their size, above (2n) or below (1n) 25 microns, on a Zeiss microscope under 400X magnification (Figure 4). The sampling was repeated over several days, and the results were averaged to estimate 2n pollen levels. Low flower production limited data collection: however the final selections were counted again to ensure accurate levels.

Field Tests:

The top 57 GFP expressers from the greenhouse study were used as a source of cuttings to be transferred into the field in June 2003. Vigor in the field combined with greenhouse vigor, GFP stability, and pollen counts were used to limit the population to the top 15 selections (Figure 5). These top 15 were propagated from greenhouse cuttings and maintained in the greenhouse.

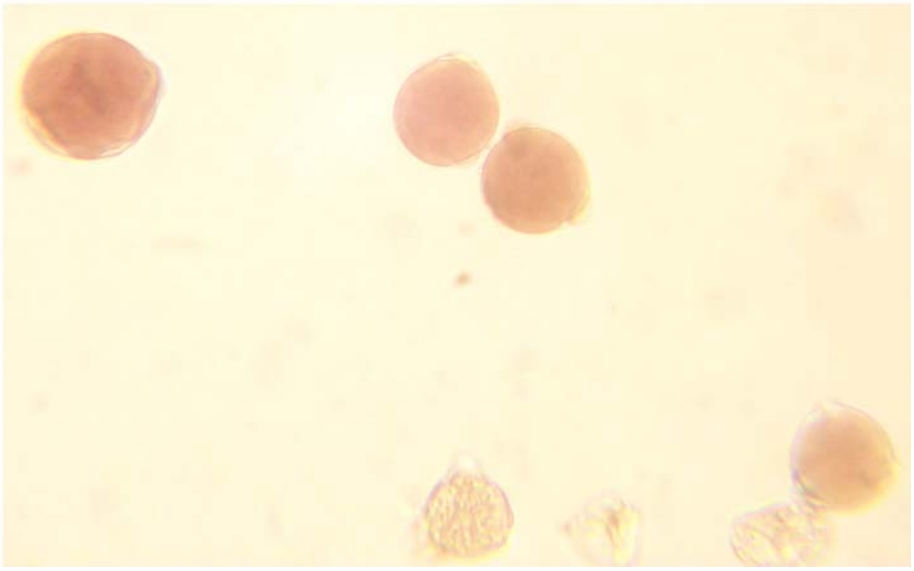


Figure 4: Pollen samples stained with acetocarmine. Oversized stained pollen (top left) is considered to be 2n, average sized stained pollen is considered to be 1n, and unstained pollen (along bottom) is considered to be dead.

Photograph taken with a Nikon digital camera on a Zeiss microsope at 400X

Visual Screening of GFP Expression

GFP was visualized in various plant parts (leaves, trichomes, corolla, stamen, embryos, seeds, tubers, seedlings) in the primary transformants and their sexual progeny derived from both self- and cross-pollination under microscopes with filters set for GFP and/or chlorophyll fluorescence. Using the Olympus SZX12 stereomicroscope with the filter for both GFP and chlorophyll (excitation 461-500nm, dichroic mirror 505nm, detection >510nm), patterns of GFP fluorescence were compared in the top 15 selected selfs of diploid transformants (Figure 5), to select the six most consistently bright GFP plants.

The seeds from the tetraploid T_0 selfs were also planted in the greenhouse and scored for GFP fluorescence in order to use segregation of the transgenic phenotype as an indirect estimate of transgene copy number. The tetraploid seedlings were scored in a destructive harvest under the Olympus SZX12 stereomicrscope with filters for GFP only (excitation 451-490nm, dichroic mirror 495nm, detection 491-540nm).

Haploid Induction:

The six final selections were crossed to Atlantic and Katahdin, to determine haploid induction capability. The seeds from these crosses were harvested after only 2-3 weeks from sterilized (1 min 100% ethanol, 15 min 100% bleach with Tween-20) fruit, and immediately planted in vitro on MS media (3% sucrose) to avoid dormancy (Singsit and Hanneman, 1991). The seedlings were visually screened for GFP fluorescence, and then tested with flow for ploidy levels.

Figure 5: Flow chart expanding Figure 1 from T_0 diploids to T_1 diploids with selections for dwarfing, curled leaves, GFP and 2n pollen levels

Chapter 3: Results

Transformation Results:

Three SMP-C leaf discs, of about nine that had been inoculated with *Agro*, produced plants from transformed callus (resistant to kanamycin) (Figure 1). Those discs were numbered 1 through 3, and each transformed plant was given a number within the group from each leaf disc. The T_0 plants are named: 1R1, 1R2, 1R3,1R4, 1R5, 1R6, 1R7, 1R9, 1R10, 1R11, 1R12, 1R13, 1R14, 1R15, 1R16, 1R17, 1R21, 2R1, 2R2, 2R3, 3R1, 3R2, 3R3, 3R4, 3R5, 3R6, 3R7, 3R8, and 3R9. In total 17 **1Rx**, three **2Rx** and nine **3Rx** plants were established in the greenhouse. Flow cytometry revealed that all of the 1Rx plants were diploid and all of the 2Rx and 3Rx were tetraploid. Under greenhouse conditions the diploid T_0 plants were as large and vigorous as the tetraploids. One difference was that the tetraploids self-pollinated without any outside influence, whereas the diploids required manual pollination.

The in vitro tissue of the original transformants (as viewed under a fluorescence stereo microscope) expressed the most GFP in the nuclei of the guard cells, in the trichomes, and along vascular traces. GFP fluorescence was quantified in the greenhouse using a prototype GFP Meter, and all the diploids fluoresced at relatively high levels, 1607-2084 tics. The GFP Meter records results in units called "tics," and using these results we selected seven **1Rx** plants from the original 17 diploid transgenics that were the most consistently high fluorescing clones (Table 1).

While sorting the seed from the seven diploid T_0 selections, we observed that dried seed, regardless of whether or not it was harvested from a transformed plant, auto-fluoresced brightly under our conditions, and so the embryo inside a dried seed may or may not be expressing GFP. The dried seed were not completely opaque; the embryo spot could still be seen through the seed

coat. However, either the autofluorescence of the seed coat overpowered the GFP in the embryo, or GFP was not expressed in dried seed. The autofluorescence became a problem very quickly if the seeds were not kept moist during sorting; however, seed that had been rehydrated allowed the GFP fluorescence from the embryo to be seen through the seed coat as if they were freshly harvested from the fruit. GFP continued to be visible through the seed coat as the seed germinated (Figure 6).

The 182 seeds from self-pollination of the selected plants were planted in vitro, but only 22.5% germinated. Of the germinated seed, 63.4% stopped growing or died before reaching a size (approximately 3 cm) appropriate for sub-culturing. This left only 15 plants in vitro, and of these only six thrived in the greenhouse. There was also obvious segregation among these plants for purple color, dwarfism, and curled leaves. Since six plants did not allow for sufficient selection against those traits, all the remaining T_1 seeds derived from self crosses of the diploid T_0 plants, which were not initially selected, (Table 1) were planted in the greenhouse, producing 879 new diploid seedlings.

GFP Seedling Selection Results:

All 879 diploid seedlings were screened visually for curled leaves (Figure 7), size (Figure 8) and purple color (Figure 9), and with a GFP Meter. Our expectation for GFP expression was that there would be distinct separation between high, medium and low GFP expression corresponding to a single insert transgene segregating into homozygous GFP, hemizygous GFP, and no GFP classes, respectively. Instead we observed a continuous range of expression (Figure 10).

Table 1: Mean GFP tics (as measured on a prototype GFP meter) of 29 primary transformants of SMP-C and a control, IVP101. Means represent 1-3 plants (N) with 3 leaves sampled per plant, depending upon the availability of germplasm.

Clone	N	Mean	Std dev
1R1	3	1903	74
1R2	2	1755	50
1R3	2	1854	311
1R4	2	1927	332
1R5	1	2084	
1R6	2	1890	36
1R7	2	2015	96
1R9	2	2041	258
1R10	2	1865	25
1R11	2	1967	54
1R12	2	1953	9
1R13	1	1607	
1R14	1	1709	
1R15	2	1953	72
1R16	2	1766	89
1R17	2	2027	511
1R21	1	2081	

Clone	N	Mean	Std dev
2R1	2	1106	380
2R2	2	719	16
2R3	2	832	240

Clone	N	Mean	Std dev
3R1	2	705	96
3R2	2	707	38
3R3	2	929	256
3R4	2	1571	282
3R5	2	1368	237
3R6	2	1149	64
3R7	2	1341	18
3R8	1	1257	
3R9	2	1168	173

Clone	N	Mean	Std dev
IVP 101	4	437	75

Clones in BLUE were initially selected for analysis of selfed seeds based on their high, stable GFP expression

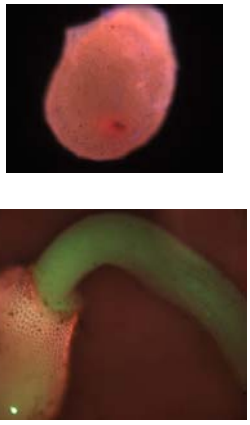


Figure 6: Once the seeds dry out (top) the seed coat auto-fluoresces too much for the presence or absence of GFP to be observed, yet the embryo spot is still visible. In a germinating seed (bottom) the seed coat becomes translucent again and GFP can be seen through it. (Nikon microscope with both GFP and chlorophyll filters)

Figure 7: Two examples of the curled leaves (left), which segregated in the T_1 progeny from diploid primary transformants of SMP-C, compared to normal leaves (right). In the bottom picture the curl is accompanied by high anthocyanin pigmentation

Figure 8: The T_1 progeny from diploid primary transformants of SMP-C also segregated for a dwarfing gene. Plants of varying height are shown in the top picture, and the severely dwarfed plants are shown in the bottom picture. Pots are 55 mm square (at inside edge) for comparison.

Figure 9: Segregation for purple color in the T_1 progeny from diploid primary transformants of SMP-C produced a wide range of expression. The top picture shows both purple and white flowers, and the bottom left picture has a seedling that inherited some purple coloring in the leaves. The three stem pictures show (from left to right): intense purple color, pale purple color, and completely green stems. Plants were only excluded that had absolutely no purple color in the seedling stage.

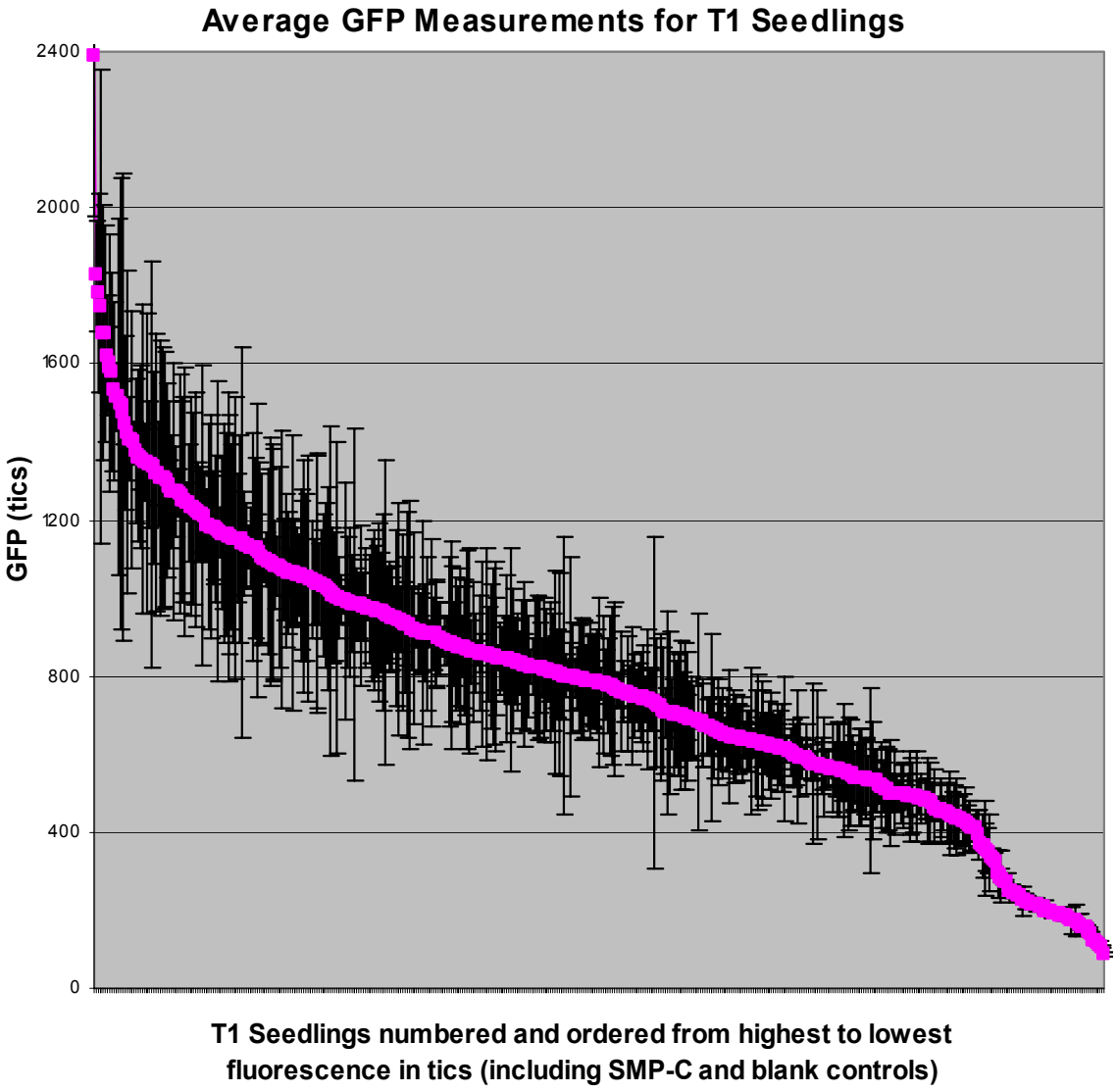


Figure 10: The mean GFP measurements based on sampling three leaves per seedling for all 879 T_1 seedlings resulting from self-pollination of 1Rx plants, ordered from highest to lowest with error bars marking the standard deviation for each mean.

High, Medium, Low Replicated Study: In order to determine the stability of GFP expression in the initial screening of single plants from each diploid T_1 genotype, we designed a replicated study using groups of three seedlings that were initially closest to 1600, 1200, 800 and 400 tics as well as the three highest and lowest levels. Three copies of each plant were vegetatively propagated and arranged in a randomized complete block design on the greenhouse bench. (One copy of each plant was in each of three blocks.) Based on the mean tics from three leaves on each plant, the six groups were significantly different at the $p < 0.0001$ level. Interestingly, the highest group based on the initial screen dropped down to just above the lowest group in the replicated study, whereas the other groups remained in the same order (Figure 11).

Low, None Replicated Study: The selections chosen to determine the cutoff between low levels of GFP expression, and no GFP expression, were the diploid T_1 seedlings between 400 and 250 tics (Figure 12). These seedlings were chosen because of the more sudden decrease in GFP fluorescence at this level. Three copies of each of the 14 seedlings were vegetatively propagated and arranged in a randomized complete block design on the greenhouse bench. (One copy of each plant was in each of three blocks.) The results showed a separation in fluorescence at approximately 300 tics (Figure 13). In the replicated study, those seedlings initially reading above 300 tics tended to increase, dramatically in some cases (2D4 and 2A5 in Figure 13), whereas those initially below 300 tics tended to decrease. Seedlings 24A5 and 2A1 had initial means only 5 tics apart and only 10 to 15 tics from 300. Although 24A5 was initially the higher of the two, 2A1 had much more variable results, and showed increased fluorescence in the replicated study.

Group	Mean	
1600	1122	a
1200	953	b
800	827	c
400	494	d
HIGHEST	338	e
LOWEST	187	f
IVP 101	202	

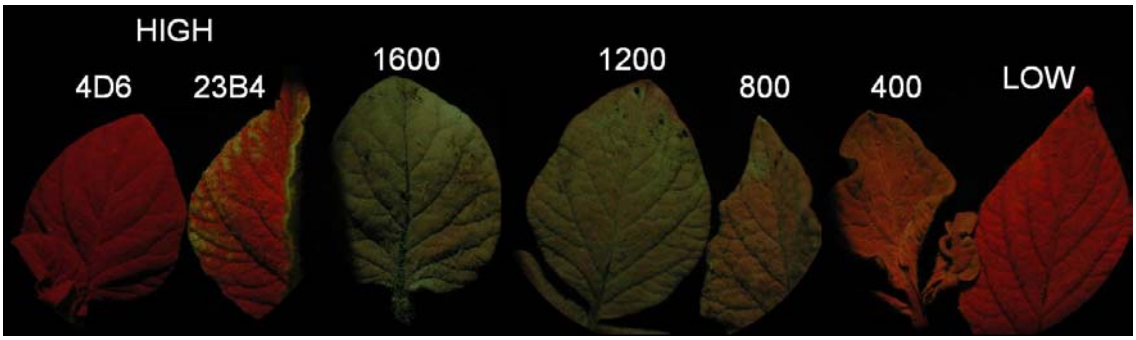


Figure 11: The selections for the High, Medium, and Low GFP expression in a replicated study were made at 400 tic intervals along with groups of the three highest and three lowest GFP expressers based on the initial screening of T_1 progeny from diploid primary transformants of SMP-C. In the top picture a leaf sample from a plant (6A4; left) in the 1600 tic group is compared to untransformed IVP 101 (right), and in the bottom photograph samples from each of the six groups are compared. All photographs were taken under an Olympus SZX12 stereomicroscope with the filter for combined GFP and chlorophyll (excitation 461-500nm, dichroic mirror 505nm, detection >510nm). The samples are taken from the replicated study rather than the initial selection; hence the Highs appear to be Lows. The highest group has two samples to illustrate the different expression patterns. The mean tics for each group are also shown; the groups were significant at the $p<0.0001$ level. IVP 101 was not included in the replicated study, but the mean value for three leaves from IVP 101 tested on the same day are displayed for comparison

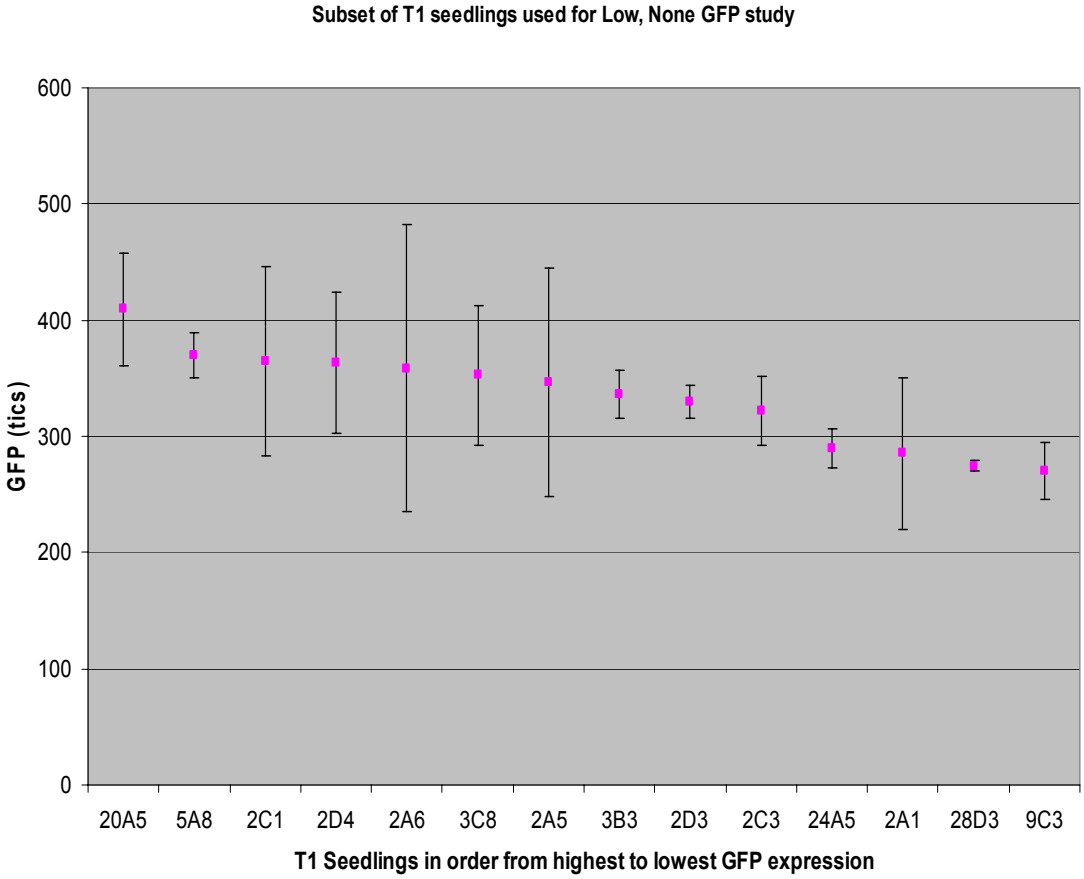


Figure 12: Selections of T_1 progeny from diploid primary transformants of SMP-C were made for the Low, None study based on a drop in mean GFP fluorescence among samples from the initial tests (Figure 10). The initial means used to make the selections are shown; these means are for 3 leaves per seedling (error bars show standard deviation). The lowest two selected means fell below the highest control result of 276 tics.

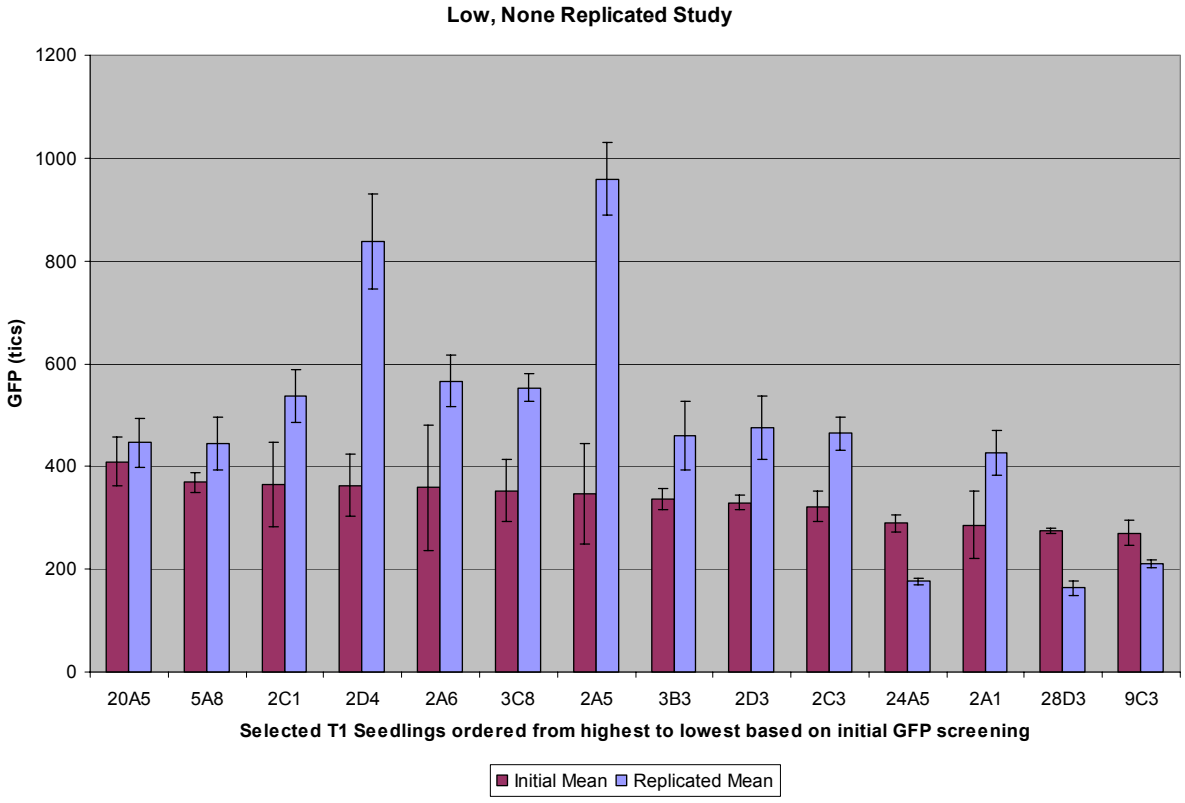


Figure 13: Mean GFP tics for the 14 T_1 diploid plants initially reading between 400 and 250 tics replicated study to define the limit between little and no GFP. Initial mean is the same mean as in Figure 10 and Figure 12, where as the replicated mean comes from three leaves on each of three plants per clone (error bars show standard deviation). Based on this data, only those remaining reliably below 300 tics would be scored as non-GFP.

Initial GFP Selections:

The mean GFP of T_1 progeny from diploid primary transformants of SMP-C were compared to see if families from different T_0 plants differed. All the families of transformants seemed to follow the same pattern, with a peak in the number of the seedlings somewhere in the range of 500-1000 tics. The bright pink bars representing controls (both untransformed SMP-C and an empty GFP meter) show that there were no controls above 300 tics (Figure 14), confirming the line drawn in the Low/None replicated study. There were three 1Rx transformants with more than 100 seedlings, and these (1R4, 1R6 and 1R10) were graphed for comparison. These individual graphs were roughly consistent with the combined results, as well as with the limit of 300 tics for no GFP and 1200 tics for high GFP (Figure 15, Figure 16, Figure 17). The segregation ratio of the selfed progeny of a single-copy transgene in a diploid would be 3:1 (Table 2), and the segregation of a double-copy transgene would be 15:1 (Table 3). Assuming seedlings with less than 300 tics are not expressing GFP, all three families (1R4, 1R6 and 1R10) with more than 100 seedlings fit the 15:1 segregation ratio expected for two copies of the transgene. Likewise, the entire T_1 diploid population also fits a 15:1 segregation ratio (Table 4). Similar ploidy and segregation ratios imply that the 1Rx transformants were all derived from the same transformation event.

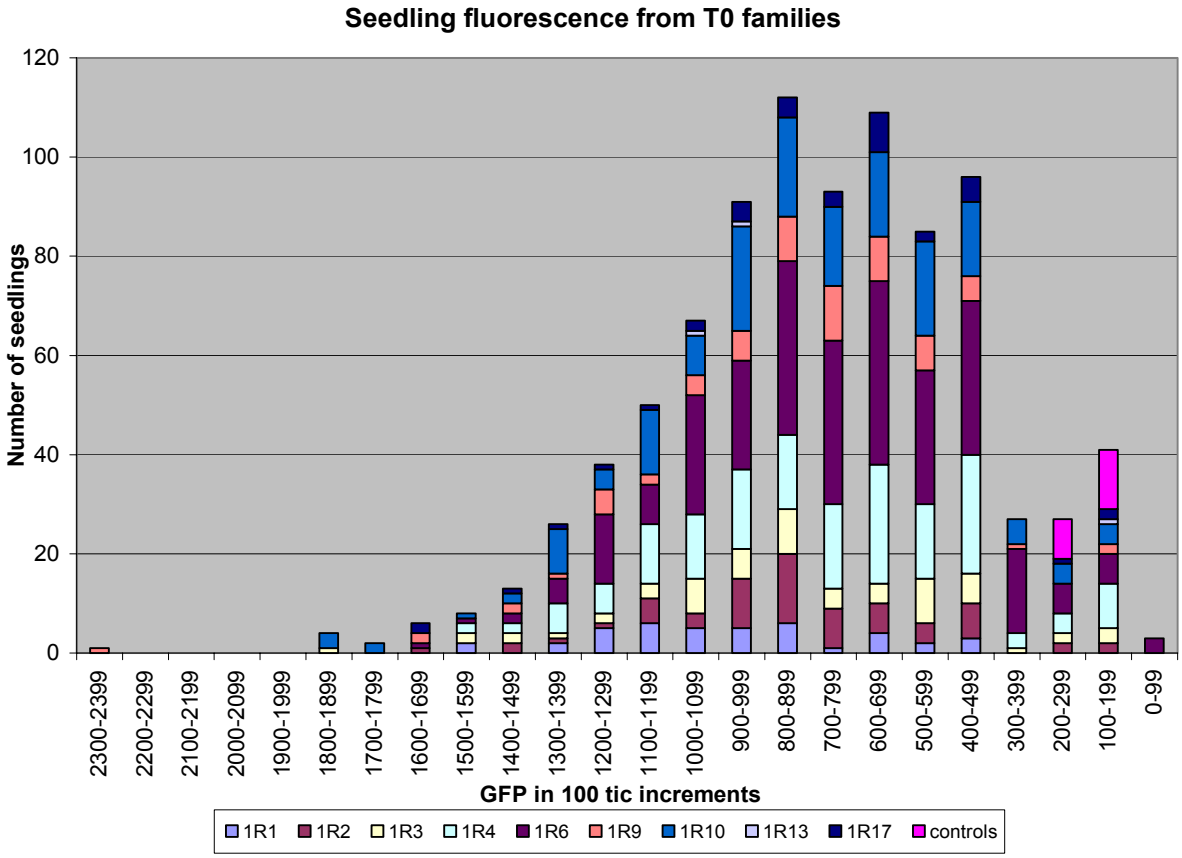


Figure 14: Number of GFP T_1 seedlings (n=879) from diploid transgenics falling within 100 tic classes of means from 3 leaves per seedling for a total of nine different T_0 families and controls (untransformed SMP-C and empty GFP meter). The families are divided by color.

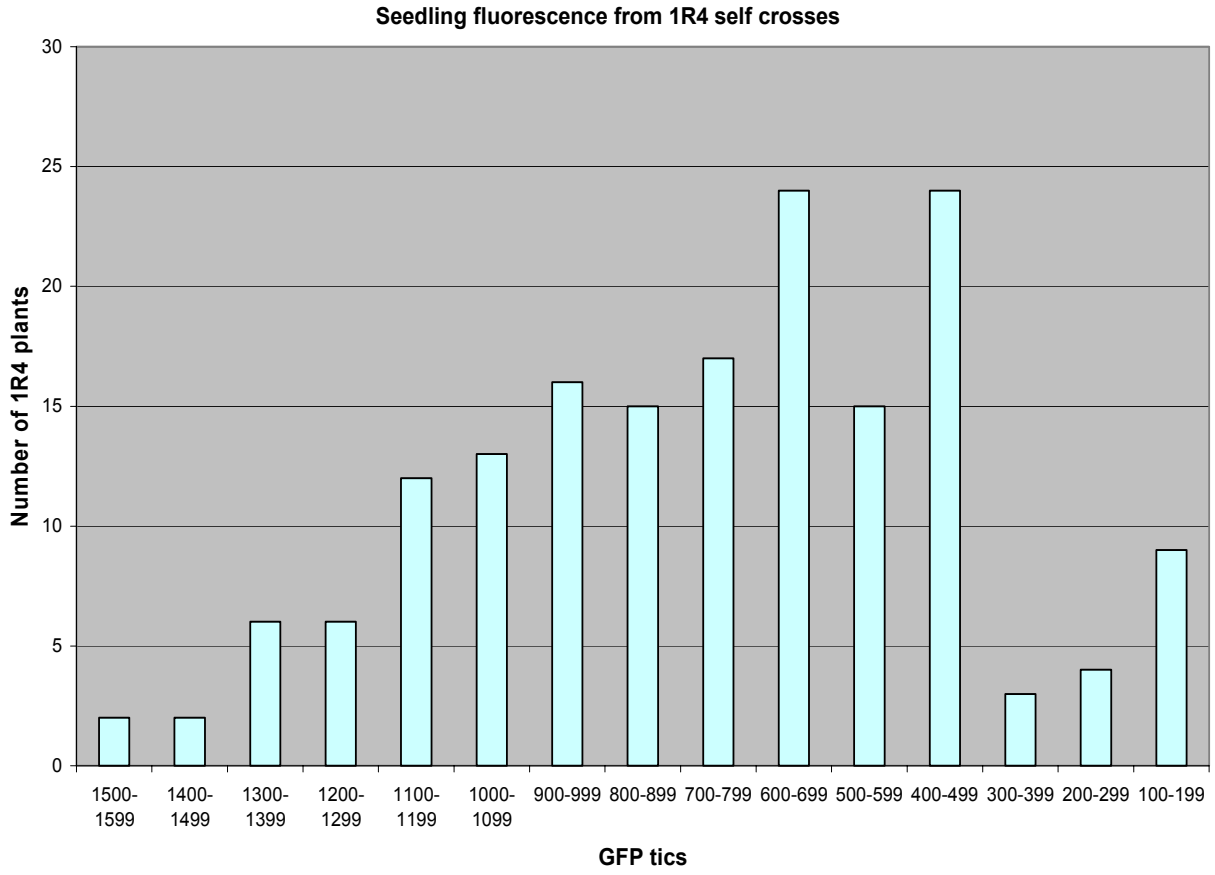


Figure 15: Number of T_1 seedlings (n=168) from 1R4⊗ (one family from Figure 14) that fall within 100 tic GFP ranges of means from three leaves per seedling

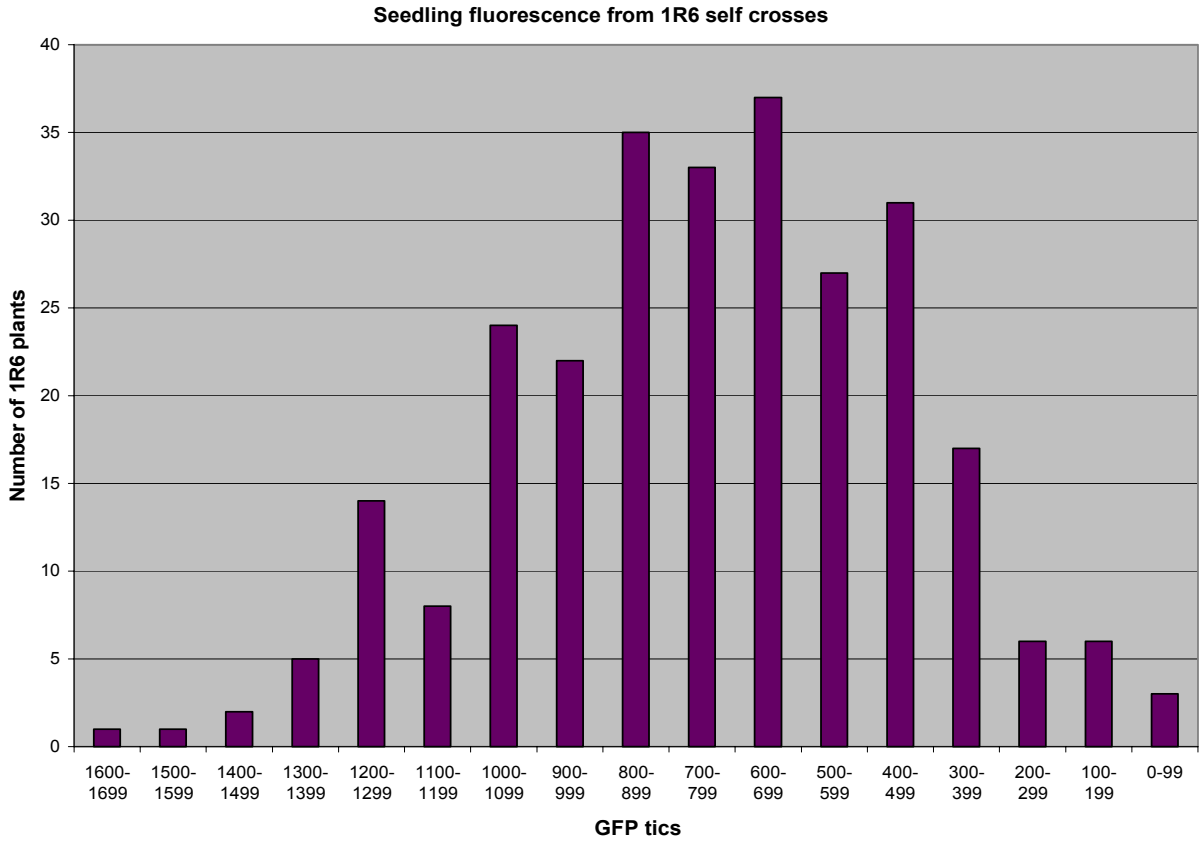


Figure 16: Number of T_1 seedlings (n=272) from 1R6⊗ (one family from Figure 14) that fall within 100 tic GFP ranges of means from three leaves per seedling.

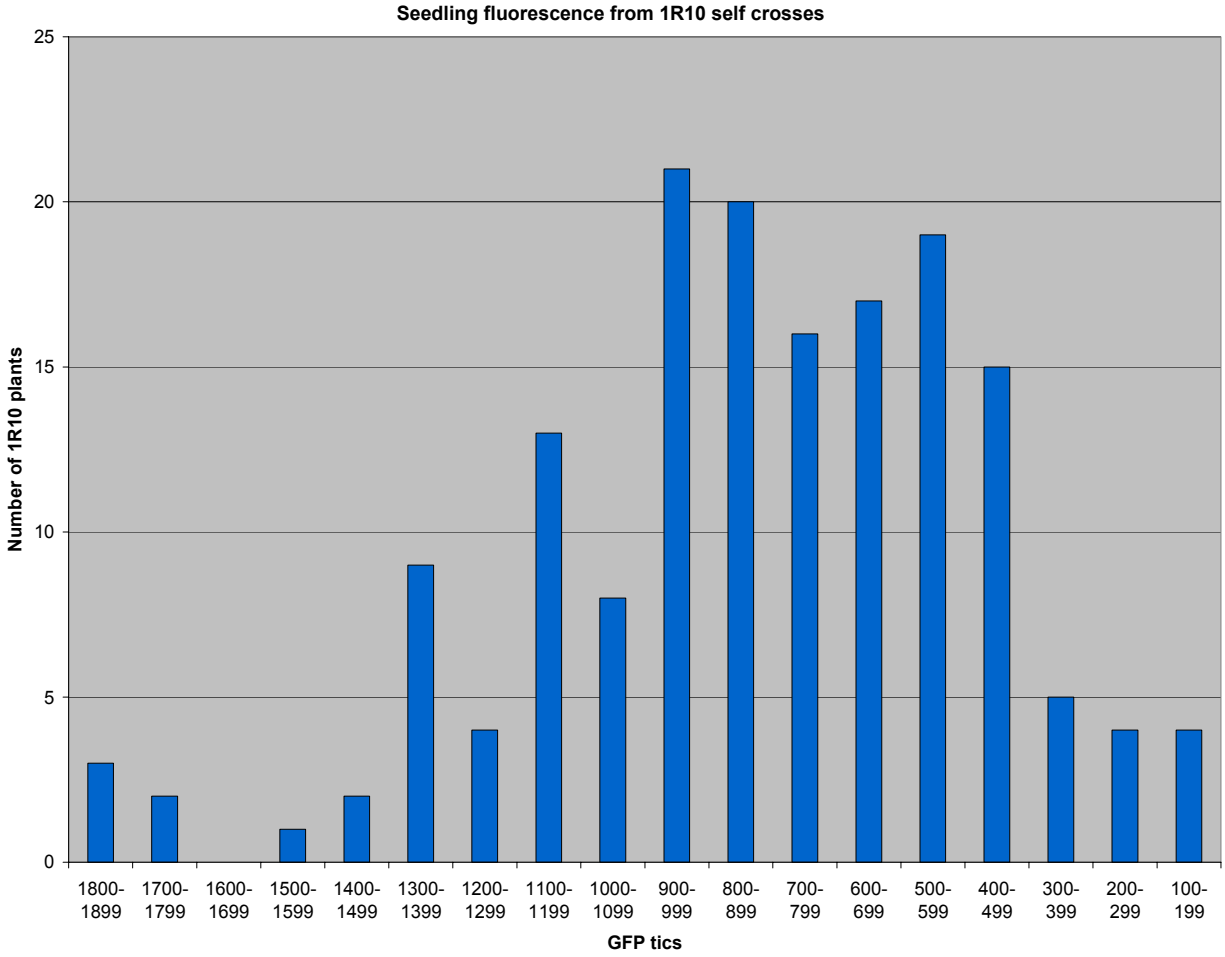


Figure 17: Number of T_1 seedlings (n=163) from 1R10⊗ (one family from Figure 14) that fall within 100 tic GFP ranges of means from three leaves per seedling

Table 2: Expected **3 transgenic:1 non-transgenic** segregation in a T_1 diploid population from a hemizygous primary transformant with a single copy of the GFP transgene (G_10); 0 = no transgene

	G_1	0
G_1	G_1G_1	G_10
0	G_10	**00**

Table 3: Expected **15 transgenic:1 non-transgenic** segregation in a T_1 diploid population from a hemizygous primary transformant with two independently segregating hemizygous copies of the GFP transgene (G_10, G_20); 0 = no transgene

	G_1 G_2	G_1 0	G_2 0	00
G_1 G_2	G_1G_1 G_2G_2	G_1G_1 G_20	G_10 G_2G_2	G_10 G_20
G_1 0	G_1G_1 G_20	G_1G_1 00	G_10 G_20	G_10 00
G_2 0	G_10 G_2G_2	G_10 G_20	00 G_2G_2	00 G_20
0 0	G_10 G_20	G_10 00	00 G_20	**00 00**

Table 4: Calculations of segregation for glowing: not glowing in diploid T_1 progeny, and total T_1 progeny from nine diploid T_0 families (1Rx) falling within 100 tic ranges

Family / GFP tics	1R1	1R2	1R3	1R4	1R6	1R9	1R10	1R13	1R17	controls	total
2300-2399	0	0	0	0	0	1	0	0	0	0	1
2200-2299	0	0	0	0	0	0	0	0	0	0	0
2100-2199	0	0	0	0	0	0	0	0	0	0	0
2000-2099	0	0	0	0	0	0	0	0	0	0	0
1900-1999	0	0	0	0	0	0	0	0	0	0	0
1800-1899	0	0	1	0	0	0	3	0	0	0	4
1700-1799	0	0	0	0	0	0	2	0	0	0	2
1600-1699	0	1	0	0	1	2	0	0	2	0	6
1500-1599	2	0	2	2	1	0	1	0	0	0	8
1400-1499	0	2	2	2	2	2	2	0	1	0	13
1300-1399	2	1	1	6	5	1	9	0	1	0	26
1200-1299	5	1	2	6	14	5	4	0	1	0	38
1100-1199	6	5	3	12	8	2	13	0	1	0	50
1000-1099	5	3	7	13	24	4	8	1	2	0	67
900-999	5	10	6	16	22	6	21	1	4	0	91
800-899	6	14	9	15	35	9	20	0	4	0	112
700-799	1	8	4	17	33	11	16	0	3	0	93
600-699	4	6	4	24	37	9	17	0	8	0	109
500-599	2	4	9	15	27	7	19	0	2	0	85
400-499	3	7	6	24	31	5	15	0	5	0	96
300-399	0	0	1	3	17	1	5	0	0	0	27
200-299	0	2	2	4	6	0	4	0	1	8	27
100-199	0	2	3	9	6	2	4	1	2	12	41
0-99	0	0	0	0	3	0	0	0	0	0	3
total	41	66	62	168	272	67	163	3	37	20	899
300 and above				155	257		155				828
below 300				13	15		8				51
observed ratio				11.9:1	17.1:1		19.4:1				16.2:1
expected ratio				15:1	15:1		15:1				15:1
X^2				0.635	0.251		0.501				0.301
P				0.426	0.616		0.479				0.583

Phenotypic Selections:

The T_1 progeny of the 1Rx primary transformants exhibited inbreeding depression in the form of segregation for dwarfed plants and curled leaves. The T_1 seedling population also segregated for anthocyanin pigmentation, which is desirable for haploid inducers as the embryo spot marker. Of the 879 seedlings 49 were purple, dwarfed and curled, 139 were purple and dwarfed, 120 were purple and curled, 14 were dwarfed and curled, 40 were dwarfed, 11 were curled, and 28 were normal but lacked purple color. That left 478 seedlings that were purple, and not dwarfed or curled. In the total seedling population the dwarfed phenotype segregated in a ratio that matched the 3:1 ratio expected for a single gene. In the three families with more than 100 seedlings, 1R4 and 1R10 matched this ratio, whereas 1R6 had an excess of dwarf plants. The curled phenotype had too few mutants to fit the 3:1 ratio in the total seedling population; however, it did fit in 1R10 and was at the 0.05 probability level in 1R4 and 1R6 (Table 5). Some of the plants that were only slightly curled may have been missed, especially if they were also dwarves. From the remaining purple, non-mutant seedlings, the 57 that had initial GFP results above 1200 tics were selected. The selected plants were grown in the greenhouse, and as they matured seven showed late signs of dwarfism or curling leaves. Those seven were eliminated, and replaced with the seven seedlings remaining from the in vitro seedlings from the first selection of 1Rx primary transformants (Table 6).

Table 5: Segregation of diploid T_1 seedlings for dwarfed size, curled leaves, and purple color

	Mutant Phenotype	wild type	mutant	total	Observed Ratio	Expected Ratio	X^2	P
Total	Dwarfed	637	242	879	2.6:1	3:1	3.00	0.08
	Curled	685	194	879	3.5:1	3:1	4.02	0.04
	Not Purple	786	93	879	8.5:1	3:1	97.48	0.00
						15:1	28.13	0.00
1R4	Dwarfed	127	41	168	3.1:1	3:1	0.03	0.86
	Curled	137	31	168	4.4:1	3:1	3.84	0.05
	Not Purple	151	17	168	8.9:1	3:1	19.84	0.00
						15:1	4.29	0.04
1R6	Dwarfed	188	84	272	2.2:1	3:1	5.02	0.03
	Curled	218	54	272	4:1	3:1	3.84	0.05
	Not Purple	234	38	272	6.2:1	3:1	17.65	0.00
						15:1	27.67	0.00
1R10	Dwarfed	119	44	163	2.7:1	3:1	0.35	0.56
	Curled	130	33	163	3.9:1	3:1	1.97	0.16
	Not Purple	142	21	163	6.7:1	3:1	12.76	0.00
						15:1	12.24	0.00

Table 6: The 57 T_1 progeny of 1Rx primary transformants, including the six seedlings from the in vitro selections, selected for normal (not curled or dwarfed), purple phenotype with high GFP fluorescence (over 1200 tics, or brightly glowing embryo for in vitro seedlings)

Plant	Family	Mean	Std Dev	n
4D6	1R9⊗	2389	414	1
16C2	1R3⊗	1826	141	1
23B4	1R10⊗	1780	254	1
24D4	1R10⊗	1748	606	1
6B5	1R17⊗	1679	326	1
28D8	1R6⊗	1676	277	1
6A4	1R17⊗	1620	152	1
12B8	1R4⊗	1590	107	1
28B2	1R6⊗	1578	255	1
1B5	1R4⊗	1530	229	1
7B2	1R1⊗	1517	87	1
8D3	1R3⊗	1515	455	1
23C7	1R10⊗	1497	579	1
20C7	1R4⊗	1489	598	1
17B8	1R3⊗	1477	81	1
16A2	1R3⊗	1425	414	1
4A2	1R9⊗	1405	328	1
7A2	1R1⊗	1376	186	1
10D7	1R10⊗	1366	218	1
7D2	1R1⊗	1362	85	1
4A7	1R9⊗	1348	97	1
21A4	1R4⊗	1346	385	1
30D8	1R6⊗	1343	162	1
11B8	1R6⊗	1342	521	1
24B4	1R10⊗	1338	99	1
18B1	1R4⊗	1334	247	1
23B6	1R10⊗	1321	356	1
24C5	1R10⊗	1319	132	1
19B5	1R4⊗	1307	354	1

Plant	Family	Mean	Std Dev	n
12A4	1R4⊗	1306	336	1
8B4	1R3⊗	1305	325	1
9B2	1R2⊗	1303	248	1
6D7	1R17⊗	1289	215	1
13A7	1R9⊗	1286	70	1
26B3	1R6⊗	1273	143	1
4B2	1R9⊗	1273	331	1
31B4	1R6⊗	1272	115	1
26C2	1R6⊗	1271	62	1
19B3	1R4⊗	1266	308	1
11C1	1R6⊗	1260	246	1
5A6	1R10⊗	1251	262	1
15C3	1R2⊗	1246	341	1
28B5	1R6⊗	1243	152	1
8C6	1R3⊗	1234	280	1
8D5	1R3⊗	1226	299	1
31A4	1R6⊗	1221	129	1
28C7	1R6⊗	1221	102	1
1R7x6	1R7⊗	1217	127	1
4B3	1R9⊗	1215	122	1
17B4	1R1⊗	1212	386	1
1R15x10	1R15⊗	1210	266	2
7A1	1R1⊗	1206	113	1
1R15x12	1R15⊗	1172	276	1
1R12x13	1R12⊗	1165	296	4
1R15x13	1R15⊗	985	440	1
1R12x14	1R12⊗	953	372	1
1R15x11	1R15⊗	909	1	2

n refers to the number of plants tested for each mean: n=1, plant mean=3leaves; n>1, clone mean= n plant means (of 3 leaves each)

Mature Plant GFP and Vigor:

The 57 T_1 plants selected as free of curled leaves and dwarfism, but with purple color and GFP, were further selected by examining GFP distribution by clipping the GFP probe on young leaves with the vein, older leaves with the vein, and large leaves without the vein. These results were compared in order to select only those plants that consistently expressed high levels of GFP. Plants were eliminated at this stage that did not have at least two tissue types (or one plus the average of all three) expressing more than 1000 tics of GFP, and nothing below 800 tics.

All 57 plants were also examined for vigor. Vigor was observed both in the greenhouse and in the field, in order to select the plants most likely to flower readily for use in pollinations. Any plant that was weak both in the greenhouse and in the field was eliminated. The mature plant GFP and vigor data combined eliminated 27 plants (Table 7).

Pollen Counts:

Effective haploid inducing pollinators should have abundant viable 1n pollen, and little or no viable 2n pollen. While the mature plant GFP fluorescence and vigor were measured, pollen samples were used to estimate 2n pollen levels and pollen viability in each seedling. Pollen was collected from at least three flowers on each plant, and each sample was collected on a separate day. Any plant that failed to shed pollen was eliminated, with the exception 1R7⊗6, which had to be removed early due to an infestation of spider mites (Table 7). Only 27 of the 54 plants flowered and released enough pollen to count while growing in the greenhouse (70^{o}F days and 60^{o}F nights) during the spring and early summer seasons of 2002. The cool, damp, overcast weather was potentially responsible for the lack of flowering and pollen release.

Table 7: GFP tests on mature T_1 selections and the vigor of those plants in the greenhouse and field. Plants with poor vigor in both the greenhouse and field (pink) were eliminated, as were any seedlings which did not have at least two GFP averages above 1000 tics (green), and none below 800 tics (blue). Seedlings meeting requirements for vigor and mature plant GFP fluorescence are in **bold**. IVP 101 is yellow for comparison.

	Greenhouse	Field	GFP Average			Average's	combined
name	plant info	Vigor	old leaves	new leaves	large leaves	average	st.dev.
8C6	flowers well		855	937	857	883	47
8D3	**flowers well**		**1507**	**1897**	**497**	**1300**	**723**
8D5	**large**		**1275**	**1663**	**983**	**1307**	**341**
9B2	flowers well		902	1084	720	902	182
10D7	large and tuberizing		1000	976	858	945	76
11B8	**medium**		**1692**	**1224**	**1431**	**1449**	**235**
11C1	**flowers well**		**1292**	**1299**	**820**	**1137**	**275**
12A4	**small**		**1575**	**1952**	**1350**	**1626**	**304**
13A7	**large**		**864**	**1424**	**1138**	**1142**	**280**
15C3	large	tiny	1101	1179	742	1007	233
16A2	large		1096	890	897	961	117
16C2	large and curled		380	172	458	337	148
17B8	flowers well		913	826	584	774	170
18B1	**medium**		**1608**	**1266**	**837**	**1237**	**386**
19B3	**large and chlorotic**		**1264**	**2737**	**1227**	**1743**	**861**
19B5	small and chlorotic	died	1287	1217	980	1161	161
20C7	**small and chlorotic**		**1168**	**1206**	**836**	**1070**	**203**
21A4	**large**		**1396**	**1334**	**990**	**1240**	**219**
23B4	**flowers well**		**1609**	**1697**	**1397**	**1567**	**154**
23B6	**medium and mildly chlorotic**		**1272**	**1353**	**897**	**1174**	**244**
23C7	**medium**		**1103**	**1406**	**874**	**1128**	**267**
7D2	medium	tiny	1486	708	211	802	643
17B4	medium and very purple		547	860	545	651	181
28C7	small and chlorotic	tiny	1412	1038	1095	1182	202
7A1	medium		675	965	635	758	180
4B3	medium		922	920	697	846	129
IVP 101	flowers well		197	214	189	200	13
24B4	flowers well		1205	701	1056	987	259
24C5	**medium**		**980**	**1297**	**901**	**1059**	**210**
24D4	**medium**		**1330**	**1941**	**1200**	**1490**	**395**
26B3	medium		899	750	1093	914	172
26C2	**small**		**1056**	**1742**	**905**	**1234**	**446**
28B5	small and chlorotic		869	925	1003	933	67
28D8	**medium**		**944**	**1234**	**1046**	**1075**	**147**
30D8	**medium**		**1113**	**1283**	**1146**	**1180**	**90**
31A4	**medium**		**1019**	**1492**	**897**	**1136**	**314**
31B4	medium/small	tiny	1439	1595	1317	1450	139
1R15#10	**medium**		**1531**	**1206**	**951**	**1229**	**291**
1R15#11	**medium**		**900**	**1565**	**982**	**1149**	**363**
1R15#12	small and chlorotic	tiny	1356	687	628	890	404
1R15#13	small	tiny	1258	985	872	1038	198
1R12#13	small	tiny	787	736	703	742	42
1R12#14	small		667	391	553	537	139
1B5	medium	tiny	1005	970	843	939	85
4A2	**medium**		**928**	**1458**	**998**	**1128**	**288**
4A7	flowers well		866	728	844	813	74
4B2	**medium**		**899**	**1311**	**851**	**1020**	**253**
4D6	**medium**		**995**	**1702**	**831**	**1176**	**463**
5A6	small	tiny	822	1065	819	902	141
6B5	**medium**		**1230**	**1104**	**1085**	**1140**	**79**
6D7	**medium**		**1090**	**1038**	**1066**	**1065**	**26**
7A2	**medium**	**tiny**	**1318**	**1371**	**1210**	**1300**	**82**
7B2	**flowers well**		**1013**	**1204**	**979**	**1065**	**122**
8B4	**medium**		**1045**	**1588**	**924**	**1186**	**354**
1R7#6	**flowers well, had spider mites**						
12B8	large		699	767	730	732	34
28B2	small	tiny	544	468	1038	684	309
6A4	large		613	577	549	579	32

Plants were also eliminated that had more than 70% dead pollen or frequencies of 2n pollen that were higher than that of IVP 101. Average pollen viability, based on staining with acetocarmine, ranged from 88.5% to 4.0% in the 27 plants that flowered in the greenhouse. Out of the pollen that was stainable, the frequency of 2n pollen ranged from 0 to 50%. All four of the seedlings with less than 30% viable pollen also had a higher frequency of 2n pollen in the living pollen than IVP 101, and these included the three with more than 20% 2n pollen in the living pollen. Seedling 12A4 had marginally more 2n pollen than IVP 101 had in the living pollen, but since 12A4's 2n pollen level was so close to being acceptable it was not eliminated. There were 18 plants eliminated by the GFP, vigor and pollen data, nine plants eliminate by only GFP and vigor data, and 15 plants eliminated by low pollen release/flowering, low frequencies of viable pollen or high frequencies of 2n pollen with in the viable pollen (Table 8).

Field GFP Results:

After the mature plant GFP, vigor and pollen observations were examined in greenhouse plants, there were only 15 of the 57 selections remaining. Three vegetatively propagated clones of each selection were grown in the field, and the GFP meter was used to collect data from three leaves of each plant, including the midvein. The GFP results continued to be inconsistent; instead of remaining above the 1000 tics they were selected for the majority drop into a range of 500-1500 tics. Only four seedlings (23B4, 28D8, 12A4, and 8D5) had field means above 1000 tics, and two (11B8 and 4B2) dropped below 500 tics (Figure 18).

Table 8: Percent 1n and 2n pollen from 200 pollen grains in each of n samples each containing pollen from at least three flowers of 57 diploid T_1 selections. Any seedlings with less than 30% living pollen, more 2n pollen than IVP 101 (yellow), no pollen release/flowers or which did not meet requirements for vigor and mature plant GFP fluorescence were eliminated (pink).

	Good GFP		% of total pollen				% of living pollen	
name	and vigor	n	1n	2n	dead	living	1n	2n
8C6	no	3	13.5%	3.8%	82.7%	17.3%	77.9%	22.1%
8D3	yes	3	20.2%	1.8%	78.0%	22.0%	91.7%	8.3%
8D5	**yes**	**3**	**29.0%**	**1.0%**	**70.0%**	**30.0%**	**96.7%**	**3.3%**
9B2	no	3	52.8%	3.8%	43.3%	56.7%	93.2%	6.8%
10D7	no	0						
11B8	**yes**	**1**	**65.0%**	**0.5%**	**34.5%**	**65.5%**	**99.2%**	**0.8%**
11C1	**yes**	**4**	**40.5%**	**0.0%**	**59.5%**	**40.5%**	**100.0%**	**0.0%**
12A4	**yes**	**1**	**40.0%**	**1.5%**	**58.5%**	**41.5%**	**96.4%**	**3.6%**
13A7	yes	0						
15C3	no	3	55.5%	2.3%	42.2%	57.8%	96.0%	4.0%
16A2	no	3	40.3%	6.8%	52.8%	47.2%	85.5%	14.5%
16C2	no	0						
17B8	no	3	63.8%	0.2%	36.0%	64.0%	99.7%	0.3%
18B1	yes	0						
19B3	**yes**	**2**	**51.3%**	**0.8%**	**48.0%**	**52.0%**	**98.6%**	**1.4%**
19B5	no	0						
20C7	yes	0						
21A4	**yes**	**3**	**53.7%**	**1.8%**	**44.5%**	**55.5%**	**96.7%**	**3.3%**
23B4	**yes**	**3**	**66.2%**	**2.2%**	**31.7%**	**68.3%**	**96.8%**	**3.2%**
23B6	yes	0						
23C7	yes	0						
7D2	no	0						
17B4	no	0						
28C7	no	0						
7A1	no	0						
4B3	no	1	56.5%	0.0%	43.5%	56.5%	100.0%	0.0%
IVP 101		4	62.3%	2.3%	35.5%	64.5%	96.5%	3.5%
24B4	no	4	70.5%	0.5%	29.0%	71.0%	99.3%	0.7%
24C5	**yes**	**1**	**78.0%**	**0.0%**	**22.0%**	**78.0%**	**100.0%**	**0.0%**
24D4	yes	1	21.5%	7.5%	71.0%	29.0%	74.1%	25.9%
26B3	no	0						
26C2	yes	0						
28B5	no	0						
28D8	**yes**	**1**	**84.0%**	**0.0%**	**16.0%**	**84.0%**	**100.0%**	**0.0%**
30D8	yes	1	2.0%	2.0%	96.0%	4.0%	50.0%	50.0%
31A4	yes	0						
31B4	no	0						
1R15#10	yes	0						
1R15#11	**yes**	**2**	**41.3%**	**0.0%**	**58.8%**	**41.3%**	**100.0%**	**0.0%**
1R15#12	no	0						
1R15#13	no	0						
1R12#13	no	0						
1R12#14	no	0						
1B5	no	2	64.3%	0.0%	35.8%	64.3%	100.0%	0.0%
4A2	yes	0						
4A7	no	3	49.2%	1.8%	49.0%	51.0%	96.4%	3.6%
4B2	**yes**	**2**	**61.8%**	**1.3%**	**37.0%**	**63.0%**	**98.0%**	**2.0%**
4D6	yes	0						
5A6	no	0						
6B5	yes	0						
6D7	yes	0						
7A2	**yes**	**1**	**33.5%**	**1.0%**	**65.5%**	**34.5%**	**97.1%**	**2.9%**
7B2	**yes**	**3**	**38.7%**	**1.2%**	**60.0%**	**40.0%**	**96.7%**	**2.9%**
8B4	**yes**	**1**	**88.5%**	**0.0%**	**11.5%**	**88.5%**	**100.0%**	**0.0%**
1R7#6	**yes**	**0**						
12B8	no	0						
28B2	no	0						
6A4	no	0						

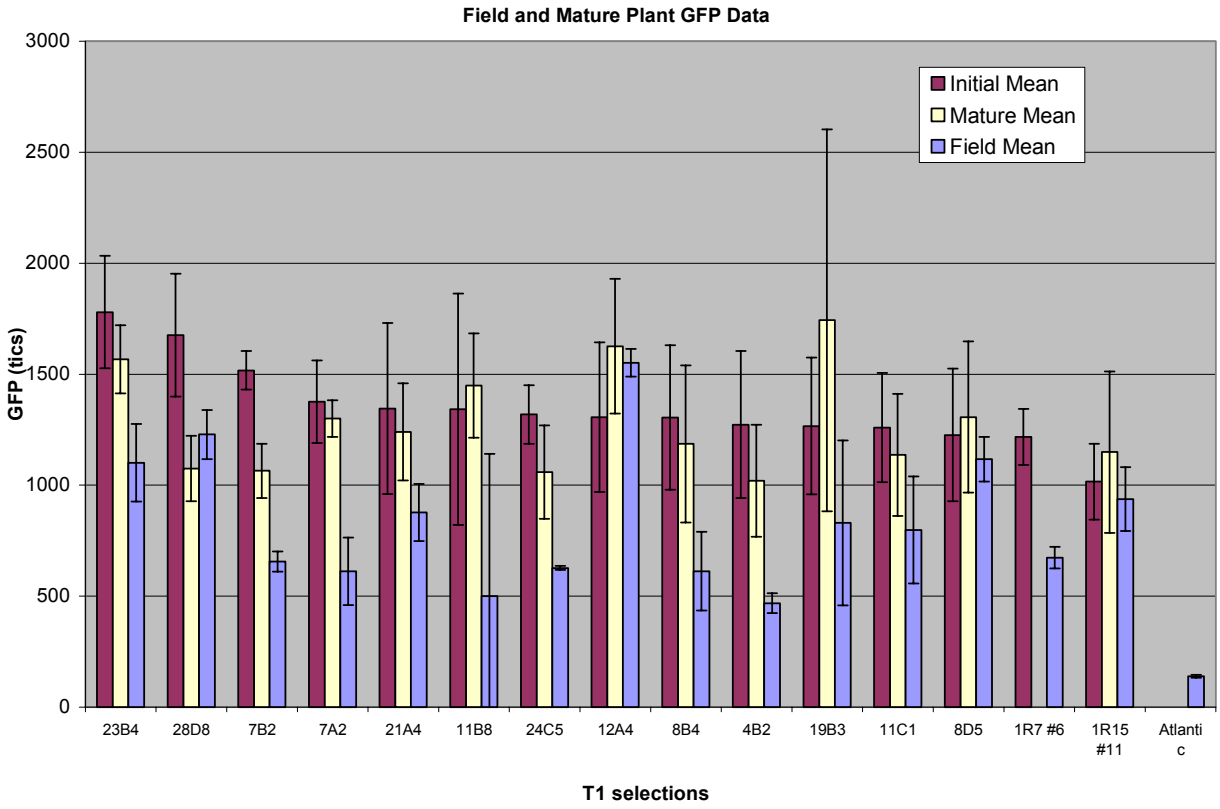


Figure 18: The top 15 T_1 diploid selections were tested in the field with a GFP meter (blue); means are for three leaves per plant. In comparison to the initial GFP screening (red) from Figure 10, and the final average of mature plant GFP fluorescence (yellow) last two columns in Table 7, the results varied but had a less scattered range than the total seedling collection. Readings were taken from Atlantic as a control from the field. Error bars for all means show standard deviation.

Visual Screening of GFP:

Greenhouse grown leaves from the final 15 selections were screened under a fluorescence microscope, in order to confirm selections after observing the variation from the field. Some of the selections were eliminated because they expressed little or no GFP and showed red chlorophyll autofluorescence instead (24C5, 11B8 and 8B4). Others were eliminated because they had no GFP fluorescence along the midvein of leaves (23B4, 28D8 and 8D5) (Figure 19). These results conflicted with the field results; however they also explain the continued inconsistencies with the GFP meter. The GFP meter takes readings from a circle approximately 2 mm in diameter, and the irregular patterns of expression in the leaves would give each part of the leaf a different value. The final selections were limited to six plants (7B2, 4B2, 21A4, 12A4, 19B3 and 11C1) that had maintained GFP fluorescence evenly and consistently according to the visual screening. Seedling 7A2 was eliminated by its dwarfed size rather than GFP fluorescence. After these selections were made, plant also 12A4 began showing the red vein that was used to eliminate other selections (Figure 20).

The tubers and flowers of these final six were also observed under a fluorescence microscope to determine the extent of GFP fluorescence throughout the plant. The tubers showed high levels of GFP fluorescence compared to controls. The tubers also showed some fluorescence in the skin; although it was more obvious with less purple pigment (Figure 21). The flowers showed high levels of GFP fluorescence in the petals, sepals, anthers and stigmas. In the control flowers the petals, sepals, and stigmas auto-fluoresced red from chlorophyll auto-fluorescence (Figure 22). A confocal microscope showed the GFP fluorescence along the outside of the leaf and in the vascular tissue, whereas chlorophyll appeared in the interior of the leaf (Figure 23).

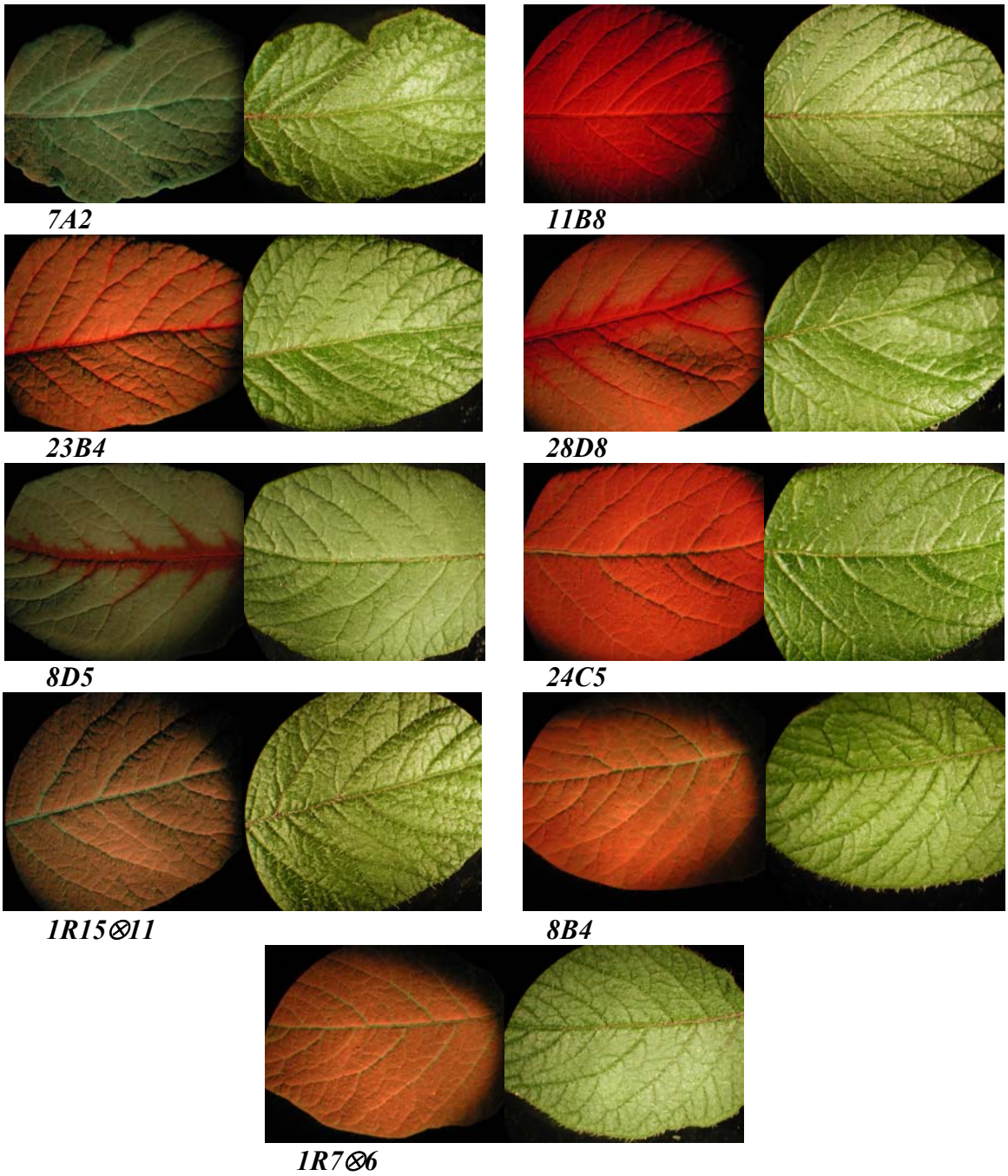


Figure 19: Fully expanded young leaves under GFP/chlorophyll filters (left) and tungsten light (right) of the nine T_1 selections eliminated from the final 15 based on visual observations of GFP fluorescence (7A2 was eliminated because of slow growth / lack of vigor)

Pictures were taken with an Olympus SZX12 stereomicroscope with the combined GFP and chlorophyll filter (excitation 461-500nm, dichroic mirror 505nm, detection >510nm).

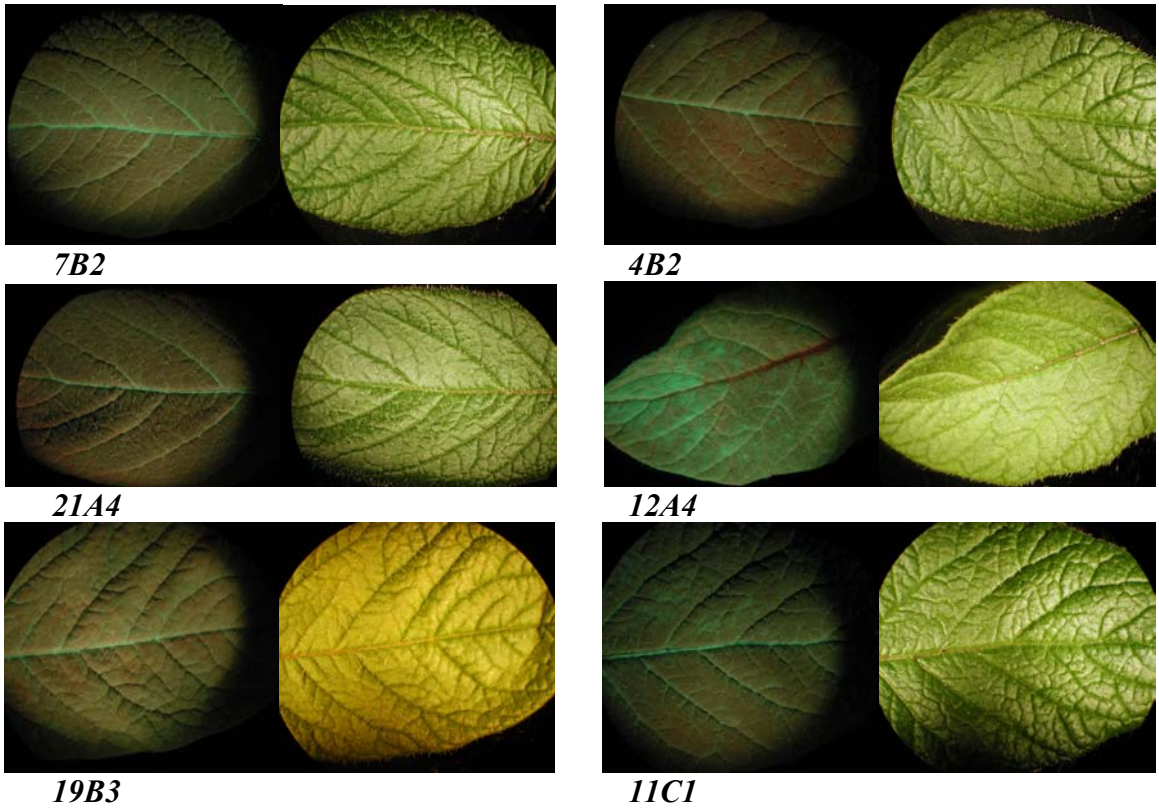


Figure 20: Fully expanded young leaves of the final six T_1 selections seen under a GFP filter (left) with a control (right) for each taken in tungsten light

All the selections have maintained relatively high, uniform fluorescence of GFP. Pictures were taken with an Olympus SZX12 stereomicroscope with the combined GFP and chlorophyll filter (excitation 461-500nm, dichroic mirror 505nm, detection >510nm).

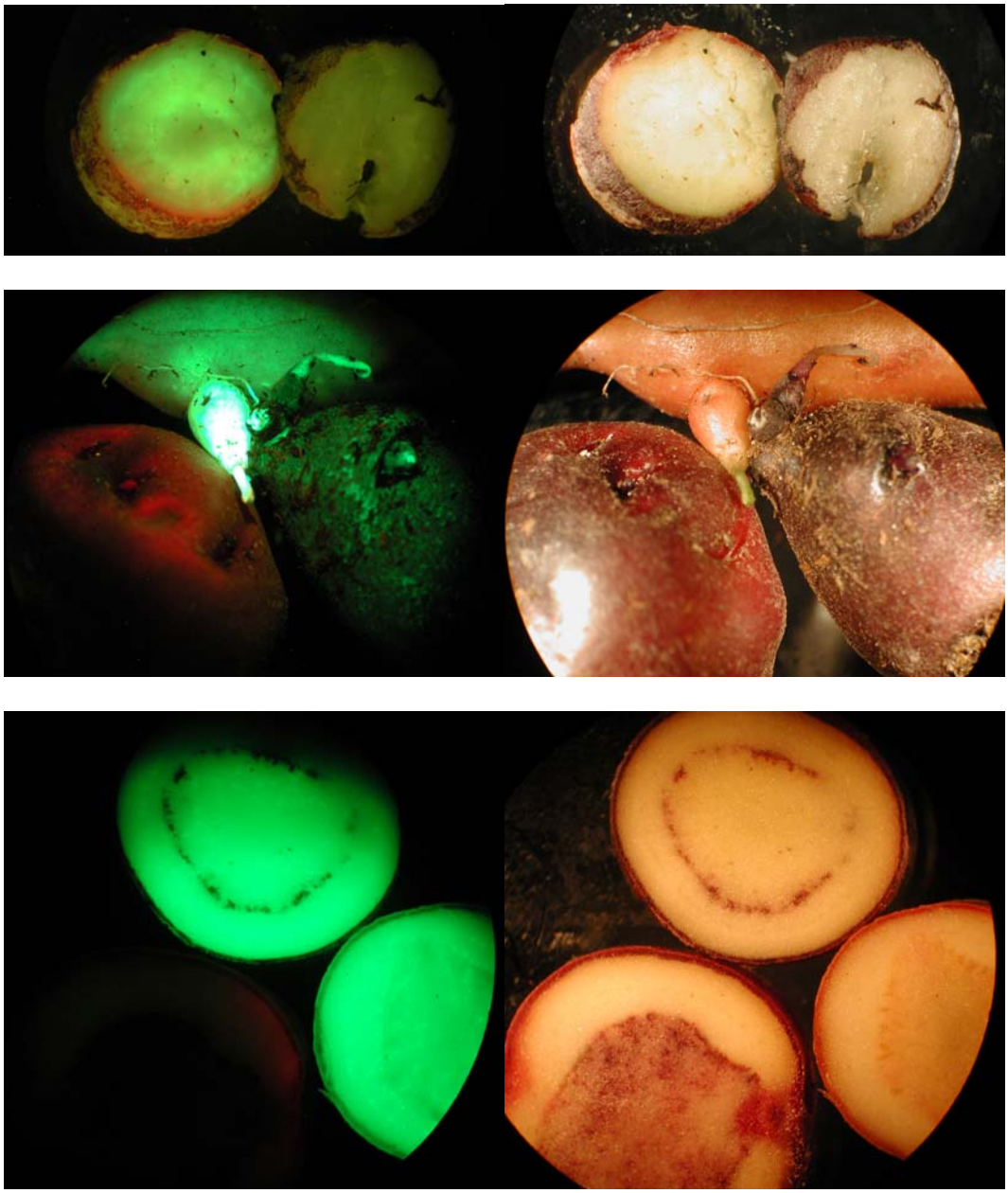


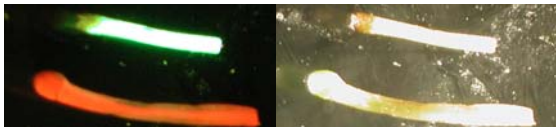
Figure 21: Pictures of diploid T_1 tubers and controls with GFP filters (left) and tungsten light (right)

The top pictures show 6A4 (left) compared to IVP 101 (right). The middle and bottom pictures show 19B3 (top), SMPC⊗1 (bottom left), and 7B2 (bottom right). Pictures were taken with an Olympus SZX12 stereomicroscope with the combined GFP and chlorophyll filter (excitation 461-500nm, dichroic mirror 505nm, detection >510nm).

21A4

11C1

IVP 101



6A4 (top) Atlantic (bottom)

Figure 22: Flowers from T_1 selections viewed with GFP filters and tungsten light, and a close up of a 6A4 stigma compared to an Atlantic stigma

Pictures were taken with an Olympus SZX12 stereomicroscope with the combined GFP and chlorophyll filter (excitation 461-500nm, dichroic mirror 505nm, detection >510nm).

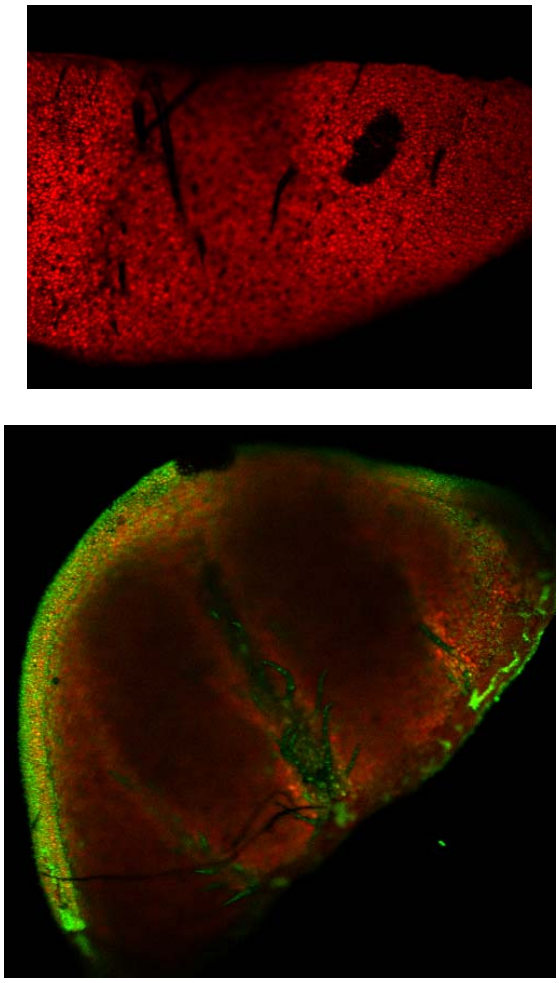


Figure 23: Leaves viewed through a Nikon confocal microscope with filters showing separate chlorophyll and GFP fluorescence.

Top: One "slice" through a control leaf. Bottom: Several "slices" of a transformed leaf stacked together for a 3D image

Tetraploids:

Anther culture:

The T_0 transformants, 2Rx and 3Rx, were tetraploids, which were most likely formed by spontaneous chromosome doubling during leaf disc regeneration. There was a possibility that the tetraploids carried a duplex copy of the transgene, so we attempted to reduce the ploidy of these tetraploids to dihaploids through anther culture. If that were the case one in six dihaploids would inherit a homozygous transgene. The first set of anther cultures produced embryos from three tetraploid T_0 selections (2R3, 3R4 and 3R7), and the second set produced embryos from 2R1 (Table 9). The antherculture treatments had significantly different numbers of embryos, with the highest numbers produced by the two treatments with 30^oC days (12h) and 20^oC nights (12h) (Table 10).

Attempts at visual screenings of embryos directly showed inconsistent expression of GFP, so once they grew large enough for subculturing the plantlets that were vigorous and purple in vitro were transplanted into the greenhouse. This produced one 2R1 AC plant, five 2R3 plants, 11 3R4 plants and 12 3R7 plants that were successfully transplanted and grown in the greenhouse. The anther regenerants intermittently expressed an extreme variation of the curled leaves seen in the seedlings. Leaves on these plants failed to expand completely (Figure 24). The GFP meter was used to screen the plants in the greenhouse, and showed that none of them fluoresced at a high level (Figure 25).

Table 9: Anther culture regenerants of 2Rx and 3Rx transformants produced and grown in the greenhouse, listed by anther culture treatment

plant	AC Media	temp	embryo media	# embryos	# plantlets	purple	vigorous invitro	transplant to GH	survived GH	
2R3	maltose	30/20	phytagel	52	9	3	1	1	2R3AR1	
2R3	maltose	30/20	agarose	23	12	10	7	5	2R3AR4,9,10,12	
3R4	maltose	20	phytagel	11	4	2	2	1	3R4AR2	
3R4	maltose	30/20	agarose	73	13	9	5	5	3R4AR17,18,20	
3R4	maltose	30/20	phytagel	36	11	7	6	4	3R4AR4-6,8	
3R4	solid	20	phytagel	2						
3R4	sucrose	30/20	phytagel	31	6	5	3	3	3R4AR11-13	
3R6	maltose	20	phytagel	8						
3R6	maltose	30/20	phytagel	20						
3R6	maltose	30/20	agarose	36						
3R6	solid	20	phytagel	6						
3R6	sucrose	20	phytagel	11						
3R6	sucrose	30/20	phytagel	35						
3R6	sucrose	30/20	agarose	36						
3R7	maltose	20	agarose	3	2	1	1	1	3R7AR12	
3R7	sucrose	20	agarose	16	2	2	2	2	3R7AR11,13	
3R7	sucrose	30/20	agarose	36	7	4	4	3	3R7AR2-4	
3R7	sucrose	30/20	phytagel	72	11	8	8	6	3R7AR1,7,8,10,16	
3R7	TM plate 2	mult. trt.	phytagel	29	1	1	1	1	3R7AR14	
3R9	maltose	20	agarose	6						
3R9	maltose	30/20	agarose	28						
3R9	maltose	30/20	phytagel	29						
3R9	sucrose	20	agarose	6						
3R9	sucrose	30/20	agarose	18						
plant	treatment	well	embryo media	# embryos						
2R1	suc. 30/20	B2	phytagel	4	3	2	2	2	2R1AR1,3	

Table 10: Analysis of variance for embryos produced by antherculture treatments, and mean number of embryos listed by treatment (3R7 plate 2 and 2R1 omitted)

Anova

Source	df	MS	*p*
AC media, temp	4	1235	0.0037
Error	18	215	

Mean embryos/treatment

Treatment	Mean	
Sucrose, 30/20	38±6	a
Maltose, 30/20	37±5	a
Sucrose, 20	11±8	b
Maltose, 20	7±7	b
Solid, 20	4±10	b

Figure 24: The curled leaf syndrome as expressed in anther regenerants of 2Rx and 3Rx transformants. Top: A mature anther regenerant with new leaves that appear to be fully opened and curled only as much as the leaves of the seedlings, and a blank spot in the middle of the plant where the abnormal leaves grew. Bottom: The abnormal leaves in the blank part of the same plant.

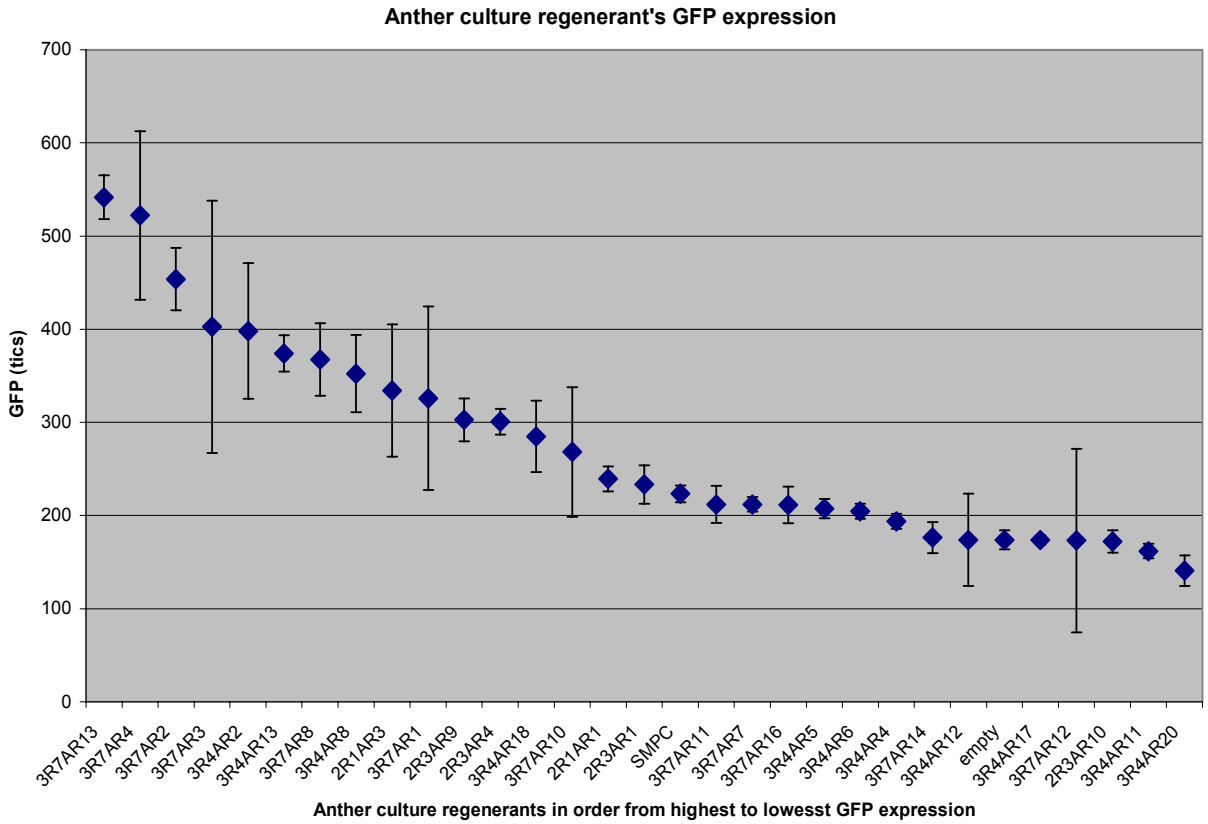


Figure 25: GFP results from anther culture regenerants of 2Rx an 3Rx in the greenhouse. None of the results were high enough to be homozygous, and most were below the 300 tic cutoff for no GFP.

Seedlings:

Any insertion of the transgene into tetraploids could occur as either simplex (G000) or duplex (GG00), depending on whether the transformation happened after or before chromosome doubling. The expected segregation ratios were 3:1 for single-copy simplex insertions (Table 11), 35:1 for single-copy duplex insertions (Table 12), and 15:1 for double-copy simplex insertions (Table 13). The selfed seed of several tetraploid T_0 plants were planted in the greenhouse, and sorted under a florescence microscope for GFP fluorescence. At least 200 seedlings were counted for each family of T_0 plants, when available. They were sorted for presence or absence of GFP; medium and high expression levels could not be distinguished visually. Of the families with over 100 seedlings, only 3R2 failed to match one of the expected segregation ratios. The 3R5 family fit the single-copy duplex segregation, whereas 3R3, 3R7 and 3R9 families matched the double-copy simplex segregation (Table 14).

Table 11: Expected **3 transgenic:1 non-transgenic** segregation in a T_1 tetraploid population from a primary transformant with a single, simplex copy of the GFP transgene (G_1000); 0 = no transgene

Alleles	1 G_10	1 00
1 G_10	1 G_1G_100	1 G_1000
1 00	1 G_1000	**1 0000**

Table 12: Expected **35 transgenic:1 non-transgenic** segregation in a T_1 tetraploid population from a primary transformant with a single, duplex copy of the GFP transgene (G_1G_100); 0 = no transgene

Alleles	1 G_1G_1	4 G_10	1 00
1 G_1G_1	1 $G_1G_1G_1G_1$	4 $G_1G_1G_10$	1 G_1G_100
4 G_10	4 $G_1G_1G_10$	16 G_1G_100	4 G_1000
1 00	1 G_1G_100	4 G_1000	**1 0000**

Table 13: Expected **15 transgenic:1 non-transgenic** segregation in a T_1 tetraploid population from a primary transformant with double, simplex copies of the GFP transgene (G_1000, G_2000) segregating independently; 0 = no transgene

Alleles	1 G_10 G_20	1 G_10 00	1 00 G_20	1 00 00
1 G_10 G_20	1 G_1G_100 G_2G_200	1 G_1G_100 G_2000	1 G_1000 G_2G_200	1 G_1000 G_2000
1 G_10 00	1 G_1G_100 G_2000	1 G_1G_100 0000	1 G_1000 G_2000	1 G_1000 0000
1 00 G_20	1 G_1000 G_2G_200	1 G_1000 G_2000	1 0000 G_2G_200	1 0000 G_2000
1 00 00	1 G_1000 G_2000	1 G_1000 0000	1 0000 G_2000	**1 0000 0000**

Table 14: Tetraploid T_1 seedling GFP segregation of Glowing: No GFP fluorescence, and proposed genotypes for families with over 100 seedlings.

Family	Glowing	No GFP	Total	Observed Ratio	Expected Ratio	X^2	P	Proposed 4x genotype
3R1	6	2	8					
3R2	167	33	200	5.1:1	3:1	7.71	0.006	
3R3	**206**	**21**	**227**	**9.8:1**	**15:1**	**3.49**	**0.062**	**G_1000, G_2000**
3R4	86	2	88					
3R5	**115**	**4**	**119**	**28.8:1**	**35:1**	**0.43**	**0.514**	**G_1G_100**
3R6	59	2	61					
3R7	**218**	**14**	**232**	**15.6:1**	**15:1**	**0.02**	**0.892**	**G_1000, G_2000**
3R9	**160**	**15**	**175**	**10.7:1**	**15:1**	**1.61**	**0.205**	**G_1000, G_2000**

Haploid Induction Results:

Repeated Pollen Counts:

The final six T_1 seedlings were planted in the greenhouse, and pollen was collected from at least three flowers of each on three separate days. The pollen was stained with acetocarmine, and sorted into categories of large stained, small stained and unstained, which corresponded to 2n, 1n and dead, respectively. The results of all three days were averaged together to confirm the initial pollen counts that were used to select these plants. Most of the selections had a higher percentage of living pollen than IVP 101, and all of the selections had a lower percentage of 2n pollen in the living pollen (Table 15).

Crosses to Cultivars:

The six final selections, along with IVP 101 and a self cross of SMP-C, SMP⊗1, were crossed with Atlantic and Katahdin cultivars. After all the pollinations only two experimental fruit were produced: Atlantic x 4B2 and Katahdin x 7B2. During pollinations, several experimental crosses began to set fruit and then fell off before ripening. All the seed was harvested after 2-3 weeks ripening from these two fruit along with all the control fruit, and planted in vitro. Each of the experimental fruit had four seeds, compared to the average of 6.5 for IVP 101. Crosses of SMP⊗1 to the cultivars also had fruit and seed sets much lower than IVP 101 (Table 16).

Table 15: Pollen counts by acetocarmine staining for three samples each taken on a separate days consisting of combined pollen from three flowers from the final six T_1 selections

	Average % of total pollen				% of living	
plant	2N	1N	Dead	Living	2N	1N
IVP 101	2.3%	60.8%	36.8%	63.2%	3.7%	96.3%
19B3	0.2%	82.8%	17.0%	83.0%	0.2%	99.8%
21A4	0.3%	70.3%	29.3%	70.7%	0.5%	99.5%
11C1	0.5%	87.8%	11.7%	88.3%	0.6%	99.4%
12A4	0.2%	94.0%	5.8%	94.2%	0.2%	99.8%
4B2	1.0%	81.5%	17.5%	82.5%	1.2%	98.8%
7B2	0.3%	54.2%	45.5%	54.5%	0.6%	99.4%

Table 16: Total fruit and seed resulting from cross-pollinations performed between the six final T_1 selections (plus IVP 101 and SMP⊗1 controls) and two cultivars, Katahdin and Atlantic

	Number of flowers on		**Total**	Number of fruit on		**Total**	Number of seed from		**Total**
Pollinator	Kahtadin	Atlantic	**flowers**	Kahtadin	Atlantic	**fruit**	Kahtadin	Atlantic	**seed**
IVP 101	58	14	**72**	18	7	**25**	92	71	**163**
SMP⊗1	29	10	**39**	0	5	**5**	0	10	**10**
11C1	66	0	**66**	0	0	**0**	0	0	**0**
19B3	32	0	**32**	0	0	**0**	0	0	**0**
12A4	3	0	**3**	0	0	**0**	0	0	**0**
7B2	50	0	**50**	1	0	**1**	4	0	**4**
4B2	9	1	**10**	0	1	**1**	0	4	**4**
21A4	8	0	**8**	0	0	**0**	0	0	**0**

Chapter 4: Discussion

The goal of creating a haploid inducing pollinator that is homozygous for GFP first required a haploid inducing pollinator that could regenerate whole plants from leaf discs. One of the most widely used haploid inducers for potato, IVP 101, was recalcitrant; therefore, we crossed it to a highly regenerable clone of *S. phureja*, BARD 1-3. A seedling selected from a cross between these two plants, SMP-C, regenerated easily from leaf discs and was selected for the transformation. After the transformation of SMP-C with the *Agrobacterium* obtained from Molinier et al. (2000), we obtained a total of 29 regenerated plants, 17 of which were diploid. The other 11 T_0 plants had doubled their chromosome number during the regeneration process. All 17 diploids came from the same leaf disc, so it was possible that they all derived from the same transformation event. The tetraploids came from two leaf discs, and therefore represent at least two transformation events.

The T_0 plants were extremely vigorous and there was little apparent phenotypic difference between diploid and tetraploid clones. All 29 clones flowered extensively. One possible explanation for the favorable growth of the diploids plants is hybrid vigor produced by the cross of IVP 101 and BARD 1-3. Although self-fertility is not expected in diploid *S. phureja* due to a gametophytic self-incompatibilty system (Cipar et al., 1964), fertile clones are relatively common and it was fortuitous that SMP-C expressed this trait. We hoped that the T_1 seedlings produced from self crosses of the original transformants would inherit some of this vigor, but instead they segregated for deleterious alleles.

Freshly extracted seeds with brightly fluorescing embryos were sorted under the microscope from diploid T_0 plants that were selected for high GFP fluorescence. Unfortunately, the selected seeds with brightly fluorescing embryos had a low germination rate. Both selected

and unselected seedlings segregated for deleterious alleles. Specifically, the T_1 seedlings segregated for curled leaves and dwarfed size. The segregation ratio of the dwarfed phenotype was what would be expected for a single recessive gene in a diploid plant. This is consistent with the results of Valkonen et al. (1999) who found that dwarves obtained in anther culture from a particular cultivar of *S. tuberosum* were the result of a recessive gene affecting the gibberellin biosynthesis pathway. Nijs et al. (1980) also reported a single recessive gene with similarly small phenotype identified as topiary. According to Nijs et al. (1980) the topiary trait produced increased branching in a compact plant.

In the total population the ratio for the curled leaves was not as similar to the expected 3:1 ratio as the dwarfed phenotype was, although in some of the individual families this ratio could not be rejected. However the leaves which were only slightly curled could be easily overlooked, especially if the plant was also extremely dwarfed. The possibility that additional curled leaves were overlooked is supported by the results of Rosin et al. (2003), who found that overexpression of a single gene produced plants which had both dwarfed size and abnormal leaves. Rosin et al. (2003) also included additional traits from the leaves, such as a heart shape. In our seedlings many of the most severe dwarves had this trait, but were not counted as curled (Figure 8).

Purple color did not segregate with ratios corresponding to either one gene (3:1) or two gene (15:1) interactions, but this was expected since it is already known that there are several genes involved in this trait (De Jong, 1991). In addition there was a wide range of purple color expression, and in spite of every effort to count the slightest tint as purple, it was easy to categorize plants incorrectly. The segregation of these three traits (dwarfed size, curled leaves and purple color) in the three families with more than 100 seedlings (1R4, 1R6 and 1R10)

generally matched the segregation of the total population, reinforcing the possibility that the diploids all came from one transformation event.

The initial screening of the diploid T_1 seeds showed that the embryos segregated for GFP fluorescence, but that fluorescence could only be seen when the seed coat was moist, diluting its autofluorescence. The interference from autofluorescence was unexpected since Molinier et al. (2000) demonstrated an obvious difference in the fluorescence of GFP and control tobacco seed under GFP filters. GFP filters showed green auto-fluorescence in the seed coat of potato that interfered with detection of GFP. Most GFP and chlorophyll combined filters also showed the seed coats fluorescing green, rather than the red auto-fluorescence of chlorophyll that was observed in most of the other potato tissues examined. However, one example was found that accurately separated the seed coat fluorescence (red this time) from the GFP in the embryo of a germinating seed (Figure 6). Unfortunately, the green fluorescence of transformed embryos within such seed coats could not be observed in the dry seed and only appeared after germination. This implies that there may not be GFP fluorescence in dry embryos. There is also the possibility that the seeds are only penetrable by UV light while hydrated, just like Mercuri et al. (2001) found some flower petals can only be penetrated by UV light from beneath. The seed coat's auto-fluorescence is a continuing problem that must be addressed before seeds can be effectively screened using GFP. For the purposes of this project a fluorescence microscope was used for sorting fresh seed; however any practical application would involve sorting dry seed with an automated seed sorter. Hydrated seed potato could not be sorted automatically, so if the dry embryos do not fluoresce this selection method will not work at the seed level.

GFP fluorescence was quantifieded with a GFP meter in the plants. The tics detected by the GFP meter were assumed to correlate directly with GFP expression, as Halfhill et al. (2003)

reported the correlation between GFP intensity and soluble GFP concentrations in *Brassica napus* L. GFP expression in the three diploid families with more than 100 seedlings all came close to fitting the 15:1 ratio expected for a double-copy insertion. This shows more evidence, in addition to the segregation patterns of the other traits, that the diploids may have all come from the same transformation event. The segregation of the total population matches the 15:1 ratio. The initial screening of the T_1 seedlings resulted in a gradual slope of expression, instead of the distinct separation expected of a single transgene for high (homozygous), medium (hemizygous), and low (none) GFP expression. There did seem to be a separation somewhere around 300 tics; this was made more obvious with data grouped into 100 tic increments with all the controls remaining below 300 tics (Figure 14). Having two copies of the transgene is consistent with the findings of Brown et al. (1991) that the majority of their *Agrobacterium* mediated transformations of potato resulted in one to three total transgene insertions.

Two replicated studies were used to better grasp the segregation. The first was the "low/none" study, which confirmed that there was a separation between low fluorescence and no fluorescence somewhere near 300 tics. However, the study continued to show variation in fluorescence levels by some genotypes. Halfhill et al. (2001) found that GFP was expressed at levels high enough to be stronger than red chlorophyll autofluorescence in canola leaves only in immature plants. In contrast, we were able to view GFP in mature leaves of the final six selections (Figure 20), and had to eliminate others because of an absence, or irregular pattern, of GFP. Jones et al. (1999) found that a similar patterns of GFP that spread to the rest of the leaf resulting from systemic silencing spread by an RNA signal. The silencing could be the result of the double-copy insertion; this would be consistent with Ma and Mitra (2002), who reported that silencing occurred at a high frequency as a result of multiple copies of the transgene (GFP).

The second replicated study was the "high/medium/low" study in which selections at 400 tic increments were used to distinguish where the separation between these categories should be. This is where the silencing became most obvious. Figure 11 shows leaves taken from each level of this study, and ironically the highest group dropped to little or no fluorescence during the 4-6 weeks between the seedling screening and the replicated study. This silencing resulted in selections being made based on the consistency of GFP fluorescence, rather than simply the highest expressers. In fact the last selection before haploid induction was a visual screening that again showed the silencing in some genotypes.

The plants with normal shaped and sized leaves, some purple pigment and the highest levels of GFP in an initial screening, were selected and added to the few plants remaining from the first selection of seeds. The pollen from these plants was screened to determine how much was viable and, of the viable pollen, how much was 1n verses 2n. A diploid pollinator with high 2n pollen, when used on a tetraploid, would produce many tetraploid hybrids in the 4x x 2x cross, instead of haploids of the mother plant. Haploid inducing pollinators have low 2n pollen, and so when a haploid inducing pollinator is used on a tetraploid, the resulting 4x x 2x cross produces mostly triploid (3x) seed. Most triploid seed abort due to the embryo/endosperm imbalance (Marks, 1966). Therefore, the few viable seeds produced by a good haploid inducing pollinator in a 4x x 2x cross are likely to be haploid.

The plants were also screened for GFP fluorescence in leaves of different maturity. In general, the older leaves expressed less brightly, and the mature plants had lower levels of fluorescence than they had as seedlings. This is consistent with the results of Halfhill et al. (2003) with GFP expression in *B. napus*. The plants that had the most viable pollen with the least 2n, and also had consistently high GFP fluorescence in the different leaf samples were

selected and tested for GFP fluorescence in the field, as well as visually under a microscope. The field results for GFP fluorescence showed that the variation was not completely eliminated, but at least the extreme highs and lows had been eliminated. The visual screening was done to double check the selections, and it identified a likely reason for expression differences. Many of the observed leaf samples showed a complete lack of GFP fluorescence, and several others showed an odd pattern in which GFP was only present at the leaf margins. This potentially shows incomplete silencing of GFP. A final selection was made based on the visual screening, and only six plants remained. Several of the plants were discarded, because of the red autofluorescence running along the mid vein of the leaves. This left GFP fluorescence visible only at the leaf margin. This would create a problem for the GFP meter, since it would be impossible to get consistent results from a leaf with that pattern. Later, when the pictures were taken for Figure 19 it was observed that most of these leaves had reverted to very little or no fluorescence over the whole leaf surface. In addition one of the selections, 12A4, began to show the red chlorophyll along the leaf's midvein that signifies silencing, and the green fluorescence of the others was less intense than it had been (Figure 20).

The other two transformed leaf discs produced tetraploids, and depending on when the chromosomes doubled, the transformations may have produced either simplex or duplex insertions. The possibilities include either a single simplex insertion segregating 3 GFP:1 NO GFP (Table 11), or a double simplex insertion segregating 15 GFP:1 NO GFP (Table 13) if the transformation happened after chromosome doubling. Alternatively, if the transformations happened before the chromosome doubling, there could be a single duplex insertion segregating 35 GFP: 1 NO GFP (Table 12).

The tetraploid screening did show one family, 3R5, which had a GFP segregation ratio among the T_1 seedlings that would be expected from a single duplex insertion. Anther culture was used in an attempt to reduce the ploidy level of tetraploid transgenics and produce dihaploids homozygous for GFP from single duplex insertions. However, the 3R5 family was the only one that fit a duplex, single-copy model, and it did not produce any anther regenerants. The anther culture results showed a significant increase in the number of embryos produced when the temperature was kept at alternating 30°C and 20°C temperatures for 12 hours each, compared to a constant 20°C. These results are consistent with the findings of Shen and Veilleux (1995) that alternating the temperature between 30°C (16h) and 20°C (8h) increased embryo production.

Three other families, 3R3, 3R7 and 3R9, had segregation ratios matching double simplex insertions, which would not have produced dihaploids homozygous for GFP. Low GFP fluorescence among the anther regenerants, shows that there is little likelihood that any of the other tetraploids have duplex insertions. Therefore, only the final six diploid selections were used in haploid induction tests.

Assuming the diploids are all double simplex insertions, the T_1 generation would segregate for homozygous and hemizygous copies of each transgene (Table 3). Nine out of 16 seedlings would have both copies of the transgene in either homozygous or hemizygous states, and so probably be subject to silencing. One in 16 would have no transgenes, and therefore express no GFP. Of the remaining six out of 16 seedlings that would have only one copy of the transgene, two would be homozygous and four would be hemizygous. The segregation of two transgenes combined with silencing could help explain the lack of distinct high, med and low categories in the initial GFP screening (Figure 10). That means that only one in eight seedlings

would have the single homozygous GFP needed for these selections. Assuming the dwarfism and curled leaves were the result of the same single gene, as found by Rosin et al. (2003), only 25% of the seedlings would be a completely normal phenotype, and so there would only be approximately 27 seedlings left out of the total 879.

Selections also have to be made for homozygous haploid induction ability (purple color, embryo spot, low 2n pollen), which would occur in 25% of the total population if only one gene were involved. However, van Breukelen (1981) reported that haploid induction has intermediate inheritance, and is linked to at least five loci. In van Breukelen's studies self-pollinations of haploid inducing pollinators, and even crosses between good and bad pollinators, produced progeny with haploid inducing abilities both better than and worse than the parent plants.

If our pollen screenings were successful the five remaining selections (after 12A4 began to show silencing) should represent the best haploid inducing pollinators from our seedlings that are also homozygous for GFP. Unfortunately, the number of fruit per pollination and the number of seed per fruit are both lower than IVP 101, and an insufficient number of fruit have been formed. The pollen counts showed that the six selections mostly have higher percentages of living pollen, and lower percentages of 2n pollen within the living pollen, than IVP 101 so it is possible that there are more fruit and seeds from the IVP101 crosses because more hybrid seed have been produced. If that is the case, the eight experimental seeds produced (Table 16) should all be haploids. The fruit that fell off the plants before ripening may be a sign that the fruit requires some hybrid (4x) seeds to "nurse" the haploid seed along, in which case our selections for extremely low 2n pollen levels may have decreased fruit set in addition to increasing the frequency of haploids.

In conclusion, GFP has the potential to be a successful marker for haploid inducing pollinators, but it will require more work to determine whether the dry seed may be sorted by GFP fluorescence. If the dry embryos do not glow, this construct will not work for sorting dry seed; however, the construct Anthony Trewavas used to develop potatoes that glow only when dehydrated (Onion, 2001) may be more successful in sorting dry seed. If the dry embryos are glowing, there is still the problem of finding a filter set or procedure that allows the embryo's fluorescence to be seen through the seed coat.

GFP may still have potential as a haploid inducing pollinator, regardless of the difficulty in sorting dry seed. The final selections have so little 2n pollen that they may produce even fewer hybrid seed than IVP 101, and so the seed screening might not be necessary at all. Very few seed were produced in experimental crosses to tetraploid cultivars, and most of those should be haploid seed. Rather than screening the seed, the seedlings could be screened either with fluorescence microscopy, or a GFP Meter. However, flow cytometry will be necessary initially to confirm that these selections are haploid inducers.

We found that in our transformations fluorescence less than 300 tics was due to autofluorescence rather than GFP, and fluorescence greater than 1700 tics was not stable. Our final selections were the plants that continually expressed over 1000 tics, and which fluoresced GFP brightly enough to overpower the red chlorophyll fluorescence. It is possible that our continued screenings and removal of selections with silenced GFP have resulted in final selections that have a single, homozygous copy of the transgene. However, southern blots will be needed to confirm that our selections only have a single copy of the transgene, and segregation for GFP, or a lack thereof, in self crosses of these selections will be needed to confirm that the GFP transgene is homozygous.

References

Allainguillaume, J., Wilkinson, M.J., Clulow S.A. and Barr, S.N.R. 1997. Evidence that genes from the male parent may influence the morphology of potato dihaploids. *Theor. Appl. Genet*. 94: 241-248.

Brown, C.R., C.P. Yang, S. Kwiatkowski and K.D. Adiwilaga. 1991. Insert copy number, chromosome-number, pollen stainability, and crossability of *Agrobacterium*-transformed diploid potato. *Am. Potato J*. 68: 317-330.

Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward and D.C. Prasher. 1994. Green Fluorescent Protein as a Marker for Gene Expression. *Science*. 263: 802-805.

Cipar, M.S., S.J. Peloquin, and R.W. Hougas. 1964. Inheritance of incompatibility in hybrids between *Solanum tuberosum* haploids and diploid species. *Euphytica* 13: 163-172.

Clulow, SA and F. Rousselle-Bourgeois. 1997. Widespread introgression of *Solanum phureja* DNA in potato (*S. tuberosum*) dihaploids. *Plant Breeding*. 116: 347-351.

De Jong, H. 1991. Inheritance of anthocyanin pigmentation in the cultivated potato: a critical review. *Am. Potato J*. 68: 585-591.

Darlington, C.D., and L. LaCour. 1942. *The Handling of Chromosomes*. London, Allen.

Gamborg, O.L., R.A. Miller and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res*. 50: 151-158.

Gelvin, S.B. 2003. *Agrobacterium*-mediated plant transformation: the biology behind the "gene-jockeying" tool. *Microbiol. Mol. Biol. R*. 67: 16-37.

Halfhill, M.D., R.J. Milwood, T.W. Rufty, A.K. Weissinger and C.N. Stewart. 2003. Spatial and temporal patterns of green fluorescent protein (GFP) fluorescence during leaf canopy development in transgenic oilseed rape, *Brassica napus* L. *Plant Cell Rep*. 22: 338-343.

Halfhill, M.D., H.A. Richards, S.A. Mabon and C.N. Stewart Jr. 2001. Expression of GFP and Bt transgenes in *Brassica napus* and hybridization with *Brassica rapa*. *Theor. Appl. Genet*. 103: 659-667.

Haynes, F.L. 1972. The use of cultivated diploid *Solanum* species in potato breeding. p. 100-110. In: French, E.R. (ed.) *Prospects for the Potato in the Developing World*. Lima, Peru.

Hawley, T.S., W.G. Telford and R.G. Hawley. 2001. "Rainbow" reporters for multispectral marking and lineage analysis of hematopoietic stem cells. *Stem Cells* 19: 118-124.

Hermsen, J. G. T. and J. Verdenius. 1973. Selection from *Solanum tuberosum* Group *Phureja* of genotypes combining high-frequency haploid induction with homozygousity for embryo-spot. *Euphytica*. 22: 244-259.

Jones, L., A.J. Hamilton, O. Voinnet, C.L. Thomas, A.J. Maule, and D.C. Baulcome. 1999. RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. *Plant Cell*. 11: 2291-2301.

Ma, C. and A. Mitra. 2002. Intrinsic direct repeats generate consistent post-transcriptional gene silencing in tobacco. *Plant J*. 31: 37-49.

Marks, G.E. 1966. The origin and significance of intraspecific polyploidy: experimental evidence from *Solanum chacoense*. *Evolution*. 20: 552–57.

Mercuri, A. A. Sacchetti, L. De Benedetti, T. Schiva and S. Alberti. 2001. Green fluorescent flowers. *Plant Science*. 161: 961-968.

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

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

Niedz, R.P. 1995. Green fluorescent protein: an in-vitro reporter of plant gene expression. *Plant Cell Rep*. 14: 403-406.

Nijs, T.P.M. den, E.F. Leue and S.J. Peolquin. 1980. Topiary, a mutant character in *Solanum infundibuliforme*. *Journal of Heredity*. 71: 57-60.

Onion, A. (ABC News Online) 2001. *One potato, new potato: a potato that glows when it's thirsty*. Retrieved Dec. 18, 2003, from Organic Consumers Association. Little Marais, MN. Website: http://www.organicconsumers.org/ge/jellyfish_potato.cfm

Owen, H.R., R.E. Veilleux, D. Levy and D.L. Ochs. 1988. Environmental, genotypic, and ploidy effects on endopolyploidization within a genotype of Solanum phureja and its derivatives. *Genome* 30: 506-510.

Olivares-Fuster, O., L. Peña, N. Duran-Vila and L. Navarro. 2002. Green fluorescent protein as a visual marker in somatic hybridization. *Annals of Botany*. 89: 491-497.

Paz, M. M. and R. E. Veilleux. 1999. Influence of culture medium and in vitro conditions on shoot regeneration in *Solanum phureja* monoploids and fertility of regenerated doubled monoploids. *Plant Breeding* 188: 53- 57.

Rosin, F.M., J.K. Hart, H.T. Homer, P.J. Davies and D.J. Hannapel. 2003. Overexpression of a *knotted*-like homeobox gene of potato alters vegetative development by decreasing gibberellin accumulation. *Plant Physiol*. 132: 106-117.

Ross, H. 1986. *Potato Breeding – Problems and Perspectives.* Verlag Paul Parey, Berlin.

Rouwendal, G.J.A. 1997. Enhanced expression in tobacco of the gene encoding green fluorescent protein by modification of its codon usage. *Plant Mol. Biol*. 33: 989-999.

Shen, L.Y. and R.E. Veilleux. 1995. Effect of temperature shock and elevated incubation temperature on androgenic embryo yield of diploid potato. *Plant Cell Tiss. Org*. 43: 29-35.

Singsit, C. and R. E. Hanneman, Jr. 1991. Rescuing abortive inter-EBN potato hybrids through double pollination and embryo culture. *Plant Cell Rep*. 9: 475-478.

Stewart Jr., C.N. 2001. The utility of green fluorescent protein in transgenic plants. *Plant Cell Rep.* 20: 376-382.

Straadt, K. and O.S. Rasmussen. 2003. AFLP analysis of *Solanum phureja* DNA introgressed into potato dihaploids. *Plant Breeding* 122: 352-356.

Valkonen, J.P.T., T. Mortiz, K.N. Watanabe and V.M. Rokka. 1999. Dwarf (di)haploid *pito* mutants obtained from a tetraploid potato cultivar (*Solanum tuberosum* subsp. *tuberosum*) via anther culture are defective in gibberellin biosynthesis. *Plant Sci.* 149: 51-57.

van Breukelen, E.W.M. 1981. *Pseudogamic production of dihaploids and monoploids in* Solanum tuberosum *and some related species.* Centre for Agricultural Publishing and Documentation. Wageningen, the Netherlands.

Vita

Rose Palumbo was born and raised in Virginia. She entered Virginia Tech in the Fall of 1998, and will be graduating with a M.S. in Horticulture in December 2003. Beginning in January 2004 she will be attending Ohio State University to earn a PhD in Plant Pathology.

Education

Virginia Tech

Two B.S. degrees from the College of Agriculture and Life Sciences (May 2002)

Majors: Crop and Soil Environmental Sciences – Biotechnology option

Horticulture – Science option

M.S. Horticulture – Breeding and Genetics (December 2003)

Experience

Graduate Research (Fall 2001-2003) –Transformations (GFP) and traditional breeding to develop a more efficient haploid inducing pollinator

Fralin Biotechnology Fellowship (Summer 2001) – research with Dr. Veilleux on anther culture of potatoes

Undergraduate Research (Fall 2000) with Dr. Veilleux on genetics of potatoes using self-pollination and backcrossing to produce better quality diploid homozygotes for future breeding