Seed germination, kanamycin sulfate selection, and the influence of nitrogen treatments on an insertional mutant population of *Fragaria vesca*

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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

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December 15th, 2010
Blacksburg, VA

Keywords: strawberry, transposition, activation tagging, knockout mutants

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ABSTRACT

With the goal of creating faster and more efficient methods of generating unique *Ac/Ds* insertional mutants in a population of *Fragaria vesca*, various methods of seed germination, kanamycin screening, and the effects of varying nitrogen fertilization on diploid strawberry have been examined. Seed germination was improved to 42% in B5 liquid medium compared to _ on MS solid medium. Kanamycin screening during germination was most effective in liquid B5 medium as well. A readily discernable phonotypic difference between sensitive (necrotic radical) and resistant (branched roots) seedlings was observed in the B5 liquid medium and the frequency of escapes was reduced from __ on solid MS to __ in liquid B5. Although there were few phenotypic differences due to nitrogen application over the tested treatments (25-300 ppm) runner initiation was suppressed and chlorophyll was increased in the high (300 ppm) nitrogen treatment. There was limited evidence to suggest an increased rate of transposition in the high (300 ppm nitrogen) treatment level compared to those plants receiving lower levels of nitrogen. The selection efficiency and greater germination of the B5 liquid medium over MS medium would be expected to reduce the cost of screening thousands of seedlings because of the need for fewer disposables and medium transfers during the 5 week germination process. The use of B5 liquid medium, as well as treating plants with high levels of nitrogen (300 ppm), may be facilitate high throughput production of transposon tagged mutants in a population of *F. vesca*.

Acknowledgments:

I would like to thank Dr. Richard Veilleux, Dr. Alex Niemiera, and Dr. Bingyu Zhao for their guidance and help in designing the experiments contained herein. I would also like to thank Kerri Mills for her invaluable assistance and advice. Last but not least, I would like to thank my wife, Tina, without whom none of this would have been possible.

Table of Contents:

Acknowledgments: iii
Table of Contents: iii
Table of Tables: iv
Table of Figures: vi
1. Introduction: 8
2. Literature review: 9
3. Objective: 14
4. Procedures: 15
Seed germination 15
MS solid medium 17
Blue Germination Paper 19
B5 liquid Medium 20
Kanamycin Sulfate selection 21
Nitrogen/ Environmental Study 22
5. Results: 26
Germination testing 26
Kanamycin Sulfate Selection 29
Nitrogen / Environmental study 30
Chlorophyll 30
Runner production 31
Height 31
Transposition 32
6. Discussion: 33
7. Conclusion: 38
8. References: 40

Table of Tables:

Table 1, Primers for the Multiplex, detailing the sequences, primer pairings, and band size. Bands refers to what the primers are treating for; EDS is the empty donor site, which will only appear if the Transposase and the EGFP genes are close enough to form a PCR product. The FDS is full donor site, and tests for the PCR product formed between the Transposase gene and the P35s promoter. The NPTII primers test for the presence of the NPTII gene found in the *Ds* element. The FVaat3 primers test for the presence of the FVaat gene that occurs naturally in *F. vesca* as a control. 43
Table 2, Nutrients applied daily to the four treatment groups of 16 plants of four *F. vesca* genotypes used in transposon tagging. The ammonium nitrate concentration varied among the four treatments while the other nutrients were held constant. 44
Table 3, Analysis of Variance of Medium v. Germination rate. Tested were 4 *F.* vecsa launch pad clones, with seeds collected and stored for app. 4 months between collection and germiantion. Significant at the 0.05 level. 45
Table 4, Germination rates for line 307 over a 6 month period and line FV10 over a similar period. Table shows number resistant and number sensitive for line 307, but not for line FV10, as Line FV10 is wild type, and does not carry the kanamycin resistance gene 46
Table 5, Germination rates of *F. vesca* lines 356 and 284 on B5 liquid medium with kanamycin in separate experiments, with the percent of seedlings resistant and sensitive to kanamycin. 47
Table 6, Spad data, the table on the left shows the ANOVA for the Mean Spad. All F values marked * are significant at the 0.05 level, and all F values marked ** are significant at the 0.01 level. 48
Table 7, ANOVA for mean runner production of three *F. vesca* launch pad clones and an untransformed control subjected to four nutrient treatments and measured four times during development. *significant at level 0.05, **significant at level 0.01 49
Table 8, ANOVA for mean height, three *F. vesca* launch pad clones and an untransformed control subjected to four nutrient treatments and measured four times during development. *significant at level 0.05 50
Table 9, date to flowering versus treatment, with 3 *F. vesca* launchpad clones treated with nitrogen levels f 25 ppm, 100 ppm, 200 ppm, and 300 ppm daily, with on *F vecsa* wild type control tested as well. No significant effect was noted. 51
Table 10, The occurrence of transposition in 215 T_2 seedlings after application of nitrogen treatments to T1 launch pads. Four fruit (f1-4) from each of three

inflorescences (I1-3) were collected with seeds of each fruit separately tested for somatic transposition. Plant (1-4) and treatment (C, L, M, H) refer to each of four T1 plants to which four treatments were applied. C is 25 ppm nitrogen, L is 100 ppm nitrogen, M is 200 ppm nitrogen, and H is 300 ppm nitrogen. The green squares represent seedlings that had a both an Ac and a dissociated Ds element. The red squares show the T2 seedlings that had only the Ds element. The black squares show seeds that were not tested for transposition. Squares with a '0' are seeds that have been tested, but had no transposition. Each character in a colored square represents a single transposed seedling. ... 52
Table 11, the total number of T2 seedlings from each family screened, with a breakdown of how many of each of the possible movement patterns are shown for the AcDs element. The column with Fvaat, NPTII, and FDS refers to those seedlings that contain a complete intact AcDs construct. Those plants with Fvaat, NPTII, FDS, and EDS have one copy of the AcDs element intact, and one that has broken into the separate Ac and Ds elements. The column containing Fvaat, NPTII, and EDS shows the number of plants that only have a fully separated copy of the AcDs element. The column named Fvaat, NPTII shows those plants that contain only the Ds element of the construct. WT refers to the plants that do not contain any element of the construct, and yet managed to make it through the antibiotic screening process. 53

Table of Figures:

Figure 1, Germination rates of seeds of four *F. vesca* launch pad clones grown on B5 liquid medium v. seeds grown on solid MS medium. 45
Figure 2, On the right Mean ± SE Spad values for four dates by nitrogen treatments (25, 100, 200, 300 ppm).....48
Figure 3 Mean ± SE runner production of three *F. vesca* launch pad clones subjected to four nutrient treatments and measured four times during development. 49
Figure 4, the *AcDs* construct used in the transformed plants. The *Ac* element consists of the RB (right border) and Tpase elements, as well as the EGFP and LB (left border elements. The two portions of the *Ac* element flank the *Ds* element, which consists of the two IR (inverted repeat) elements, the Pmas and P35s promoters, and the Pns-NPTII-Tnos gene and promoter terminator complex. The construct is supposed to function a follows: The EGFP gene is supposed to cause the plant to glow under a UV light, this function is driven by the Pmas promoter across the IR. The Tpase gene is activated by the P35s promoter and produces Transposase, which cuts the *Ds* element out of the *AcDs* construct at the IRs, and then inserts the *Ds* element into the plant genomic DNA at a random location. 54
Figure 5, Shown above are the three germination methods, from left to right; Blue Germination Paper, MS solid medium, and B5 liquid medim in a DeLong flask. Also shown in the center image, that of the MS solid medium, is the difference between a kanamycin sensitive seedling(top center) and a kanamycin resistant seedling (bottom Center). Both seedlings have both primary and secondary roots, with the major difference being a retardation of growth in the sensitive seedling 55
Figure 6, The rate of germination over a three week period of the three medium types tested. The B5 medium starts seeds fastest. The graph for the third week only contains the data points for germination paper and for MS solid medium, as the seedlings sprouted in the B5 medium could not be accurately counted in the flasks. B5 medium overall shows the highest germination rate. 56
Figure 7, The tangled shoot and root mass that is the result of letting the seedlings grow for more than about 2 weeks in Liquid B medium. This also demonstrates the curled, tangled, and short nature of the stems of seedlings grown in this germination medium. These seedlings are 4 weeks old, and are only starting to reach a length and total size that can survive planting. 57
Figure 8, The distinct phenotype of a sensitive seedling of six weeks of age grown on B5 liquid medium. Of note is that the main root on this plant is shriveled and blackened, as a result of the effects of the kanamycin root inhibition. This

effect is not visible in the germination paper method, and can best be seen in Liquid B5 medium. 58
Figure 9, A kanamycin sensitive seedling (left) and a kanamycin resistant seedling (right), both of which are 6 week old T2 seedlings from plant 311.5, and were grown in the same batch in liquid B5 medium in a Delong flask, orbital shaker set at 90 rpm. 59
Figure 10, Mean height of four genotypes of F. vesca in four different nitrogen treatments. Because there was no significant effect due to the nitrogen treatments the data across treatments was combined for each genotype; genotype 347+8 was significantly shorter than the other three genotypes tested. 60
Figure 11, Date and treatment effects on inflorescence occurrence. No inflorescences were present on 16 October, 2009. Results after the first two data reads consistently show that higher levels of nitrogen in the daily fertilizer have the effect of increasing inflorescence occurrence. 61
Figure 12. Multiplex PCR analysis of crown tissue and tissue isolated from leaf tissue on three different infructescences of five launch pad T_1 311 plants. The top band at 1 kb shows the presence of the empty donor site. The band located at 600 bp shows the presence of the Full Donor Site, the Tpase and P35s elements in place in the full construct. The Band at 300 bp is the *NPTII* band, showing the presence of the *Ds* element. The bottom band, at 179 bp, shows the presence of a native control gene *FVaat* that is present in all *F. vesca* plants. The Numbers at the top refer to the plants from which tissue was taken. 311-5.11 is plant M3, 311-5.12 is plant M4, 311-5.14 is plant H2, 311-5.15 is plant H3, and 311-5.16 is plant 4. A refers to tissue taken from the crown of the plant, and B, C, and D are tissue samples taken from the first, second, and third infructescences. The tissue samples were taken to test for somatic transposition in the different tissues of each plan 62

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1. Introduction:

With the growing importance and use of transposon tagging DNA constructs, a better understanding of the efficiency of the systems in different crops or model systems is required if high throughput methods are to be attempted in the development of large populations with the possibility of recovering insertional mutations or activation tagging in every gene. While current methods in many plant species include the use of independent insertions of T-DNA constructs to create large populations of insertional mutants and the resultant insertional mutant lines, in several species, such as rice, maize, and *Arabidopsis*, a more efficient method has been developed. This method involves the use of a transposing element in the DNA construct, which allows the production of unique insertional mutants derived from a single DNA transformation event. A transposing element makes use of the plant's ability to mobilize alien transposable elements using maize transposase to generate new insertional mutants. However, most of the working transpositional elements have been developed in monocot crops, and the

constructs do not always transfer to dicot species effectively. One such transposing cut and paste construct is the *Ac/Ds* construct, which has had limited success in *Fragaria vesca* as compared to transpositional rates in crops such as corn and rice (Chen et al. 1992; Greco et al. 2003; Luan et al. 2008; Pastori et al. 2007b). This construct approaches a transpositional effectiveness of roughly 30% in selected insertional lines. This efficiency level, though respectable, is not yet at the level of efficiency demonstrated in monocot crops, where a transpositional efficiency of 62% has been reported (Greco et al. 2003). In addition, the recovery of unique mutations after meiotic transposition in T1 plants has been hampered by somatic transposition resulting in the recovery of the same transposon tagged line in many seedlings.

As described in Boyko et al.(2009), transformation efficiency in *Arabidopsis* containing a similar *Ac/Ds* construct has been demonstrated to be affected by environmental conditions. Specifically, Boyko et al. (2009) proved that an increased level of nitrogen increased the transformation rate up to 4 fold that of plants not treated with nitrogen increases.

2. Literature review:

The commercial strawberry is one of the more economically important fruit crops in the world, with more than 600,000 acres harvested in 2007, and a harvest

weight of 4.1 million tons (FAO 2009). This, including the 1.05 million tons produced in the United States annually, serves as an indicator of the relative economic importance of this crop, both worldwide and domestically (USDA 2005). However, like many food crops, the strawberry that is most commonly grown, *Fragaria ×ananassa*, has a narrow genetic base (Gullino et al. 2008). Unfortunately, this species, along with the related *F. vesca* species, cannot be easily expanded through traditional breeding methods (Galletta and Maas 1989).

The commercial strawberry, *F. ×ananassa*, is octoploid, which renders it difficult to analyze by standard molecular and genetic methods. However, through the use of a proposed model species, *F. vesca*, the analysis can be simplified due to the diploid nature of *F. vesca*. This species of strawberry has a short life cycle (16 weeks from seed to seed), produces seed in abundance, and is compact, all of which are desirable traits in a model organism (Ontivero et al. 2000). Two important factors in choosing *F. vesca* as a model species are that *F. vesca* can be inbred for several generations without significant loss of fertility, and that it reacts well to *Agrobacterium*- mediated transformation (Gruchala et al. 2004; Haymes and Davis 1998). This ability to self-pollinate, coupled with the diploid genome, allows the generation of homozygous lines fairly easily.

One method of studying gene function in *F. vesca* is via genetic transformation, the insertion of a DNA element into the host chromosome to form

insertional mutants (Oosumi et al. 2006). The different insertional methods that are commonly used range from polyethlene glycol, electroporation, and microinjection to microprojectile and *Agrobacterium*-mediated transformation (Akhond and Machray 2009). Of these, the best suited to use with *F. vesca* is *Agrobacterium*-mediated transformation, due to *Fragaria vesca's* ease of transformation via *Agrobacterium tumefaciens* mediated transformation. This susceptibility, in addition to the ease of forming callus and shoots from infected tissue, imply that the most efficient method for producing many transformant *F. vesca* plants is via *A. tumefaciens* mediated transformation (Haymes and Davis 1998).

Agrobacterium tumefaciens is a commonly occurring bacterium that can be found in most soils, and houses a plasmid DNA capable of inserting into a host cell's genome. In the wild, this commonly results in the diseases of crown gall and cane gall (Kado 1991). In *Agrobacterium*-mediated transformation, *Agrobacterium* has been disarmed so that the transfer of DNA into the host's genomic material does not result in disease expression, but instead is capable of transferring genes from any source into host cells which may grow into transformed callus and eventually regenerated plants (Frenkiel-Krispin et al. 2007; Haymes and Davis 1998; Kado 1991; Oosumi et al. 2006). The resultant transformed plants can be stable insertional mutants, unstable insertional mutants,

or transposing insertional mutant lines (Frenkiel-Krispin et al. 2007; Haymes and Davis 1998; He et al. 2007). Of the methods for producing insertions, the most efficient at producing unique insertional mutants is the transposing mutant type of insertion (Charng et al. 2007; Ito et al. 2005).

The transpositional insert system is based on two components: a version of the transposase gene, found in many different organisms but usually isolated from maize for transposon tagging purposes, and a pair of inverted repeats that are recognizable as excision and ligation points by the transposase. These factors are often built into a T-DNA construct that contains promoters and selective markers that enable positive selection of successfully transformed plants. Several variations of *Ac/Ds* constructs have been employed for transposon tagging (Greco et al. 2003; Pastori et al. 2007a; Qu et al. 2009).

The system is supposed to work like this: 1) Plants are transformed with the complete *Ac/Ds* construct and fluoresce green due to GFP expression (Figure 4). Single insertion lines are characterized at the site of insertion. These are putative "Launch Pad" genotypes because of their anticipated ability to transpose the *Ds* element to other sites within the genome during meiosis, resulting in stable transposant lines, which are the result of expression of the transposase gene that recognizes and cuts the T-DNA at the IR sites; 2) Progeny are screened for the presence of an intact *Ac/Ds* element (full donor site or FDS) and a detached *Ac*

from *Ds* element (empty donor sites or EDS); 3) Plants that lack the FDS but still retain components of the *Ds* element are putative transposants; they should no longer express GFP because the promoter has detached from the gene, but still express kanamycin sulfate resistance; 4) putative transposants are characterized as to their position within the genome to determine the independence of transformants; 5) Interesting phenotypes are identified due to gene interruption or change in expression pattern. This method of using the transposition of the *Ds* element itself is the basis of the efficiency and the effective advantage that such a system has over the use of a non-transposing construct (He et al. 2007).

Because transposition efficiency varies with insertion site, gene expression, and environmental factors, launch pads are not all created equal. Even "good" launch pads that have greater than 20% transposition can vary in their efficiency depending upon environmental conditions. Inducible transposons and the nutrition of the plants can affect the rate of transposition (Qu et al. 2009). Because transformation and transposition have similarities with regard to alien DNA integration into sites within host plant DNA, and because Boyko et al.(2009) have shown that nitrogen fertilizer can affect the rate of *Agrobacterium*-mediumted transformation in tobacco, our hypothesis is that the rate of fertilization of strawberry plants may affect the transposition efficiency of launch pad clones.

In addition to the increases in transformational rate that are demonstrated by Boyko et al. (2009), other efficiencies can be found, most notably by affecting the stringency of the selection method. In the case of the *Ac/Ds* construct, one of the selectable markers confers resistance to the antibiotic kanamycin sulfate (Alsheikh et al. 2002; Greco et al. 2003; Ipek et al. 2006). While kanamycin has been used for many years as a bacterial and callus selectable marker, little effort has been made to tailor both the seed germination and selection processes to maximize the effectiveness of kanamycin selection on seedlings. Recent experiments have proven that kanamycin can be applied in a number of different ways, but application of kanamycin to the germination medium of seeds from T0 plants containing the *NPTII* gene is the most cost effective. This type of kanamycin selection works not by killing wild type seedlings, but by stunting their growth, an effect that is difficult to distinguish from the growth of plants that are resistant (Duan et al. 2008) (Figure 5B).

3. Objective:

Methods of *F. vesca* germination available will be tested to determine which method provides the highest number of germinated seedlings for the least amount of input. Another area of interest is the effectiveness of using kanamycin sulfate screening during seedling growth. Transposants and plants with a full donor site

should be resistant to kanamycin whereas wild type and plants with only an empty donor site should be susceptible. By analyzing the effects of kanamycin selection on seedlings in different media, the most effective method will be found for screening seedlings and for reducing the amount of wild type escapes that must be screened by multiplex PCR. To explore the feasibility of improving the rate of transposition in *Fragaria vesca,* plants that have been transformed with an *Ac/Ds* construct will be manipulated using nutritional factors. Four different levels of ammonium nitrate will be given to the transformed plants, to test the effect of varying levels of nitrogen on the rates of transposition in the *Ac/Ds* construct inserted into the *F. vesca* genomes. The effects of nitrogen level on the vegetative growth and the fruiting and flowering characteristics of the transformed plants, as well as for untransformed plants will also be assessed. By utilizing these approaches, we endeavor to increase the percentage of transposant plants and the total number of transposants.

4. Procedures:

Seed germination

Methods of seed germination were assessed with the intent of finding an acceptable balance between the amount of effort needed for each batch of seeds and germination rate per batch. This was done by assessing different seed

germination techniques, including: MS solid medium with vitamins in disposable plates, disposable plates with dampened Anchor Germination paper, with seedlings planted after screening, and B5 liquid medium in flasks. As a control, all methods were tested on seeds from transgenic lines containing the *Ac/Ds* construct conferring kanamycin sulfate resistance and FV10 wild type seeds. This allowed an assessment of whether the presence of the construct affected the germination rate of seeds in different germination techniques. Also included in this study was a small assessment of the germination rates of T1 seedlings from line 284 and 356 to determine if germination rates varied between lines. Rate of germination was determined by dividing the total number of seedlings germinated by the total number of seeds added to the germination media for each batch.

Seeds were collected from mature *F. vesca* fruit, with seed ages ranging from 1 week after picking to eight months after fruit picking, with the majority of the seeds used being collected in the spring and summer from greenhouse grown plants. All fruits were dried in a food desiccator at 95◦f for 24 hours, after which the seeds were separated from fruit and stored in 2 ml vials with a desiccating agent in low light conditions at 10◦C. All seeds were counted, and then surface sterilized by the following procedure;

1. soak for 5 min in 70% ethanol, drain

2. rinse with 1 ml autoclaved DI water
3. add 1 ml autoclaved DI water for overnight soak
4. drain water and add 1 ml 1% sodium hypochlorite and let soak for 15 min, shaking every 5 min, drain
5. rinse seeds with 1 ml autoclaved DI water
6. repeat step 5 (twice) for a total of 3 water rinses
7. add 1 ml 50% filter sterilized PPM-Preservative for Plant Tissue Culture Medium (Plant Cell Technology, Washington, DC) and let sit for 15 min, shaking every 5 min
8. drain PPM and place seeds in/on incubation medium using sterile forceps.

MS solid medium

Germination on MS medium (Murashige and Skoog, 1962) with vitamins in a disposable plate was done by the following method:

1. 10 ml of autoclaved media, containing 50 mg/l kanamycin sulfate (filter sterilized) were poured into each disposable plate, 20 x 100 mm, from Fischer Scientific, in a sterile hood.

2. Surface sterilized seeds were placed on the surface of the media, arranged in a grid pattern. The plate was sealed with parafilm and then labeled with the start date and seed line.
3. Plates were placed in a darkened germination chamber set to 25°C and 50% RH.
4. After 2 weeks, seeds were re-plated via sterile forceps to fresh MS + vitamins solid medium with 50 mg/l kanamycin sulfate. The fresh plates were sealed with new parafilm and record was taken of the start date and transfer date.
5. Unsprouted seeds were re-plated to fresh B5 medium every 2 weeks.
6. Plates were checked for sprouted seeds every 2 days. All sprouted seedlings were screened for kanamycin resistant phenotypes (if applicable) and then moved to fresh media. The fresh plates were then sealed and placed in a lighted incubator with a 16 h photoperiod, and 22/18 C temperature range.
7. Sprouted seeds were re-plated once a week and then returned to the lighted incubator until they had two true leaves.
8. Mature sprouts were planted in cell packs with Promix Media and placed in a growth chamber with a 16 h photoperiod, 16/22 C temperature range, and 70 % humidity.

9. All seeds which were not sprouted within 8 weeks elapsed time were assumed to be non-viable. These seeds were discarded.

Blue Germination Paper

Germination on moistened germination paper (Anchor Paper Co., St. Paul, Minn.) was conducted using the following procedure;

1. Circles (20 mm diameter) of blue germination paper were cut and autoclaved for 20 min on the vacuum setting.
2. Individual circles of germination paper were placed into Fischer Scientific disposable plates, 20 x 100 mm (pre-sterilized).
3. Germination paper was moistened with a solution containing autoclaved DI water and 50 mg/l kanamycin sulfate.
4. Surface sterilized seeds were spread out over the surface of the germination paper using sterilized forceps.
5. Plates were sealed using 3M Micropore tape (3M, St. Paul, Minn.), and labeled with line, date, and number of seeds.
6. Plates were placed in a dark germination chamber with a temperature of 25 C and a RH of 50%.
7. Plates were checked every 3 days and water was added as needed to keep the germination paper moist for the first week. (Note, care was taken to avoid

washing the seeds under the germination paper, as it would complicate seedling retrieval.)

8. After the first week, seeds were checked daily for sprouting.

   Sprouted seeds were planted in plastic cell packs with Promix Media and placed in a growth chamber with a 16 h photoperiod, 16/22 C temperature range, and 70 % humidity.

9. All seeds which were not sprouted within 8 weeks elapsed time were assumed to be non-viable. These seeds were discarded.

B5 liquid Medium

Seeds were sprouted in liquid B5 medium (Gamborg et al. 1968) according to the following procedure.

1. Liquid B5 medium was made with 3.2 g B5 Basal salts and 20 g sucrose per liter of DIH_2O, pH ~5.75.
2. Liquid B5 medium was autoclaved, and once cooled, filter-sterilized kanamycin sulfate was added to reach a final concentration of 50 mg/l.
3. 10 ml of B5 liquid medium was added via serological pipette to previously autoclaved 125 ml Delong flasks.
4. With sterile forceps, surface sterilized seeds were placed in each flask. The flask was then capped and sealed with 3M Micropore tape.

5. Flasks were placed in an environmental shaker with 180 rotations per minute, with a 16 h photoperiod, and a temperature of 25 C.
6. After 5 weeks, the sprouts were decanted and sorted according to kanamycin sensitivity. Resistant sprouts were planted in cell packs and placed in a growth chamber with a 16 h photoperiod, 16/22 c temperature range, and 70 % humidity. Kanamycin sensitive sprouts were discarded.
7. All seeds which were not sprouted within 5 weeks elapsed time were assumed to be non-viable. These seeds were discarded.

Kanamycin Sulfate selection

Kanamycin sulfate selection was tested for efficacy on strawberry seedlings in transgenic plant lines containing the *Ac/Ds* construct. Various concentrations of kanamycin sulfate have been tested for efficacy, with 50 mg/l found to be the most effective for seed screening without stunting seedlings containing the gene conferring resistance (Nathan Presnell, personal communication). Selection efficacy was tested simultaneously with the germination medium testing, and consisted of a preliminary visual screening, followed by PCR screening of all apparently resistant seedlings for the presence of the construct.

The visual screening consisted of examining the root structure of each seedling, specifically looking for secondary roots and branching of roots, which would indicate a resistance to the root inhibiting effects of kanamycin sulfate. All

seedlings which did not show at least a tap root and one secondary root were assumed to be sensitive to kanamycin sulfate and were discarded.

The PCR screening consisted of collection of leaf tissue for purposes of DNA extraction from each plant, followed by PCR analysis of the DNA using a series of primers in multiplex. By looking for the presence specifically of any plants that were missing both the *AC* and the *Ds* elements of the construct, the multiplex PCR, which is detailed in Table 1, confirmed whether there were any FV10 wild type plants that were not caught by the initial screening.

To ensure that the screening was not biased in favor of older plants with more developed root systems or against seed lines that had a phenotype that consisted of a small root structure, germination rates of the seeds and of wild type seeds were examined and compared to the expected baseline as part of this study to ensure that the initial screening techniques were not overly stringent.

Nitrogen/ Environmental Study

Four test plants were chosen and vegetatively propagated from runners into sixteen clones each, for a total test population of 64 plants.

This population was created to test the effects of nitrogen fertilizer on phenotype and transposition of the *Ds* element in the pAc-DsNEn-EG construct (3). Three T1 transgenic lines were chosen because they were derived by self-pollination of

single insertion Launch Pad lines that have proven to be actively transposing lines (Kerri Mills, personal communication).

1. fourth generation inbred FV10
2. FV10- 307
3. FV 10- 311
4. FV 10- 347

The specific T1 plants used as progenitors, excluding the fourth generation inbred FV10, were chosen by examining multiplex PCR reactions of segregating T1 populations to ensure that the selections retained an intact and untransposed full donor site (FDS) of the *Ac/Ds* element. Clones were propagated from nodes on runners of each T_1 plant; 18 nodes per T1 line were planted in four inch pots of soil-less media (Promix Media), with the
runners still attached to the parent plant. Once the planted runners were 4 cm tall, they were cut from the mother plant. After the runners had been cut, the 16 most uniform plants from each line were subjected to different nitrogen fertilizer treatments, added in soluble form as part of the daily watering. The clones were divided into four blocks, with each block containing four clones of each plant (16 plants per block, 4 lines), which were further subdivided into four treatment groups. Each different group received a different concentration of nitrogen

fertilizer, with each treatment group replicated four times (one replication from each block).

The fertilizer treatments consisted of the following; 25 (0.892 mM), 100 (3.569 mM), 200 (7.139 mM), and 300 (10.707 mM) ppm nitrogen in the form of ammonium nitrate. The fertilizer solutions also contained macro- and micronutrients which were standard for all test plants (Table 2). Each plant received 250 ml of fertilizer solution daily, from when the runners were cut until seed was collected. This volume of fertilizer solution was adjusted to take into account greenhouse temperature and light conditions, to avoid over-watering with the fertilizer solutions, with a minimum of a twice weekly flow through. The treatment time was 5 months, and consisted of a normalization period of 2 months, during which all inflorescences were discarded, followed by a collection period, during which fruit was allowed to mature and seeds collected. Seed germination and transposition rates were then analyzed and determined for the T_2 seedlings.

The effects of the different treatments were measured by the following metrics; chlorophyll readings, height, runners produced, and the percentage of progeny in which the *Ac/Ds* element had independently transposed. Tissue samples were taken from each plant, in order to test for somatic transposition of the construct.

Chlorophyll measurements were taken using a Minolta Spad 1703, with readings recorded on four dates. All plants had readings taken from five leaves, starting with the youngest leaf, and the fifth read on the oldest leaf available but still green. These five reads were averaged together to yield the MeanSpad reading for each individual plant, with the means of the MeanSpad readings being taken from all 16 plants in each treatment level.

When seed was collected, tissue was also collected from each inflorescence, to ensure that no somatic transposition had taken place. All tissue samples were tested via the FDS/EDS (empty donor site) PCR multiplex designed to detect the occurrence of transposition.

Seed was collected and stored separately from each fruit on each individual infructesence. All seeds were germinated on B5 medium with kanamycin sulfate. This allowed screening for the presence of the *Ds* element, which confers resistance to kanamycin sulfate. After germination and kanamycin selection, seedlings were transplanted and grown in a Conviron growth chamber with conditions of 16/8 h day, 22/20◦c to reach a size (approx. 4 cm diameter with 6 true leaves) which could survive tissue collection. After tissue collection of two small folded leaflets, a CTAB DNA extraction procedure, as described in Oosumi et al. 2006, modified by Kendall Upham; (Kendal Upham, personal communication) and

multiplex PCR analysis were conducted to test for transposition of *Ds*, using the previously mentioned FDS/EDS multiplex.

5. Results:

Germination testing

ANOVA revealed a significant difference in germination rate between seeds germinated on liquid B5 medium compared to those on solid MS medium, both imposing kanamycin selection (Table 3). The germination rate of the *F. vesca* seeds on blue germination paper (Figure 5A) was 19±4% after 8 weeks. The germination rate of the seeds grown on MS solid medium in petri plates was 30 ±3% (Figure 5B) after 8 weeks. The germination rate of the seeds grown in liquid B5 medium in Delong flasks on a shaker was 42.5±4% after 5 weeks (Figure 5C). There is a statistically significant difference between the germination rate of seeds grown on the MS medium and those grown in B5 liquid medium (Table 3). There was a 42% increase in germination rate when Liquid B5 medium was used compared to MS solid medium even though the experiment was terminated after 5 weeks for liquid medium compared to 8 weeks for solid medium. The early termination of the liquid medium treatment was due to the difficulty of separating the tangled mass of seedlings; a longer experimental period would have made

seedling separation unmanageable. While germination techniques affected the germination rate of seedlings, there are many uncontrolled factors that affect the germination rates of *F vesca* seed. These factors include, but are not limited to; the age of the seed, germination conditions, age of the fruit/seed when collected, plant line, and environmental conditions of the parent plant (Baturin 2009; Bringhurst and Voth 1956; Scott and Ink 1955). Many of the seeds used in the germination tests were collected at least 6 months before the experiments were started. However, testing done by Bringhurst and Voth (1956) showed that seed stored for up to 6 months from drying before germination did not differ in germination rate significantly from seed planted immediately after drying. (Scott and Ink 1955) The age/development stage of the fruit and seed at collection were not recorded although these factors have been reported to impact the germination rate (Andersen 1964). Another controllable variable is genotype. In our studies with transposon tagging mutant lines, all derived from seedlings of the same accession of *F vesca* seeds of line 284 exhibited germination rates of 60 to 70%, whereas those of line 356 consistently germinated at a rate of only 2-6% for T1 seeds (Table 5). However, these results may have been confounded with other factors that cause low germination rates, such as seed maturity at collection. Another variable that could affect the germination rate of the T1 seedlings is the environment that seeds have been subjected to over the course of extraction and storage. An examination

of T2 germination rates from these lines could differentiate genotypic from environmental factors.

The time to germination varied among the three germination methods tested, with the initial germination rates showing a clear advantage to using liquid B5 suspension for the first 14 days (Figure 6). The germination results shown in Figure 6 at 21 days demonstrate the similar germination rates for germination paper and MS medium; however, germination in liquid suspension could not be measured by this time because the many small seedlings had become tangled and were impossible to accurately count (Figure 7), this was the reason for the missing data regarding liquid suspension at 21 days (Figure 7). The early results of the superiority of liquid suspension shown in Figure 6 for the second week were confirmed by the overall results that showed a 30% increase in germination in the liquid B5 medium over either the MS solid medium or the germination paper. This difference in germination time held true over several different lines of *F. vesca*. Seedlings in the B5 liquid medium did not elongate for several weeks, and instead remain curled up and fairly compact (Figure 7) compared to those on germination paper, where seedlings tended to lengthen more rapidly, and were ready for planting within 1 week of emergence. Seedlings on solid MS medium, which had a comparable germination rate to the germination paper, were compact for several weeks before achieving a transplantable size.

Kanamycin Sulfate Selection

The liquid B5 medium was highly effective for screening using kanamycin as the antibiotic on transgenic plants harboring the *nptII* gene. Of the seedlings grown on the liquid B5 medium that survived the initial antibiotic screening, 99% (142 of 144 tested) were shown by PCR to have the *NPTII* gene carried on the *Ds* element of the *Ac/Ds* construct. MS solid medium was almost as effective, with approx. 90% (59 of 65 tested) of seedlings that passed the antibiotic screening proven to contain the antibiotic resistance gene. Of the seedlings sprouted on moist germination paper, PCR testing revealed that only 75% had the antibiotic resistant gene. This three to one ratio (3 *nptII* positive; 1 wild type) is the standard Mendelian segregation ratio expected of a single insertion hemizygous transgenic plant carrying *nptII*, expected of a population that is not subject to selective screening. This lack of screening efficacy on the blue germination paper means that dampening the germination paper with kanamycin sulphate at the same concentration used in the other two tissue culture methods was ineffective and unsuited to a high-throughput plant production process. It is probable that the wild type plants that escaped the screening process may have been misclassified as resistant due to an unremarkable phenotype or mistakenly planted along with the resistant seedlings. The kanamycin sensitive phenotype of FV10 seedlings that

were the basis of the stock from which all insertional mutants were derived is shown in Figure 8 for the MS basal selection. On liquid selection medium, the phenotype of sensitivity to kanamycin was extremely different from that of a plant bearing the *nptII* gene (Figure 9) (Duan et al. 2008). Kanamycin works by inhibiting the growth of the roots, slowing down or stopping the growth of the center root, and stopping the growth of secondary roots in sensitive individuals (Figure 8) (Duan et al. 2008).

Nitrogen / Environmental study

Several plant variables were measured in the nitrogen application experiment, including chlorophyll measurements, runner growth patterns, height differences, time to flower, inflorescence occurrence, and the rate of transposition in T2 seed.

Chlorophyll

ANOVA (Table 6) shows the effect of nitrogen treatment and date on the chlorophyll MeanSpad measurements. The date also had a significant effect on the chlorophyll measurements, possibly due to either the age of the plants when the data were collected or because of the time that the treatments had to affect the growth patterns of the plants. Although the accompanying bar chart in Figure 2 shows the treatment means by date, there was no significant difference in

chlorophyll patterns between treatments for the first two dates. However, the third and fourth measurements revealed that the SPAD measurements of the 25 ppm nitrogen treatment were significantly lower than those of the 100 and 200 ppm nitrogen treatments (Table 6).

Runner production

As can be seen in Table 7, treatment and date has a significant effect on the runner growth initiation patterns, but not on other factors. This may be attributable to the fact that the runners are removed after each measurement, and since the dates of the measurements are not a set number of days apart, the date is not a meaningful significant factor in this measurement. However, it should be noted that Table 7 showing runner growth initiation patterns, does show that the first measurement has no significant difference in the runner growth initiation patterns from treatment to treatment, however, for measurements two through four, there is evidence that the higher nitrogen levels suppressed runner initiation. The last day, January 5th, does not show a significant difference between treatments.

Height

Treatment levels had no significant effect on the height of the plants tested (Table 8). However, genotype did have an effect on the plant height, with plants from line 347+8 exhibiting a mean height of approx. 2 cm less than all other plants,

as can be seen in Figure 10. Genotype 347+8 consistently exhibited reduced height throughout the experiment.

Transposition

Transposition was detected in T2 seedlings grown from seeds collected from different fruit and inflorescences of the T1 FDS line 311 by the presence or absence of the FDS PCR band along with the presence of the *Ds* element with or without the EDS PCR band using the multiplex PCR reaction with the primers detailed in Table 1, and a resulting sample of the gel shown in Figure 3. The multiplex screening showed that transposition appeared to have occurred in 11 of 215 T2 seedlings tested, or 5%. The greatest apparent frequency of transposition was found in the first fruit on the first inflorescence in the 300 ppm treatment (Table 10). However, transposition also occurred sporadically in the other nitrogen treatments. The preferred type of transposition is when the seedling retains only the *Ds* element of the *AcDs* insertion, excluding the EDS of the *Ac* element. While there was one such transposition event in the 25 ppm, the 100 ppm, and the 200 ppm treatment groups, the 300 ppm group exhibited five such transpositional events in addition to two transpositional events in which the *Ds* element was also found with the EDS (Table 10).

The frequency of transposition was too low to make global statements about the influence of nitrogen treatments on transposition (Table 11); however nearly 13% of the T2 plants in the high nitrogen treatment showed evidence of transposition compared to 5%, 2.7%, and 2.1% in the control, low and moderate nitrogen treatments, respectively. In the 36% of the T2 plants that exhibited both an EDS and a FDS in all treatment levels, transposition may have occurred in either gamete but would not have been detected after self-pollination because the *nptII* primers were used to amplify both *Ds* and FDS, so the two are confounded. Due to this factor, the evidence of transposition was underestimated. Ideally, cross-pollination between an FDS plant and a wild type plant would allow detection of more transposants.

6. Discussion:

The germination testing shows data that can be used in two different fashions, either with plantable seedlings produced within two weeks, or a slightly larger number of seedlings produced over a period of five to six weeks. This is the result of the twin facts that the Anchor Blue Germination paper method of germinating seed produced unscreened plantable seedlings faster than the liquid B5 medium and with more ease than production via the solid MS media germination

technique. However, this rate of germination is lower than has been reported in Bringhurst and Voth (1956), which showed a germination rate of over 80% for some treatments, or Scott and Ink's (1955) rate of between 50 and 80%. This difference in germination rates can be attributed to several different factors, such as the population from which the seeds were collected, as well as the treatments that Bringhurst and Voth (1956) used (Scott and Ink 1955). These treatments differ from the blue germination paper treatments in that Bringhurst and Voth (1956) used. One such treatment is call stratification, which entails a form of low temperature pre-incubation to help the seed germinate better. The main problem with this technique is that the most effective stratification times tend generally entail several months of treatment prior to the actual germination. Because of this, stratification adds a significant amount of time to the generation of screenable seedlings produced by the germination paper method. While not tested here, due to the selection of the FV10 population as a population that does not need or require stratification for germination, use of the stratification period of two to three months may be effective in increasing germination rate when used in other germination methods, such as the liquid B5 medium or with other strains of *F vesca*.

B5 liquid medium in flasks for germination of strawberry seeds allowed concomitant easy screening of the seedlings for resistance to kanamycin sulfate, as opposed to germination of seeds on kanamycin soaked germination paper. With

the germination paper, the seedlings were removed soon after germination, before a distinct phenotype could be developed by the seedling and hence observed (Duan et al. 2008); sensitive seedlings could then recover from any stunting effects of the kanamycin in the absence of selection. More effective selection for kanamycin resistance yielded fewer wild type escapes that would have been subjected to PCR (and the cost involved in the process) than batches of seedlings produced via the use of germination paper. The kanamycin screening in B5 liquid medium produced seedlings with pronounced sensitive or resistant phenotypes, making resistance easy to determine, reducing the amount of wild type escapes that were subjected to PCR screening. This reduction of wild types that underwent PCR screening reduced the cost to process each batch of seedlings by approximately one dollar per sample, counting materials and time.

My results were inconclusive on whether high nitrogen levels influence the rate of transposition. Although several phenotypic effects of nitrogen fertilization were observed, including an increase in runner initiation and an increase in chlorophyll content, the rate of transposition was only marginally higher and may not be repeatable. An increased rate of transposition as a function of nutritional treatment would be a facile method to develop *Ds* insertional mutants, and is in line with both the hypothesis of the experiment and work done on transgenesis. (Boyko et al. 2009; Qu et al. 2009; Yan and Rommens 2007) Adjusting fertilizer

levels to affect transposition has not been previously done, and opens up a new aspect of improving transposition by affecting the plant environment. Use of this fertilization technique has the potential to increase the expected number of transposants that can yielded from a given number of seedlings by hopefully double what is currently the case, especially when the seedlings are grown on B5 medium with proper kanamycin selection. This is the case when that transposition occurs in unique transpositional events, and can be tested for via PCR. This testing can be further streamlined by storing seed from each infructescence on each plant separately, so as to reduce the number of somatically transposed seeds that must be screened and processed.

The potentially increased rate of transposition needs to be further investigated as the results were inconclusive due to the low overall number of transposants. Ideally, research could be done either in a crop with a higher rate of transposition, or with a much larger study so as to yeild a higher number of transposants. The mechanism by which transposition may be enhanced has been hinted at in Boyko et al (2009), that of an increased rate of recombination due to the effects of the nitrogen on the host cells. Boyko et al (2009) further hypothesize that this increase is brought about by an increase in the recombination rate of the host cell, leading to a more active environment in which transformation events can occur. This is also in line with the results and hypotheses of CA Cullis (1979)

from studies of flax genotrope. CA Cullis (1979) discovered that the environment can and does affect the genome in a heritable fashion, and is further supported by S DeBolt (2010), who found that the copy number of genes is affected by the environment, and contributes to phenotypic differences. The copy number on either stress genes or of the NO_3- ion transporters may be related to the potential increase in host cell genome recombination, which in turn is supposed by Boyko et al (2009) to increase the rate of transformation, and the possibility exists that an environment that supports an increased level of transformation will also support an increased rate of transposition. This potential effect of the excess inorganic nitrogen supply on the genome and on DNA repair mechanisms is yet unclear, despite the documented phenotypic effects. There has been documentation of the effects of high nitrogen sulfate levels in humans leading to increased rate of recombination due to an inducement of Rad51 in humans, and there is no convincing research to document if this is the same effect that may increase transposition, and if it works by a similar pathway or mechanism (Shim et al, 2006; Sigurdsson et al, 2001). This research needs to be further tested before a better understanding of the potential effects of inorganic nitrogen amounts on transgene activity in plants can be achieved.

7. Conclusion:

Insertional mutant or transposon tagged mutant collections need to number in the hundreds of thousands to have a chance of generating a mutant line for each of the 25,000 to 30,000 functional genes estimated to occur in most plant genomes. Hence, an efficient methodology needs to be implemented to develop such populations. The *AcDs* construct used in the present study was designed to make this objective feasible through the incorporation of two selectable markers, kanamycin resistance on the *Ds* element and GFP on the *Ac* element with its constitutive promoter adjacent to but on the *Ds* element. The way the system is supposed to work is for GFP to be used for initial selection of transgenics and for selection of Launch Pad genotypes with an intact *Ac/Ds* construct. Transposition during gametogenesis should remove the promoter from the GFP, allowing a dual selection of transposants that are still resistant to kanamycin and no longer express GFP (i.e., they are no longer launch pads). The B5 liquid medium with kanamycin in Delong flasks described in this study provide an effective method of producing kanamycin resistant seedlings with few escapes, thus facilitating one part of the selection scheme. Unfortunately, the GFP screening was not possible in this study because of an unexpected lack of GFP expression in the strawberry population.

Hence, we used PCR, a slow but generally reliable way to select potential transposants.

The nitrogen experiment was an attempt to increase the rate of transposition based on its published influence on transgenic plant production. There was some evidence that the transpositional rate in *F. vesca*, line 311 was improved in the high nitrogen treatment. We would need to repeat the experiment with additional lines to demonstrate a consistent trend. With improved dual selection and environmental manipulations that favor transposition, generating the population size required for creation of a high coverage mutant library may be possible at a lower cost in time and materials than has previously been possible in *F. vesca*.

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Table 1, Primers for the multiplex, detailing the sequences, primer pairings, and band size. Band refers to what the primers are amplifying; EDS is the empty donor site, which will only appear if the Transposase and the EGFP genes are close enough to form a PCR product. The FDS is Full Donor Site, and tests for the PCR product formed between the Transposase gene and the P35s promoter. The *NPTII* primers test for the presence of the *NPTII* gene found on the *Ds* element. The FVaat3 primers test for the presence of the *FVaat* gene that occurs naturally in *F. vesca* as a control.

Primer	Sequence	Pairs with	Band size	Band
TpaseR1	GGTGAAATGCTGCCATAC	EGFPR1 P35SF1	1000 bp 600 bp	EDS FDS
EGFPR1	TTGTACAGCTCGTCCATG	TpaseR1	1000 bp	EDS
P35SF1	ACGCACAATCCCACTATC	TpaseR1	600 bp	FDS
NPTIIF1	TATGACTGGGCACAACAG	NPTIIR1	300 bp	DS
NPTIIR1	GCATCAGCCATGATGGATAC	NPTIIF1	300 bp	DS
FVaat3F	GTGACTTGGTTAACTTGCTC	FVaat3R	179 bp	FVaat
FVaat3R	AAATTAGTCCAGCTCGTGAA	FVaat3F	179 bp	FVaat

Table 2, Nutrients applied daily to the four treatment groups of 16 plants of four *F. vesca* genotypes used in transposon tagging. The ammonium nitrate concentration varied among the four treatments while the other nutrients were held constant.

Nutrient	Concentration (mM)
K2SO4	9.4
CaCl2 • 2H2O	3
KH2PO4	1.25
MgSO4 • 7H2O	1.5
Boric Acid	0.0154
MnCl2 • 4H2O	0.00914
ZnSO4 • 7H2O	0.000765
CuSO4 • 5H2O	0.000200
Na2MoO4 • 2H2O	0.000496
Nitrogen	
25 ppm	0.892
100 ppm	3.569
200 ppm	7.139
300 ppm	10.707

Table 3, ANOVA for the influence of two media on germination rate of four *F. vesca* launch pad clones, with seeds collected and stored for app. 4 months between collection and germination. *Significant at the 0.05 level.

Figure 1, Germination rates of seeds of four *F. vesca* launch pad clones grown on B5 liquid medium v. seeds grown on solid MS medium.

Source	DF	Mean Square	F Ratio
Medium	1	0.14	4.27*
Error	43	0.03	
C. Total	44		

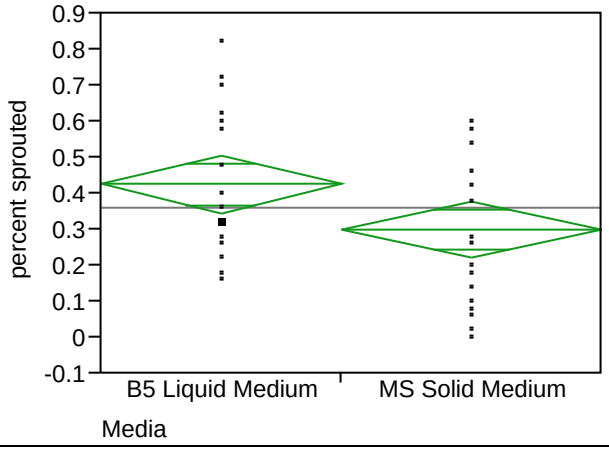

Table 4, Germination rates over time for line 307 over a 6 month period and line FV10 over a similar period. Table shows number resistant and number sensitive for line 307, but not for line FV10, as Line FV10 is wild type, and does not carry the kanamycin resistance gene.

Line	Date Started	# Seeds	Resistant	Sensitive	Total sprouts	% resistant	% germinated
307	19-Jun-09	150	36	0	36	100%	24%
307	11-Sep-09	300	40	0	40	100%	13%
307	18-Sep-09	200	39	0	39	100%	20%
307	04-Dec-09	100	20	10	30	67%	30%
307	04-Dec-09	100	28	9	37	76%	37%
307	04-Dec-09	100	27	9	36	75%	36%
307	04-Dec-09	100	20	6	26	77%	26%

Line	Date Started	# of seeds	Percent germinated
FV 10	30-Sep-09	500	37%
FV 10	6-Oct-09	250	58%
FV 10	29-May-09	50	44%
FV 10	1-Mar-10	100	8%
FV 10	1-Mar-10	100	54%
FV 10	1-Mar-10	100	12%
FV 10	28-May-09	150	59%
FV 10	28-May-09	150	56%

Table 5, Germination rates of *F. vesca* lines 356 and 284 on B5 liquid medium with kanamycin in separate experiments, with the percent of seedlings resistant and sensitive to kanamycin.

Line	Date Started	# Seeds	Resistant	Sensitive	Total sprouts	% resistant	% germinated
356	17-Apr-09	50	2		2	100%	4%
356	18-Sep-09	50	1		1	100%	2%
356	17-Apr-09	150	6		6	100%	4%
356	3-Mar-10	100	3	0	3	100%	3%
356	3-Mar-10	100	5	1	6	83%	6%
284	28-May-09	150	99	1	100	99%	66%
284	28-May-09	150	112	5	117	96%	75%
284	1-May-09	500	341		341	100%	68%

Table 6, Spad data, the table on the left shows the ANOVA for the Mean Spad. All F values marked * are significant at the 0.05 level, and all F values marked ** are significant at the 0.01 level.

Figure 2, On the right Mean ± SE Spad values for four dates by nitrogen treatments (25, 100, 200, 300 ppm)

ANOVA for spad data

Source	DF	MS value	F value
Trt	3	94.05	12.24**
Genotype	3	17.54	2.28
Date	3	1303.75	169.65**
Date*Trt	9	40.155	5.22**
Genotype*Trt	9	12.41	1.62
Date*Genotype	9	16.22	2.11*
Date*Genotype*Trt	27	8.01	1.04
Error	128	7.68	

Mean spad graph with standard error

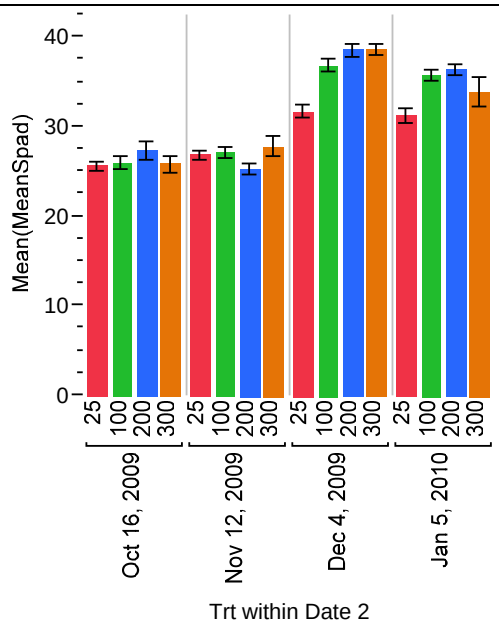

Table 7, ANOVA for mean runner production of three *F. vesca* launch pad clones and an untransformed control subjected to four nutrient treatments and measured four times during development. *significant at level 0.05, **significant at level 0.01

Figure 3 Mean ± SE runner production of three *F. vesca* launch pad clones subjected to four nutrient treatments and measured four times during development.

ANOVA for Runner data, experiment 2

Source	DF	MS value	F value
Trt	3	5.51	4.74*
Genotype	3	2.65	2.28
Date	4	18.33	15.76**
Genotype*Trt	9	2.11	1.82
Trt*Date	12	1.24	1.07
Genotype*Date	12	1.77	1.52
Genotype*Trt*Date	36	1.22	1.05

Mean Runner initiation graph with standard error

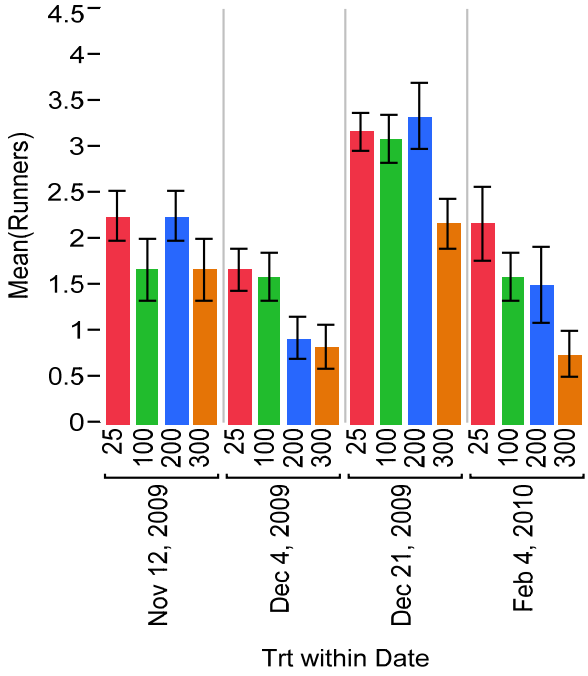

Table 8, ANOVA for mean height, three *F. vesca* launch pad clones and an untransformed control subjected to four nutrient treatments and measured four times during development. *significant at level 0.05

ANOVA of Height			
Source	DF	MS value	F value
Model	15	8.75	1.43
Error	47	6.11	
Total	62		
Effects			
TRT	3	3.98	0.65
Genotype	3	21.96	3.59*
Genotype * TRT	9	5.54	0.91

Table 9, Date to flowering versus treatment, with 3 *F. vesca* launchpad clones treated with nitrogen levels f 25 ppm, 100 ppm, 200 ppm, and 300 ppm daily, with on *F vecsa* wild type control tested as well. No significant effect was noted.

Source	DF	Mean Square	F Ratio
Trt	3	0.1875	0.3944
Error	44	0.4754	.
Cumulative. Total	47	.	.

Table 10, The occurrence of transposition in 215 T_2 seedlings after application of nitrogen treatments to T1 launch pads. Four fruit (f1-4) from each of three inflorescences (I1-3) were collected with seeds of each fruit separately tested for somatic transposition. Plant (1-4) and treatment (C, L, M, H) refer to each of four T1 plants to which four treatments were applied. C is 25 ppm nitrogen, L is 100 ppm nitrogen, M is 200 ppm nitrogen, and H is 300 ppm nitrogen. The green squares represent seedlings that had a both an Ac and a dissociated Ds element. The red squares show the T2 seedlings that had only the Ds element. The black squares show seeds that were not tested for transposition. Squares with a '0' are seeds that have been tested, but had no transposition. Each character in a colored square represents a single transposed seedling.

		Plant and treatment															
Inflorescence	Flower	C1	C2	C3	C4	L1	L2	L3	L4	M1	M2	M3	M4	H1	H2	H3	H4
	f1						0	0			0	0		*ddddd*			
	f1						0	0			0	0		*xx*			
	f2			*d*						*d*		0				0	
	f3		0				0	0		0		0		0			
I1	f4							*x*		0		0		0			
	f1						*x*			0		0					
	f1						*d*					0					
	f2					0	0	0		0		0		0		0	
	f3		0	0		0						0					
I2	f4		0	0								0				0	
	f1																
	f2						0										
	f3															0	
I3	f4			*x*													

Fruit not tested

x= EDS, NPTII

d =NPTII

Table 11, the total number of T2 seedlings form each family screened, with a breakdown of how many of each of the possible movement patterns shown for the AcDs element. The Column with Fvaat, NPTII, and FDS refers to those seedlings that contain a complete intact AcDs construct. Those plants with Fvaat, NPTII, FDS, and EDS have one copy of the AcDs element intact, and one that has broken into the separate Ac and Ds elements. The column containing Fvaat, NPTII, and EDS shows the number of plants that only have a fully separated copy of the AcDs element. The column named Fvaat, NPTII shows those plants that contain only the Ds element of the construct. WT refers to the plants that do not contain any element of the construct, and yet managed to make it through the antibiotic screening process.

				Transposing categories		
N trt	**Total plants**	**nptII FDS**	**nptII FDS EDS**	**nptII EDS**	**nptII**	**WT**
Control	**39**	**6**	**29**	**1**	**1**	**2**
Low N	**75**	**60**	**9**	**2**	**2**	**2**
Medium N	**47**	**34**	**10**	**1**	**0**	**2**
High N	**55**	**18**	**29**	**2**	**5**	**1**
Total plants per category	**216**	**118**	**77**	**6**	**8**	**7**
Percent	**100%**	**54%**	**35%**	**2.7%**	**3.7%**	**3.2%**

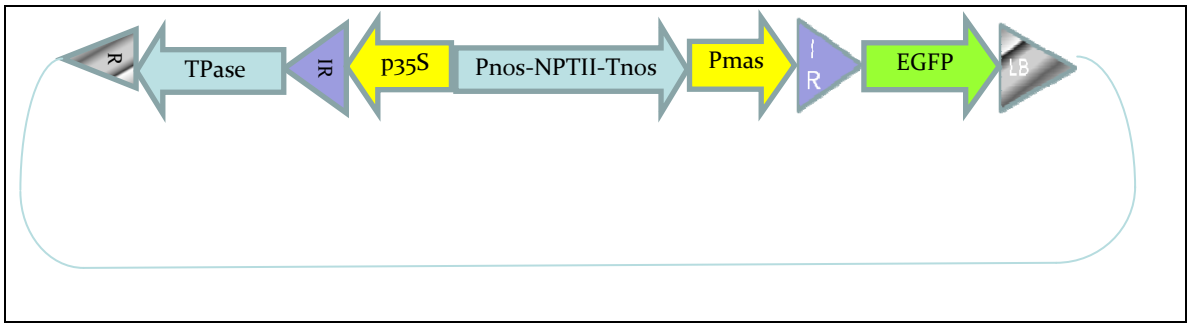

Figure 4, the *AcDs* construct used in the transformed plants. The *Ac* element consists of the RB (right border) and Tpase elements, as well as the EGFP and LB (left border elements. The two portions of the *Ac* element flank the *Ds* element, which consists of the two IR (inverted repeat) elements, the Pmas and P35s promoters, and the Pns-NPTII-Tnos gene and promoter terminator complex. The construct is supposed to function a follows: The EGFP gene is supposed to cause the plant to glow under a UV light, this function is driven by the Pmas promoter across the IR. The Tpase gene is activated by the P35s promoter and produces Transposase, which cuts the *Ds* element out of the *AcDs* construct at the IRs, and then inserts the *Ds* element into the plant genomic DNA at a random location.

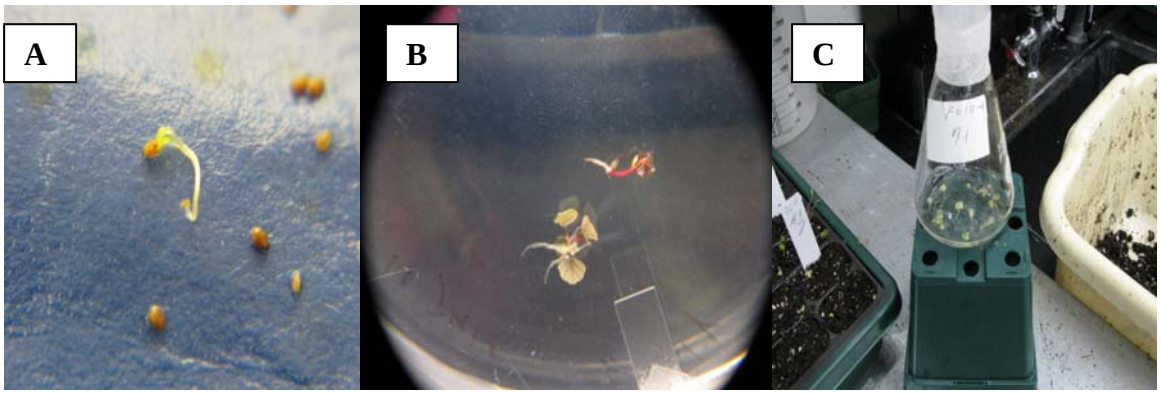

Figure 5, Shown above are the three germination methods, from left to right; Blue Germination Paper, MS solid medium, and B5 liquid medim in a DeLong flask. Also shown in the center image, that of the MS solid medium, is the difference between a kanamycin sensitive seedling(top center) and a kanamycin resistant seedling (bottom Center). Both seedlings have both primary and secondary roots, with the major difference being a retardation of growth in the sensitive seedling

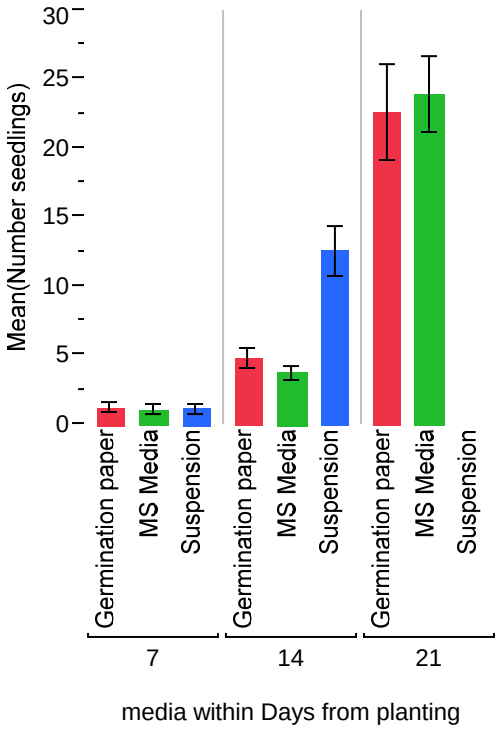

Figure 6, The rate of germination over a three week period of the three medium types tested. The B5 medium starts seeds fastest. The graph for the third week only contains the data points for germination paper and for MS solid medium, as the seedlings sprouted in the B5 medium could not be accurately counted in the flasks. B5 medium overall shows the highest germination rate.

Figure 7, The tangled shoot and root mass that is the result of letting the seedlings grow for more than about 2 weeks in Liquid B medium. This also demonstrates the curled, tangled, and short nature of the stems of seedlings grown in this germination medium. These seedlings are 4 weeks old, and are only starting to reach a length and total size that can survive planting.

Figure 8, The distinct phenotype of a sensitive seedling of six weeks of age grown on B5 liquid medium. Of note is that the main root on this plant is shriveled and blackened, as a result of the effects of the kanamycin root inhibition. This effect is not visible in the germination paper method, and can best be seen in Liquid B5 medium.

Figure 9, A kanamycin sensitive seedling (left) and a kanamycin resistant seedling (right), both of which are 6 week old T2 seedlings from plant 311.5, and were grown in the same batch in liquid B5 medium in a Delong flask, orbital shaker set at 90 rpm.

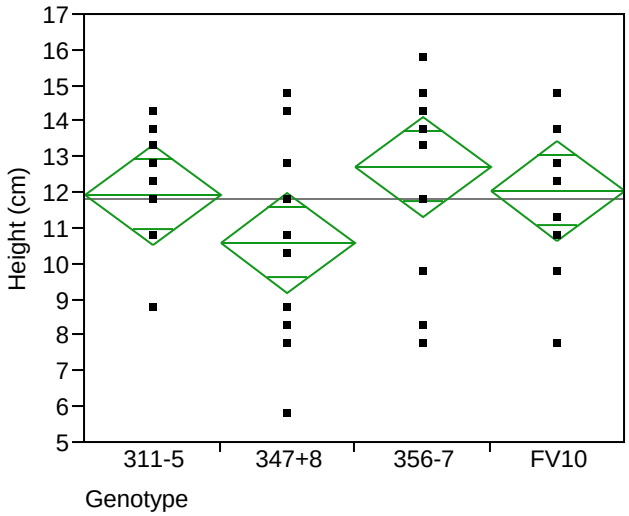

Figure 10, Mean height of four genotypes of F. vesca in four different nitrogen treatments. Because there was no significant effect due to the nitrogen treatments the data across treatments was combined for each genotype; genotype 347+8 was significantly shorter than the other three genotypes tested.

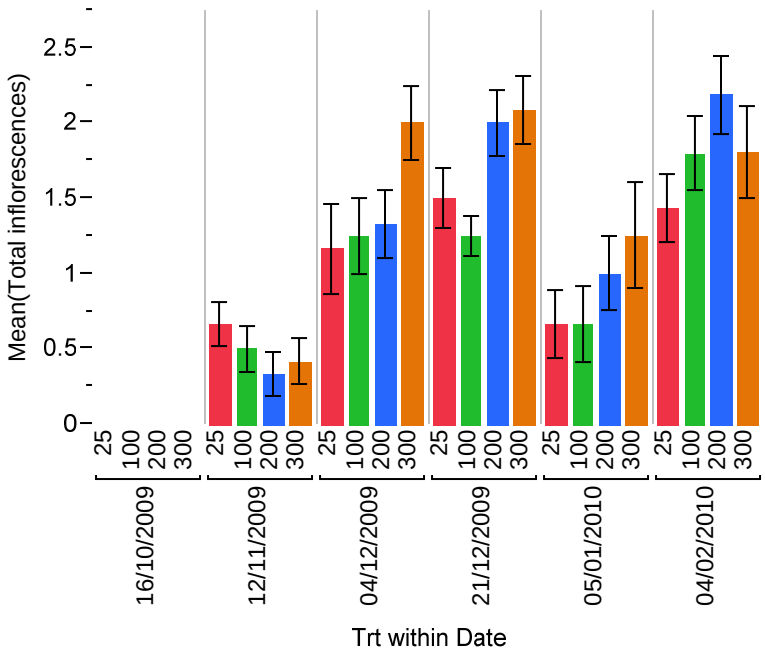

Figure 11, Date and treatment effects on inflorescence occurrence. No inflorescences were present on 16 October, 2009. Results after the first two data reads consistently show that higher levels of nitrogen in the daily fertilizer have the effect of increasing inflorescence occurrence.

Figure 12. Multiplex PCR analysis of crown tissue and tissue isolated from leaf tissue on three different infructescences of five launch pad T_1 311 plants. The top band at 1 kb shows the presence of the empty donor site. The band located at 600 bp shows the presence of the Full Donor Site, the Tpase and P35s elements in place in the full construct. The Band at 300 bp is the *NPTII* band, showing the presence of the *Ds* element. The bottom band, at 179 bp, shows the presence of a native control gene *FVaat* that is present in all *F. vesca* plants. The Numbers at the top refer to the plants from which tissue was taken. 311-5.11 is plant M3, 311-5.12 is plant M4, 311-5.14 is plant H2, 311-5.15 is plant H3, and 311-5.16 is plant 4. A refers to tissue taken from the crown of the plant, and B, C, and D are tissue samples taken from the first, second, and third infructescences. The tissue samples were taken to test for somatic transposition in the different tissues of each plan