FINAL REPORT

10 CFR 600.151

DOE/ER/20190-71

Genetic Analysis of Embryo Dormancy

Glenn Galau
Botany Department
University of Georgia

DE-FG05-95ER20190 \$135,000 for 18 months (09/01/95 - 02/28/97) No-Cost Extension to 02/28/98

Original Proposal Abstract (for 36 months of funding):

Primary dormancy is the inability of mature seed to immediately germinate until specific environmental stimuli are perceived that predict that future conditions will support plant growth and seed set. The analysis of abscisic acid deficient and insensitive mutants, in particular in *Arabidopsis*, suggests that embryo abscisic acid may be directly involved in the development of primary dormancy. Other studies implicate the continued accumulation of LEA proteins as inhibiting germination in dormant embryos. The results of these physiological, molecular and genetic approaches are complex and equivocal. There is a real need for approaches that test the separate nature of vivipary inhibition and primary dormancy and deliberately seed to decouple and dissect them. These approaches should be of help in understanding both late embryo development and primary dormancy.

The approach taken here is to directly isolate mutants of *Arabidopsis* that appear to be deficient *only* in primary dormancy, that is fresh seed that germinate rapidly without the normally-required cold-stratification. We have isolated at least 8 independent, rapidly germinating RGM mutants of *Arabidopsis* from a collection of T-DNA induced mutants from Ken Feldmann. All other aspects of plant growth and development appear normal in these lines, suggesting that the *rgm* mutants are defective only in the establishment or maintenance of primary dormancy. At least one of these may be tagged with T-DNA. In addition, about 50 RGM isolates have been recovered from EMS-treated seed.

The specific goals for the project are as follows:

- 1) The *rgm* loci in the current collections will be defined. This will be through mapping and tests for allelism within the collections and with existing mutants altered in ABA and GA function. Priority will be given to any *rgm* mutants that are linked to T-DNA and any that are not ABA deficient or insensitive. Double mutants will be constructed with the expectation that their phenotypes may indicate the order of action of the *RGM* genes in dormancy.
- 2) The physiological phenotypes of the *rgm* mutants will be evaluated, in particular as an aid in above genetic analysis and in understanding their function. Characters will include: the time in embryo development at which rapid germination is acquired; ABA contents and sensitivity of germination to exogenous ABA during development; light and temperature requirements for germination.
- 3) If genetic analysis proves difficult with current collections, a uniform, very highly dormant line will be used to obtain additional EMS-induced RGM mutants.
- 4) Isolation of second-site suppressors of selected *rgm* mutations will be attempted.

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

MASTER

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

**Portions of this document may be illegible
electronic image products. Images are
produced from the best available original
document.**

RESULTS

Strategies and Methods

The primary objective was to acquire and characterize as complete a set of dormancy mutants as possible. These were isolated on the basis of their rapid germination in light, without stratification, and their phenotypes operationally defined as "Rapid Germinating" (RGM). Some of these should be deficient only in the wild-type requirement for stratification and thus be true dormancy mutants. We choose growth conditions that routinely produced high levels of seed dormancy, growth under cool fluorescent light at 16°C. Bulk seed of 3 ecotypes are consistently 100% dormant for many weeks; Cvi-0 (Cape Verdi Islands, CS902), C24 (Columbia derivative, CS906), and Wu-0 (Wurzburg, from Barbara Kunkel). We used the Ws-2 ecotype because of the T-DNA lines in that background. As described below, dormancy in Ws-2 is silique-position dependent, which provides a nondormant wild-type controls against which mutant and dormancy-broken wild-type seeds can be compared.

Germination assays are as follows: 5-10 day-old seed is used to minimize after-ripening contributions to nondormancy, and they are sown on MS medium solidified with phytigel and incubated under cool fluorescent light with a low far-red component in order to activate the phytochrome system required for germination. These conditions give reproducible rates and extents of germination in parental wild-type seeds and derivative mutants. Plates are examined daily, indicating on the bottom of the plate, beneath each seed the day in which germination occurs. After 6 days, the plate is removed to 4° for 4 days to stratify nongerminated seed, and germination of each seed is observed upon return to 22°. Seed that germinates before stratification are scored as nondormant, and those germinating only after stratification are scored as dormant. Total germination routinely exceeds 97% in Ws-2 and Col-0.

Goal 1

a. Isolation of RGM Lines from T-DNA and EMS-Treated Seed

We screened 4900 T-DNA lines released by the *Arabidopsis* Biological Resource Center. These were made by Ken Feldmann (Feldmann 1991) in Ws-2. About 600 T4 seed (equivalent to M3 seed) from each of 49 pools of 100 T-DNA lines were evaluated after 1 day imbibition. Germinating seed were termed RGM-T for Rapid Germination, T-DNA derived, and given pool and isolate numbers. A total of 18 RGM-T isolates from 8 of the 49 T4 family pools were taken to S4 to S6 through single-seed descent with maintenance of the RGM phenotype, a frequency of at least 8 M3 mutants from 4,900 M2 plants (>0.15%).

M2 seed from EMS-treated Ws-2 were also screened. A total of 21 RGM-E isolates from 9 pools were taken to S5 to S74 with maintenance of the RGM phenotype, a frequency of at least 9 M2 mutants from 12,000 M1 plants (>0.08%).

b. Dominance Relationships of the *rgm* Mutations

Most of the RGM isolates have been crossed in both directions with parental Ws-2. Nondormancy in the F1 seed (seed resulting from the cross) was sometimes higher than predicted from subsequent knowledge of dominance relationships, possibly due to most crosses being with early flowers, which may produce less dormant seeds even as heterozygotes. Seed development may also be altered in siliques resulting from manual pollinations. Consequently, the phenotype of F1 seed is now considered unreliable. Representative nondormant and dormant F1 individuals (as seed) were transferred to soil and the germination of F2 seed (seed produced on F1 plants) from late siliques on each F1 individual was then evaluated. Mutations were scored as recessive if about 25% of the F2 seed were nondormant. When examined, F3 seed from late siliques of individual F2 plants had the expected phenotypes; F3 seed made by virtually all nondormant F2 seed were entirely nondormant and F3 seed from individual

dormant F2 seed were either completely dormant or about 25% nondormant. Mutations were scored as dominant if 75% of F2 progeny were nondormant.

Several mutants routinely have 40-70% nondormant F2 seed. This was shown in several cases to be due to semidormant mutants with some, but not all heterozygous seed being nondormant. Again, F2 individuals produced F3 seed with predicted phenotypes, but with a significant fraction of dormant F2 individuals having segregating F3 seed indicative of some heterozygous F2 seed being dormant.

c. Complementation Tests Identify at Least 11 *RGM* Genes

Crosses have been made between individual *RGM* lines, usually in both directions, to identify if independent isolates were different mutant alleles at the same locus, and if isolates from the same parental pool contained the same mutation. As noted above, the phenotype of the F1 was not considered to be definitive, so F2 and F3 seed from representative F1 individuals was also examined. The two *RGM* isolates were scored as being in different complementation groups (mutant in different *RGM* genes) if a significant fraction of the F2 seed was dormant, the fraction being close to that predicted from independent knowledge of the dominance relationship of each isolate to wild-type. In most comparisons where probable wild-type, dormant F2 individuals were observed, they were recovered and a reasonable fraction shown to produce only dormant F3 seed. This analysis was even able to identify all three dominant mutant isolates from T-DNA pool 19 as containing the same mutation; F1 seed from all 6 possible crosses was 99-100% nondormant, as was F2 seed produced from representative F1 individuals from each cross.

There are 11 *RGM* genes so far identified by mutation. The assignments in Table 1 are still somewhat tentative. Only half have been mapped to chromosome, and 8 independent isolates are still not assigned. However, there are clearly a large number of different mutant loci in the current group of isolates with most represented by only a single allele. This suggests that there are a significant number of additional *RGM* genes not yet identified (Jürgens et al. 1991).

d. Mapping the Mutations

Most SSLPs distinguish Ws-0 and Col-0 (Bell and Ecker 1994; SSLP web page), as do most CAPS (Konieczny and Ausubel 1993) where tested (CAPS web page). Most of the *rgm* mutants have been crossed with Col-0, which has a short after-ripening period similar to Ws-2. The spectrum of germination rates of F2 seed from F1 plants with Col-0 or Ws-2 as a parent is very similar, indicating that the dominance relationship of the Ws-2 mutant alleles are the same with both Ws-2 and Col-0 wild-type alleles. The most rapidly germinating F2 seed should be homozygous mutant where the mutant is recessive or both homozygous and heterozygous mutant where the mutant is dominant or semidominant.

Dormancy in Cvi-0 is very high; up to at least 30 days after harvest, <0.5% of bulked seed germinate without stratification and repeated stratification still leaves 80-90% of the seed dormant. When crossed with Ws-2 or Col-0 in either direction, <0.5% of F1 or F2 seed are nondormant, but at least 80% of the dormant seed germinates after one standard stratification. Furthermore, F2 seed from crosses between *RGM* isolates and Cvi-0 contain 15-60% nondormant seed, depending on isolate.

In all cases, the genotypes of F2 seed were confirmed by examination of the germination its F3 seed. In several cases, representative F3 seed were grown to plants and their F4 seed examined to confirm the genotypes of the original F2 seed. DNA was extracted from F3 or pooled F4 seed derived from individual F2 seed of confirmed genotype. Some polymorphisms between Cvi, Col, and Ws have been reported (CAPS web page), and we have confirmed that a significant fraction of CAPS can be used to distinguish Ws-2 from either or both Col-0 and Cvi-0.

About half of the mutant loci in Table 1 have been mapped at least to chromosome by these techniques. As predicted, their map assignments 1) confirm the assignment, by complementation tests above, of each *RGM* isolate to gene, 2) identify complementation tests to be used to assign remaining *RGM* isolates to gene, and 3) identify other genes that might be the same as a mapped *RGM* gene.

e. Phenotypes of Double Mutants

Several probable double mutants have been recovered from F2 seed, with ongoing confirmation of their identity from analysis of their selfed progeny. About half of the double mutants are very similar to the parents in the extent and rate of germination as immature and mature seed, suggesting these pairs of mutants are in the same pathway. Six double mutants have additive or unexpected phenotypes. Additive rates of germination are seen in 4 combinations, suggesting that *rgm1*, *rgm4*, *rgm8*, and *rgm9* are in the same pathway. Unexpected phenotypes include *rgm2,rgm4* which is identical to null alleles of *ABI3*. Because *rgm4* is ABA insensitive, this suggests that *rgm2* may be weakly ABA deficient.

f. T-DNA Mutants are Probably Not Tagged with T-DNA

Unfortunately, probably none of the RGM-T isolates from the T-DNA pools contain mutant *RGM* genes tagged with T-DNA. Only two isolates have retained resistance to kanamycin throughout the several generations of selfing, where selection has been only for the RGM phenotype. But when crossed to Ws-2, segregation of kanamycin resistance in F2 seed clearly indicates that one and possibly both of these lines are homozygous for a single resistance locus unlinked to the mutation.

Goal 2

a. Dormancy Dependence on Silique and Ovule Position in Wild-Type Plants

Bulked seed of Ws-2 plants contain both dormant and nondormant seed. This hindered our ability to do genetic analysis of the RGM lines, so the reasons Ws-2 had variable dormancy was examined. First, Ws-2 has a short after-ripening period. About 5-15% of initially dormant seed will become nondormant after 15-20 days storage. Remarkably, there are also significant position effects. About 35-40 siliques develop on the primary inflorescence. The first 10-20 siliques contain nearly all nondormant seeds while the last 15-20 siliques contain nearly all dormant seeds. There is also a gradient of dormancy within the silique, from nondormant seeds near the pedicel to dormant seeds near the residual stigma. Removing the meristem at flower 20-25 results in fewer siliques in the nondormant zone without greatly changing the number of siliques in the dormant zone. These results indicate that siliques contain dormant seed if they are within 15-20 siliques of a nonfunctional or ablated meristem. Similar tests with 7 independent RGM isolates failed to detect any position dependence; all seed were nondormant. There must be a maternal signal, possibly predicting success in seed set, to which the embryo responds. Some mutant *rgm* plants could then be deficient in maternal signal (maternal-effect mutant) or contain embryos that do not perceive or transduce these signals (zygotic-acting mutant). Additionally, virtually all wild-type seed are dormant if recovered from late siliques and sown within 10 days, greatly increasing the accuracy of genetic analysis of the *rgm* mutations.

b. Time of Dormancy Establishment

The rates and extents of precocious germination of immature seeds are the same for wild-type and 8 independent RGM isolates up until at least the late postabscission and possibly desiccation stages, at which time RGM seeds germinate rapidly and wild-type seeds germinate even more slowly, if at all. Dormancy then must be established quite late, as this is when the rates and extents of stratification-independent germination diverge in wild-type and most RGM lines.

However, several mutant lines have seeds that germinate rapidly even at an early post-abscission stage. This suggests that establishing dormancy in wild-type embryos is at least a two step process. It should be noted that all mutants have seed that are desiccation tolerant, clearly making them very different in phenotype than null or near-null alleles of *abi3*.

c. Three *rgm* Mutants May have Maternal Effects

Recessive maternal-effect mutations are identified by reciprocal crosses of homozygous mutant individuals to wild-type, and the phenotype of all F1 progeny are mutant and only so when the maternal parent is homozygous mutant. F2 progeny should all have wild-type phenotype, regardless of their genotype. Dominant or semidominant maternal-effect mutants would result in a large fraction of F2 progeny having a mutant phenotype, regardless of direction of the original F1 cross. Although the phenotype of F1 progeny is potentially compromised for reasons noted above, there are three mutants, *rgm3*, *rgm6*, and *rgm10*, that may be maternal-effect. In crosses to Ws-2 and other *rgm* mutants, more nondormant F1 seed were routinely produced when these mutants were the maternal parent than when they were the paternal parent. In crosses to Ws-2, however, a significant fraction of F2 seed were nondormant. These mutants are probably semidominant and also zygotic-acting, although alternative explanations have not yet been eliminated.

d. *rgm* Mutants Still Require Light to Germinate

The *rgm7* mutant does not require light to germinate, all others retain the wild-type strict light requirement. This single mutant must be altered in a pathway or endpoint common to both light-sensitive and stratification-sensitive components of dormancy.

e. Most *rgm* Mutants are Sensitive to ABA

We have taken pains to conduct reasonable tests of the sensitivity of the mutants to exogenous ABA during imbibition and seedling growth. The method of choice is to cold-stratify all mutants and controls on various ABA concentrations. This increases the fraction of seed competent to germinate and increases the rate of germination. For 12 genotypes tested, the quality of the seedling on a particular concentration of ABA is more informative than the fraction that germinate, and this quality is not dependent on cold-stratification or whether ABA is present during stratification or only during the germination test. Controls are included in all assays, especially *abi1*, *abi3-1*, a null *abi3*, and Ws-2. Only 6 isolates out of 36 tested did not have parental sensitivities to ABA, but had sensitivities the same as *abi1* and *abi3-1* and are thus classified as ABA insensitive. These identify *rgm4* and one unassigned dominant mutation as ABA insensitive.

Goals 3 and 4

Project Goal number 3, obtaining more EMS mutations in a highly dormant line like Cvi-0, was not attempted because genetic analysis of the current mutants is progressing nicely, although sometimes difficult by current methods because of physiological variation in dormancy, especially in heterozygotes of semidominant mutants. It is also clear that dormancy in Cvi-0 cannot routinely be broken by stratification except in very old seed. Thus, wild-type seed cannot be easily germinated, which is required for genetic analysis of mutants. We will evaluate other backgrounds for levels of dormancy between Ws-2 and Cvi-0, as well as try different growth and stratification conditions that might render Cvi-0 more useful. The latter would help the mapping project in confirming genotypes of F2 individuals from crosses of mutants with Cvi-0.

Project Goal number 4, a search for second-site suppressors of selected *rgm* mutants, was not attempted, because it is probably premature. But we will continue to consider approaches that would be direct, fairly easy to do, and that would yield mutants that could be germinated reliably. In summary, the genetic and physiological characterization of the current *rgm* mutants should retain the highest priority.

Table 1. Results of Initial Characterization of *rgm* Mutants

Gene	Mutant Allele and Isolate	Response to ABA	Comments
<i>RGM1</i>	-1 E2.1	Sensitive	
	-2 E6.3	Sensitive	
	-3 E9.15	Sensitive	
<i>RGM2</i>	E5.12/19/23	Sensitive	Slow to green
<i>RGM3</i>	E1.29	Sensitive	Maternal influence
<i>RGM4</i>	E3.2/3/8	Insensitive	
<i>RGM5</i>	-1 E3.7/12	Sensitive	
	-2 E4.10/11	Sensitive	
<i>RGM6</i>	E4.16	Sensitive	Maternal influence
<i>RGM7</i>	E6.11	Sensitive	Dominant; Germinates in dark; spindly plant
<i>RGM8</i>	E8.59		
<i>RGM9</i>	E9.17	Sensitive	
<i>RGM10</i>	E9.28		Maternal influence
<i>RGM11</i>	E12.1	Sensitive	

Mutations are recessive or semidominant unless shown otherwise. Among the 14 isolates not yet assigned is a dominant, ABA-insensitive mutation (T19.1/3/4) and a dominant, ABA-sensitive mutation (T12.1)

Table 2. Phenotypes of Selected Double Mutants

Double mutant	Phenotype
<i>rgm1-1 rgm9</i>	Nonadditive
<i>rgm1-2 rgm9</i>	Nonadditive
<i>rgm4 rgm8</i>	Nonadditive
<i>rgm8 rgm9</i>	Nonadditive
<i>rgm1-1 rgm4</i>	Additive
<i>rgm1-2 rgm4</i>	Additive
<i>rgm10 rgm4</i>	Additive
<i>rgm10 rgm6</i>	Additive
<i>rgm2 rgm4</i>	Null <i>abi3</i> -like mutant
<i>rgm2 rgm10</i>	White embryo

Publications

At least 3 publications should result from the work performed so far. These will concern 1) gradients of seed dormancy in wild-type plants, 2) characteristics of dominant alleles at different loci, 3) use of mutants to understand the development of dormancy in wild-type.

References to Final Report

- Bell, C.J. and J.R. Ecker (1994). Assignment of 30 microsatellite loci to the linkage map of *Arabidopsis*. *Genomics* 19:137-144.
- CAPS web page: <http://genome-www.stanford.edu/Arabidopsis/aboutcaps.html>
- Feldmann, K.A. (1991). T-DNA insertion mutagenesis in *Arabidopsis*: mutational spectrum. *Plant J.* 1:71-82.
- Jürgens, G., U. Mayer, R.A. Torres Ruiz, T. Berleth and S. Misér (1991). Genetic analysis of pattern formation in the *Arabidopsis* embryo. In *Molecular and Cellular Basis of Pattern Formation*. (K. Roberts et al., eds.) The Company of Biologists Limited, Cambridge. pp. 27-38. [Development Supplement 1].
- Konieczny, A. and F. Ausubel (1993). A procedure for quick mapping of *Arabidopsis* mutants using ecotype specific markers. *Plant J.* 4:403-410.
- SSLP web page: http://chil.humgen.upenn.edu/~atgc/SSLP_info/comming-soon.html