

Cause for reduced seed viability of maize haploid inducing lines and counter-measure

Sayareh Irani¹, Allen D. Knapp², Thomas Lubberstedt^{2*}, Ursula Frei² and Ehsan Askari¹

¹Isfahan University of Technology, Isfahan, 8415683111, Iran

²Department of Agronomy, Agronomy Hall, Iowa State University, Ames, IA 50011, USA

*Corresponding Author

sayarehirani@yahoo.com

adknapp@iastate.edu

thomasl@iastate.edu

ufrei@iastate.edu

ehsanaskarii@gmail.com

Abstract

Some haploid inducing lines used in the production of maize doubled haploids (DHs), express germination problems and reduced vigor. In this study, haploid inducing lines RWS, RWK-76 and their reciprocal hybrids RWS/RWK-76 and RWK-76/RWS were examined for viability by Tetrazolium (TZ) and germination ability by standard germination tests. Evaluation based on TZ tests showed that 59% of the seed of RWK-76/RWS were not viable, compared with only 12% dead seed in RWS/RWK-76. Similarly, the percentage and speed of germination in RWK-76/RWS (25%, 1.53) was lower than for RWS/RWK-76 (74%, 4.30). In an effort to develop a quick method for assessing seed viability in these lines, the TZ test was repeated in a different way. Seed from each genotype was placed in beakers containing distilled water. Seed would either float or sink. Subsequent TZ testing confirmed that seed that floated was dead, and seed that sank was alive, although some of them had defective embryos. The dead seed in both genotypes failed to develop an embryo, leaving an empty cavity that would fill with air and cause seed to float on water. This feature can be exploited for a simple and practical method to separate living from dead seed. In addition, we surveyed the *igl* (indeterminate gametophyte) gene as a candidate for germination problems in inducer lines. Sequencing data from the *igl* region showed that RWS and RWK-76 differed in one nucleotide and amino acid in the first exon of *igl*. Segregation of *igl* alleles from RWS and RWK-76 was significantly ($P=0.01$) distorted in the respective F_2 population relative to the expected Mendelian segregation ratio (1:2:1). Thus, either *igl* or a linked gene in the *igl* region affect seed viability.

Introduction

Maize (*Zea mays* L.) is one of the most important crops globally. Inbred line production is increasingly done using doubled haploid (DH) technology (Geiger, 2009). Some of the most effective inducer lines in the production of maize DHs at present are RWS (inducer derived from a cross KEMS \times WS14) and its sister line, RWK-76, and crosses of hybrids RWS \times RWK-76 and RWK-76 \times RWS (Rober et al., 2005; Geiger and Gordillo, 2009). The cross RWS \times RWK-76 with an induction rate of about 90 to 100 haploids in every 1000 seeds is the preferred inducer over parental lines RWS and RWK-76 because it has more vigor and sheds more pollen (Geiger, 2009). Prigge et al. (2012) reported that a major QTL on chromosome 1 (*qhir1*) controls 66% of the genetic variance for haploid induction ability. Prasanna et al. (2012) mentioned, however, that inducers UH400, RWS, and RWS \times UH400 display poor vigor, poor pollen production, poor seed set, and high susceptibility to tropical maize diseases. Moreover, germination rates of seed from RWS and RWK-76 are substantially lower compared to seed

harvested from most other inbreds (unpublished data). Maize caryopses are complicated structures with three genetically distinct parts: the embryo (diploid), the endosperm (triploid), and the seed coat (pericarp). Seed quality is determined by the ability to germinate under “normal” environmental conditions, and is influenced by genetic, physical, physiological, and health aspects (Burriss, 1977). Among many factors controlling crop productivity, seed quality and its viability are very important. Knowledge of germination rates of planted seeds is crucial to achieving desired stand densities and expected yields. It is thus necessary to have suitable methods for testing seed vigor (Milosevic et al., 2010). Macdonald (1975) divided vigor tests into three groups. Physical tests are based on physical seed characters and are quick and inexpensive. Physiological tests use germination under favorable (standard germination) or stress (e.g., cold test) conditions. Biochemical tests evaluate seed viability, such as Tetrazolium (TZ) tests (Macdonald, 1975). In TZ tests, seed is first hydrated to restore metabolic activity. Obviously, at this stage seed is dependent on fixed carbon sources and derives energy production from the flow of electrons. The activity of dehydrogenase enzymes is thus crucial to the germination process. Colorless 2,3,5-triphenyl tetrazolium chloride solution is able to interact with the dehydrogenases and is reduced in this process into a red compound called formazan. Formazan stains living cells with red color and dead cells remain colorless. TZ tests work well for both dormant and non-dormant seed (Copeland and McDonald, 1995). Paliwal et al. (1990) evaluated three seed quality methods in sorghum including tetrazolium, soil cold and standard germination tests. They demonstrated that TZ tests were closely correlated with emergence in soil cold tests. Another seed quality method is a specific gravity test that has been used to distinguish viable from unviable seed. In this procedure, individual seed with low specific gravity is considered non-viable (Van De Venter and Barla-Szabo, 1994).

Maize has been studied broadly for many genetic aspects. However, there are only few genetic studies related to seed quality regarding germination ability (Eagles, 1982; Barla-Szabo et al., 1989; Odiemah, 1989). Also, expression of genes related to seed quality could be assayed by germination and vigor tests (Coimbra et al., 2009). Causse et al. (1996) determined quantitative trait loci (QTL) related to germination efficiency, grain size, and weight. Revilla et al. (2009) identified several genes related to germination in aged maize seed. They found polymorphic SSR (simple sequence repeat) markers for six known genes discriminating between living and dead seed. A quantitative trait locus (QTL) mapping study (Limami et al., 2002) identified three QTL related to germination trait T50 (T50 is the time at which 50% of the kernels germinated) that explain 18.2% of the phenotypic variance, three QTL related to a trait linked to germination performance, kernel size and weight (thousand kernel weight), that explain 17% of the

phenotypic variance, two QTL related to glutamine synthetase (GS) activity at early stages of germination that explain 17.7% of the phenotypic variance, and one QTL related to GS activity at late stages of germination that explains 7.3% of the phenotypic variance.

Jones (1920) and Mangelsdorf (1923, 1926) studied defective kernel (*dek*) mutations, with effect on endosperm and embryo development. These mutations are recessive and cause development of non-viable kernels. Various mutations have been recognized that act genetically during the haploid phase in angiosperms. Indeterminate gametophyte (*igl*) or female gametophyte mutants lead to abnormalities in embryo sacs and the fertilization process. *Igl* mutants produce both viable and defective seed, abnormal embryos and endosperm. Additionally, *igl* decreases the embryo genic ability of cells that miss one of the two parental genomes, so that mutant embryo sacs produce haploid progeny, of both maternal and paternal origin, at a higher rate compared to wild-type embryos (Kermicle, 1969; Evans, 2007). In this study, we determined seed vigor and germination rates of the two reciprocal F₁s of the haploid inducing lines RWS and RWK-76, and both inbred lines. We evaluated, whether previously mapped QTL affecting induction ability or the gene *igl* are likely to cause germination issues in the reciprocal F₁s, and established a simple technique for enrichment of viable inducer seed.

Materials and Methods

Tetrazolium Test

Crosses between haploid inducing genotypes RWS, RWK-76, RWS/RWK-76, and the reciprocal cross RWK-76/RWS, were examined for viability by TZ tests. TZ tests included four replications with 50 kernels each. Each replicate of 50 seeds was placed between moist germination paper (Anchor Paper, St. Paul, MN), overnight at constant 22°C (in dark). The next day, kernels were cut in half and placed in 0.1% TZ solution. After 30 minutes at 35°C, kernels were examined for staining patterns using a dissecting microscope according to the AOSA (2010) guidelines.

Germination Test

Haploid inducing F₁ genotype RWS/RWK-76 and the reciprocal F₁ RWK-76/RWS were assayed for standard germination performance. Germination tests were performed using a paper roll test according to AOSA (2010) guidelines. Kernels were placed on brown germination paper (Anchor Paper, St. Paul, MN), pre-moisturized with distilled water, and rolled up afterwards. Rolled germination papers were kept in bins. The bins were incubated in

growth chambers at 22°C (in dark). Genotypes were grown in a completely randomized design with four replications. Each paper roll containing 50 seeds was considered an experimental unit. After 14 days, total germination and speed of germination were evaluated as described below (Anjumiand Bajwa, 2005).

$$\text{Total Germination (\%)} = \frac{[N_T \times 100]}{N}$$

N_T : number of germinated kernels

N: Number of seeds

Speed of Germination (S)

$$= (N_1 \times 1) + (N_2 - N_1) \times \frac{1}{2} + (N_3 - N_2) \times \frac{1}{3} + \dots + (N_n - N_{n-1}) \times \frac{1}{n}$$

$N_1, N_2, N_3, N_{n-1}, N_n$: number of germinated kernels observed after one, two, three, (n - 1), (n) days

Float Test

To determine, if seed density tests could approximate seed viability more rapidly than germination tests, haploid inducing F₁S, RWS/RWK-76, RWK-76/RWS and F₂ seed from the cross of RWS×RWK-76 were tested. Kernels were placed into beakers filled with distilled water. Kernels would either float or sink. TZ testing was subsequently carried out to determine whether this method separated viable from non-viable seed.

Molecular Methods

Since the two QTL on chromosomes 1 and 9 affecting haploid induction (Prigge et al. 2012) did not affect germination rates when introduced in B73 background (unpublished data), we evaluated whether the gene *igl* may be a likely cause of germination issues. Leaves from the two inducer lines RWS and RWK-76 were harvested and DNA isolated. DNA extraction was conducted according to the CTAB protocol (Saghai-Marroof, 1984). For cloning of parts of *igl* (Evans, 2007), primers were designed with Primer3 Software (<http://frodo.wi.mit.edu/primer3/>). The left and right primers were 5'-TCATGTGATCTCTACAACATGTGC-3' and 5'-AGATTCTCTATTCTCCCCCTGATT-3', respectively. DNA amplification was performed in a 10µl reaction mixture containing 1 µl 5×buffer, 0.5µl DNA (~50ng), 0.2 µl of dNTPs (10mM), 0.2 µl of each left and right primer (10µM), 1 µl MgCl₂ (25 mM), 1 µl *Taq* (~1 unit) (Beijing Transgen Biotech Co., LTD) and 5.4

μ l distilled water. The PCR program included an initial denaturation step at 94°C for 2 min, followed by 39 amplification cycles: 94°C for 30s, 61°C for 30s, 72°C for 4 min, and a final extension step at 72°C for 10 min. Two percent agarose gels in 1× TBE buffer with 0.15 μ g ethidium bromide/mL were used to separate the PCR products. PCR products were separated by gel electrophoresis at 100 V for 3 h. Purifying DNA excised from the gel was done by QIAquick Gel Extraction Kit (QIAGEN, USA) according to its protocol. Products were cloned into pGEM-T vector (Promega, USA) and transformed into DH α 52 competent cells by heat shock at 42°C for 90 seconds. Transformed DH α 52 competent cells were cultivated on solid Lysogeny broth (LB) medium overnight. Plasmid DNA was extracted by QuickLyse Miniprep Kit (QIAGEN, USA) according to its protocol. DNA sequencing was carried out using an AB 3730xl DNA Analyzer. The resulting sequences were evaluated with BLAST against the NCBI nucleotide database (<http://www.ncbi.nlm.nih.gov/>). Alignment of the *igl* region in two inducer lines RWS and RWK-76 revealed minor differences, which were used to develop allele specific PCR primers. Two allele-specific markers with different lengths and reverse primers for analysis of the F₂ population derived from RWS/RWK-76 were designed with the Batch Primer3 software at <http://probes.pw.usda.gov/batchprimer3/> to generate a GC% between 40% and 70% (You et al., 2008). The two allele-specific primers were 5'-GTGATCACCGACCCCG-3' and 5'-ACGCCACGGTGATCACCGACCCCA-3'.

F₂ seed derived from F₁ RWS/RWK-76 (n=50) was divided into two fractions, using the floating method in water. Sunken seed was germinated and DNA was extracted from leaves according to the CTAB protocol. DNA from floated seed (dead seed) was extracted from endosperm according to Gao et al. (2008). All DNA samples were analyzed with the allele specific PCR marker for *igl*.

If *igl* has no impact on seed viability, we expected to see equal segregation ratios: 1/4 RWS/RWS + 1/2 RWS/RWK + 1/4 RWK/RWK in the sunken (DNA was extracted from leaf) and floated seed (DNA was extracted from endosperm) fractions. However, if *igl* affects female gamete development, we expected to obtain a significant deviation from the 1:2:1 segregation ratio in at least one of the two fractions.

Statistical Methods

The TZ test and germination experiment were conducted in a completely randomized design with four replications. Analysis of variance was done using SAS software (SAS Institute, 1999). The statistical model was:

$$Y_{ij} = \mu + \tau_i + \varepsilon_{ij}$$

Where, Y_{ij} is the j^{th} observation of the i^{th} treatment, μ is the population mean, τ_i is the treatment effect of the i^{th} treatment, and ε_{ij} is the random error.

Phenotypic correlations for seed viability based on TZ tests, and percentage and speed of germination were calculated as described by Falconer and Mackay (1996).

The significance of the null hypothesis (that *igl* has no impact on seed viability) was assessed using a X^2 test, which compares expected (1:2:1) with observed frequencies in two fractions of an F_2 population. Based on X^2 tests for comparing observed ratios in the two fractions (floated: 4:35:1; sunken: 0:1:9), another X^2 test was conducted. The null hypothesis was that floated fractions are not different from sunken fractions.

Results

Tetrazolium and Standard Germination Tests

The reciprocal crosses RWS/RWK-76 and RWK-76/RWS differed ($P=0.05$) in seed viability, percentage, and speed of germination (Table 1). Viability of seed as determined by TZ, was 88% for RWS/RWK-76 compared to only 41% in RWK-76/RWS (Figure 1-A). Percentage and speed of germination in RWK-76/RWS (25%, 1.53) was lower than for RWS/RWK-76 (74%, 4.30) (Figure 1-B and -C). There was a close correlation between the results of germination and seed viability, as estimated by the TZ test ($r=0.81^{**}$, and $r=0.82^{**}$, respectively) (Table 2). A floating test along with subsequent TZ testing confirmed that caryopses that floated were dead, while those that sank were alive, although some of them had defective embryos.

The *igl* Gene as Likely Cause of Germination Issues in Inducer Lines

The *igl* gene is located on chromosome 3 of the maize genome (Evans, 2007). After cloning and sequencing the *igl* region in the two parents of RWS \times RWK-76, the sequence alignment showed that RWS and RWK-76 are different in one nucleotide (Figure 2). This SNP (single nucleotide polymorphism) was located in the exon of *igl* on chromosome 3 of maize. This SNP changes the amino acid composition of the resulting protein (changing the respective amino acid from serine to leucine).

If this SNP in *igl* or another SNP in linkage disequilibrium with this SNP affects female gamete development, segregation at the *igl* locus in the F_2 population of RWS/RWK-76 should be affected after separating seed into sunken and floated. The X^2 value of 22.9 within the sunken and 22.6 within the floated seed fractions are significantly different from a 1:2:1 Mendelian

segregation ratio ($P=0.01$) (Table3). Therefore, the null hypothesis that *ig1* has no impact on seed viability was rejected. In other words, 90 percent of the endosperm sample was skewed towards the RWK parent and 87.5 percent of leaf samples were heterozygous (Figure3). Based on a χ^2 test for comparing the observed ratios in the two fractions (floated: 4:35:1; sunken: 0:1:9), the null hypothesis that floated fractions were not different from the sunken fractions was rejected.

Discussion

Two inducer genotypes (RWS/RWK-76 and RWK-76/RWS) differed significantly for seed viability, percentage and speed of germination. The assessment of seed viability by TZ tests is routinely used for determining seed quality in several species, including sorghum (Noll et al., 2010), wheat (Carvalho, 2013), and Triticale (Souza et al., 2010). 88% of the seed of RWS/RWK-76 were viable compared with 41% in RWK-76/RWS. Prasanna et al. (2012) reported that inducer RWS had a poor vigor. Germination tests are widely used across species (Van De Venter and Barla-Szabo, 1994; Carvalho, 2013). The percentage and speed of germination in RWK-76/RWS (25%, 1.53) was lower than for RWS/RWK-76 (74%, 4.30). Comparison of reciprocal F_1 hybrids provides an excellent system to study genes associated with parent-of-origin effects in diploid tissue that influences various traits of agronomic interest. In hybrid seed production systems such as maize, identification of genes associated with imprinting provides an opportunity to select parents (male or female) through which to introduce the desired alleles (Kollipara et al. 2002). In this study, the differential response of the reciprocal F_1 hybrids for germination rate indicates maternal dominance (parent-of-origin effect). This pattern of maternal influence attributable to parent-of-origin effect was previously observed by Kollipara et al. (2002) in maize. They reported that germination of the F_1 s with the high parent (i.e., parent with high germination score) as the maternal parent was usually higher in their germination tests compared to those with poor germinating parents as maternal parent. In other words, *Ig1* is expressed in egg cells – and the defective *ig1* allele causes abortion of the egg cell and thus lack of embryos in one but not the other direction of the cross. Alternatively, *ig1* is expressed later (in embryo/diploid cells) – in that case the difference can only be explained by epigenetic effects, as the two directions of the cross are genetically identical. Thus, in this latter case it would be a “parent of origin effect”, with “memory” provided by an epigenetic mechanism.

The significant and positive correlation between germination and TZ tests suggests that using the TZ test as a reliable indicator of maize viability and also showed the potential of the TZ

test for assessing seed quality in maize haploid inducers. Noll et al. (2010) reported a positive and significant correlation between TZ viability and germination in sorghum. Also, Paliwal et al. (1990) reported that TZ viability was closely correlated with emergence of sorghum in cold soil tests.

After dividing seed into floating or sunken fractions in water, subsequent TZ testing confirmed that seed that floated was dead, and seed that sank was alive. The dead seed in both inducers failed to develop an embryo, leaving an empty cavity that would fill with air and cause seed to float on water. Xu et al. (2013) reported that defective kernels differed in their appearance and showed a wide range in size. In particular, kernels with aborted embryos with well developed endosperm resulted in lighter kernels than normal kernels. Sivakumar et al. (2007) explained the application of petroleum ether as a separation medium in *Casuarina equisetifolia*. Petroleum flotation resulted in 90% germination in the sunken fraction and 4% in the floating fraction. Van De Venter and Barla-Szabo (1994) evaluated various methods for their potential as rapid viability tests for kernels of grain sorghum including specific gravity tests (series of ammonium sulphate solutions), a conductivity test, the assay of glutamic acid dehydrogenase activity (GADA), oxygen uptake rate of kernels, and a TZ test. Specific gravity and oxygen uptake methods showed the highest potential as rapid viability tests (Van De Venter and Barla-Szabo, 1994). In conclusion, the new method (gravity test) facilitates separating viable from dead seed. Also, this simple method would save a lot of time and effort in screening, especially large numbers of seeds. In agreement with our results, Van De Venter and Barla-Szabo (1994) also mentioned that the specific gravity test has the advantage of being rapid, simple and inexpensive.

The source of the *ig1* allele in RWS (inducer derived from a cross KEMS × WS14) and its sister line, RWK-76, come from the inbred line WS14 derived from the cross W23 (carrying the *ig1* gene) × Stock6 (Lashermes and Beckert, 1988). The *ig1* gene is located on chromosome 3 of the maize genome. The *ig1* gene contains four exons that constitute an mRNA 1264 bp in length. The first exon contains most of the 5' untranslated region (UTR), the second exon contains a lateral organ boundaries (LOB) domain, the third exon contains the C-terminal domain, and the fourth exon contains most of the 3' untranslated region (Evans, 2007). Evans (2007) reported that the C-terminal domain of the *ig1* protein may be critical for *ig1* function in the embryo sac, whereas the LOB domain is critical for lateral organ function. The sequence alignment showed that RWS and RWK-76 are different in one nucleotide. This SNP (single nucleotide polymorphism) was located in the first exon of *ig1* on chromosome 3 of maize. F₂ populations derived from crosses of RWS × RWK-76 showed significant (P = 0.01) segregation

distortion. The χ^2 tests for a ratio of 1:2:1 at the *igl* locus of the F₂ population indicated significant deviation from the expected Mendelian segregation ratios (X^2 values of 22.9 within the sunken and 22.6 within the floated seed fraction). Therefore, *igl* or a gene closely linked with *igl* affects seed viability. This is in agreement with Evans (2007) who reported that *igl* produces both viable and defective seeds, abnormal embryos and endosperm. Indeed, this SNP changes the amino acid composition of the resulting protein in the first exon (5' UTR) of *igl* (changing the respective amino acid from serine to leucine). Serine is a polar (hydrophilic) and leucine is a non polar (hydrophobic) amino acid. Untranslated regions of mRNAs have crucial roles in many aspects of gene regulation. UTRs are known to play critical roles in the post-transcriptional regulation of gene expression, including modulation of the transport of mRNAs out of the nucleus and of translation efficiency, subcellular localization and stability (Mignone et al. 2002).

In conclusion, we introduced a simple and practical method (gravity test) to separate living from dead seed, which is beneficial for separating non viable from viable inducer seed prior to sowing. Sequencing of *igl* showed that the two parents of RWS×RWK-76 differ in one nucleotide in the first exon leading to a non-conserved amino acid change. Distorted segregation in F₂ suggests that allelic variation at *igl* may affect seed viability.

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Table 1. Analysis of variance for TZ test and total and speed of germination characteristics in inducer lines

	df	Mean Square		
		Viable seed(TZ test)	Total Germination	Speed of Germination
Inducer lines	1	4418.00***	4802.00**	15.32**
Error		87.33	164.67	0.69

,* Significant at $P = 0.01, 0.001$, respectively.

Table 2. Correlation coefficients between TZ test, total, and speed of germination

	Viability Seeds (%)	Total Germination (%)	Speed of Germination
Viability Seeds (%)	1		
Total Germination (%)	0.82*	1	
Speed of Germination	0.81*	0.99**	1

*: Correlation is significant at the 0.05 level.

** : Correlation is significant at the 0.01 level.

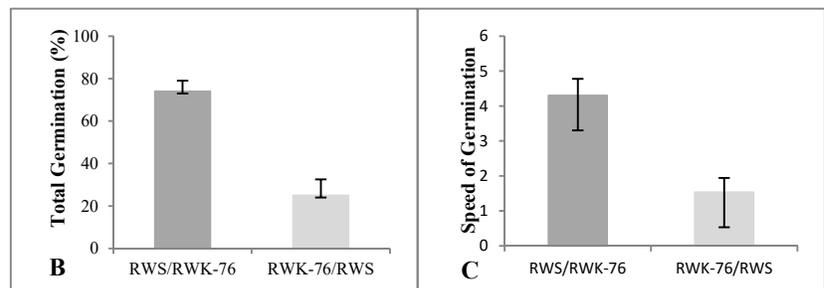
Table3. Chi-Square tests for segregation of expected ratios in the F₂ population of RWS/RWS-76

Genotype in the F ₂ Population	Mendelian Ratio	Expected Ratio	Observed Ratio	χ^2
Sunken Seeds (seedling) (n=40)				
RWS/RWS (A/A)	1	10	4	22.95**
RWK/RWS or RWS/RWK (H)	2	20	35	
RWK/RWK (B/B)	1	10	1	
Floated Seeds (endosperm) (n=10)				
RWS/RWS (A/A)	1	2.5	0	22.60**
RWK/RWS or RWS/RWK (H)	2	5	1	
RWK/RWK (B/B)	1	2.5	9	

$$\chi^2_{0.01,2} = 9.21$$

** : χ^2 is significant at the 0.01 level.

A



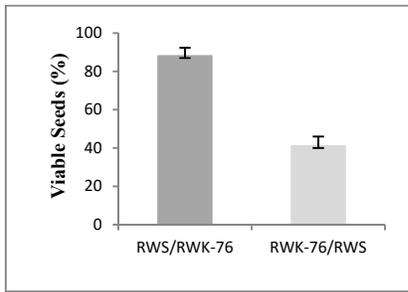


Figure1. Percentage of viable seed (A), total germination (B), and speed of germination (C) in inducer lines (bars show standard errors of sample means).

Score	Expect	Identities	Gaps	Strand
1020 bits(552)	0.0	554/555(99%)	0/555(0%)	Plus/Minus
Query 40	GATTAGATTCTCTATCTCCCCCTGATTTCCAGCAGCTTCCAAAACTTTCTAAAGAAACA	99		
Sbjct 596	GATTAGATTCTCTATCTCCCCCTGATTTCCAGCAGCTTCCAAAACTTTCTAAAGAAACA	537		
Query 100	AAAGAAAGTCTAACACAGCTCGTGATCCTTCGCCAGGGCAGCAGCGGATGGCTTCGT	159		
Sbjct 536	AAAGAAAGTCTAACACAGCTCGTGATCCTTCGCCAGGGCAGCAGCGGATGGCTTCGT	477		
Query 160	CGGTGCCGGGCCATCGGGGTCGGTGATCACCCTGGCGTCTTCTTCTCAGCAGCCG	219		
Sbjct 476	CGGTGCCGGGCCATTGGGGTCGGTGATCACCCTGGCGTCTTCTTCTCAGCAGCCG	417		
Query 220	CGGCCCGGTTGTGCGGCACGGGCTCCCCATGCGGTGCGTGCAAGTTCCTGCGTCGCAAGT	279		
Sbjct 416	CGGCCCGGTTGTGCGGCACGGGCTCCCCATGCGGTGCGTGCAAGTTCCTGCGTCGCAAGT	357		
Query 280	GCCAGCCGGACTGCGTGTTCGCGCCCTACTTCCACCAGGACAAACCCGAGAAAGTTCGTGC	339		
Sbjct 356	GCCAGCCGGACTGCGTGTTCGCGCCCTACTTCCACCAGGACAAACCCGAGAAAGTTCGTGC	297		
Query 340	ACGTGCACCGGCTTTCGGCGCAGCAACGTGACCAAGCTGTGAACGAGCTCCACCCT	399		
Sbjct 296	ACGTGCACCGGCTTTCGGCGCAGCAACGTGACCAAGCTGTGAACGAGCTCCACCCT	237		
Query 400	TCCAGCGCAGGACGCCGTGAACCTCCCTCGCCTACGAGGCCGACATGCGCCTCCGCGACC	459		
Sbjct 236	TCCAGCGCAGGACGCCGTGAACCTCCCTCGCCTACGAGGCCGACATGCGCCTCCGCGACC	177		
Query 460	CCGTCTACGGCTGCGTCGGGCTCATCTCCATCCTCCAGCACAACTACGACAGCTCCAGC	519		
Sbjct 176	CCGTCTACGGCTGCGTCGGGCTCATCTCCATCCTCCAGCACAACTACGACAGCTCCAGC	117		
Query 520	AGGACCTCGCCCGCCCAAGTACGAACTTCCAAGTACCAGGTGATGTGACGATCGTCGA	579		
Sbjct 116	AGGACCTCGCCCGCCCAAGTACGAACTTCCAAGTACCAGGTGATGTGACGATCGTCGA	57		
Query 580	TCGACATATATATAT	594		
Sbjct 56	TCGACATATATATAT	42		

Figure2. BLAST results of *igl* allele sequence comparison between RWK-76 (Query) and RWS (Subject).



Figure3. Segregation in sunken and floated seed of F₂ population