Metabolomic Discrimination of Near Isogenic Low and High Phytate Soybean [*GLYCINE MAX* (L.) MERR.] Lines

by
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Abstract

Phytate is the major storage form of phosphorus in seeds of soybeans. Because phytate chelates mineral cations including calcium, iron, and zinc, these mixed salts are often excreted by non-ruminant animals such as humans, swine, poultry, and fish. While this causes iron and zinc deficiencies**,** phytate is also considered a water pollutant due to the excess phosphorus excreted in animal waste. These negative environmental and nutritional effects, create a need for low phytate soybeans. While several low phytate soybean lines have been developed, a major drawback is the reduced seedling emergence of these lines resulting in low yields. Therefore, understanding the genetic and molecular bases of the low emergence trait in relation to seed phytate content in major crops such as soybean is of great economic importance.

This PhD project worked towards the long term goal of developing low phytate soybean cultivars with good seedling emergence and high-yield. This dissertation focused on metabolomic differences between low and normal phytate lines and how these could relate to the low emergence phenotype. The genetic materials used here include four near isogenic lines that differ in mutations in two multi drug resistance-associated proteins (*MRP*s). Only the line with both mutations was low phytate.

The phytate levels, field- and lab-based emergence rates were determined for these lines, their parents and a control line through replicated field experiments for three consecutive years. The

emergence rates of the low phytate lines were not always reduced. This showed that the environment the seeds were produced in is highly important, especially when breeding and commercially growing low phytate lines.

A protocol was developed for successful metabolomic discrimination of these closely related soybean lines. The polar and non-polar metabolite profiles were determined using ultra performance liquid chromatography mass spectrometry and metabolomic differences between the low and normal phytate lines were identified. The low phytate double mutant did not contain C22 glucose terminated Group A soyasaponins and almost exclusively contained C22 xylose terminated Group A soyasaponins (A4, A5 and A6). Compared to the normal phytate lines, the low phytate soybean line showed a higher concentration of storage lipids (triacylglycerols and diacylglycerols) and certain phospholipids.

Dedication

Max, Sox and Rex

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ABC	ATP-binding cassette transporter
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BEH	ethylene bridged hybrid
DGs	diacylglyceride
DNA	deoxyribonucleic acid
ECGT	extended cold germination test
ECT	extended cold test
EMRT	exact mass retention time
EMS	ethyl methane sulphonate
ESI	electrospray ionization
H2O	water
HILIC	hydrophilic interaction liquid chromatography
HPLC	high performance liquid chromatography
HSS	high strength silica
KASP	Kbiosciences competitive allele-specific PCR
LC	liquic chromatography
m/z	mass-to-charge ratio
MeOH	methanol
MIPS	myo inositol phosphate synthase
mRNA	messenger ribonucleic acid
MRP	multidrug resistance-associated protein
MS	mass spectrometry
NIL	near isogenic line
OPLS-DA	orthogonal partial least squared discriminate analysis
PC	phosphatidylcholine
PCA	principal component analysis
PCR	polymerase chain reaction
PE	phosphatidylethanolamin
PI	phosphatidylinositol
PS	phosphatidylserin
PSV	protein storage vacuole
QTL	quantitative trait locus
RIL	recombinant inbred line
RNA	ribonucleic acid
RPC	reverse phase column
RT	retention time
SNP	single nucleotide polymorphism
TGs	triacylglyceride
UPLC	ultra performance liquid chromatography

Chapter 1

Low Phytate and Soybean Seed Composition (Literature Review)

1.1 Importance of Soybean

Soybean, a legume of the Fabaceae family, is considered to be one of the most economically important oilseed crops in the world (Singh, 2010). Soybean seeds contain approximately 40% protein and 20% oil (Singh, 2010), making them a valuable source of both of these agricultural commodities. The US, Brazil and Argentina are the main soybean producing countries (Wilson, 2008). Worldwide, 218 million tons of soybeans were produced in 2005 allowing for a worldwide contribution of an estimated $48.6 billion (Wilson, 2008). Commercial soybean production in the US has increased dramatically since the 1950's, now more than 28 million hectare produce ~80 million tons of soybean (Singh, 2010). Soybeans have a wide variety of uses including human food, animal feed, and fuel (Ortega, 2009). They are also an important source of vitamins and anti-oxidants (Wilson, 2008). They further contain about 35% carbohydrates, 10% moisture, and around 5% minerals and ash, as well as phytic acid (phytate), essential fatty acids, isoflavones and saponins (AraúJo et al., 2013). While to some degree all of these components are required for successful plant growth and development, several downstream uses of soybean seeds are negatively impacted by the presence of high levels of phytate (Saghai Maroof et al., 2009). A goal for researchers and breeders is to develop low phytate soybean lines and thus reducing the negative impact while maintaining or increasing soybean seed yield (Raboy, 2001).

1.2 Phytate

Phytate, also known as *myo*-inositol (1,2,3,4,5,6)-hexakisphosphate or phytic acid, belongs to the class of inositol phosphates (Raboy, 2009a). It is mainly known as the major storage form of phosphorus in seeds of legume and cereal crops (Raboy, 2009b). In fact, about 75% of the seed's phosphorus is stored in phytate compounds (Raboy, 2001). Free phytic acid is acidic and thus stored in seeds as a salt, and hence referred to as phytate (Yuan et al., 2009). If deposited in the seeds as mixed salts of mineral nutrients such as K and Mg, phytate can be referred to as phytin (Raboy, 2009a). Phytate and its mineral nutrients are stored in protein storage vacuoles known as globoids (Raboy, 2009a).

Phytate's major role is to serve as a nutrient reservoir during seed germination (Raboy, 2007c), as it stores phosphorus, *myo*-inositol and mineral cations. Other roles are described as well. Phytate is ubiquitous in eukaryotes and heavily involved in development and signaling (Raboy, 2009a). For example, it plays a role in hormone signaling as a cofactor for an auxin receptor that regulates gene expression (Kim & Tai, 2011) and is involved in DNA repair and RNA editing (Raboy, 2009a). Phytate and its pyrophosphate containing derivative also play a role in ATP generation from ADP (Raboy, 2001). It is considered to be the most abundant inositol phosphate in a eukaryotic cell (Raboy, 2001).

1.2.1 Negative Effects of Phytate

As soybean seeds are predominantly used for animal feed (Ortega, 2009), it is of great importance that the composition of the seed is optimal. The majority of phosphorus that the animals need to take up with their diet is bound to phytate. Monogastric animals like poultry, swine or fish lack an endogenous phytase enzyme that helps break down the phytate, so that the

phosphorus is unavailable to them (Yuan et al., 2009). Moreover, phytate chelates mineral cations, making them nutritionally unavailable to the animal and also the human consumer (Yuan et al., 2009). Phytate in rice is associated with micronutrient deficiencies in Asians for whom rice is the staple food (Zhao et al., 2008b). Therefore, phytate is considered to be an anti nutrient (Raboy, 2007b). Farmers need to supplement animal feed with inorganic phosphorus (Adeola et al., 1995) or phytase enzyme (Lei et al., 1993), both being expensive options.

Apart from the negative nutritional effect, phytate in crops also has a negative environmental impact (Raboy, 2007a). Because the phosphorus uptake is inefficient, it leads to an increased excretion of phosphorus by the animals, which in turn increases the phosphorus transfer to the environment and thus environmental pollution, such as eutrophication (Walker et al., 2006). When excess phosphorus is excreted in animal waste, which is often used as fertilizer, it has the potential to run off into streams and lakes (Sharpley et al., 1994), and there increased amounts of phosphorus are seen as pollutants in bodies of water resulting for example in increased algae growth (Yoo et al., 2005).

Due to these negative effects on nutrition and the environment, many scientists work on the generation and implementation of low phytate crops, as they are highly desirable (Raboy, 2002). However, a major drawback with low phytate crops is reduced seedling emergence (Gao et al., 2008). Previous studies in soybean, barley, rice, wheat and maize have shown that not only the emergence is negatively impacted, but low phytate mutants have many inferior agronomic traits, leading to reduced yield compared to the wildtype lines (Bowen et al., 2007; Guttieri et al., 2004; Oltmans et al., 2005; Rasmussen et al., 2010).

1.2.2 Low Phytate Lines

Low phytate lines were developed through chemical or physical mutagenesis and by genetic transformation (Raboy, 2007c). Maize (*Zea mays*) has at least four different low phytate mutants (Pilu et al., 2003; Raboy & Gerbasi, 1996); barley has more than twenty low phytate mutations in at least six different loci (Oliver, 2009). Most low phytate lines have a decreased amount of phytate, but also an increased amount of inorganic phosphorus and thus unchanged levels of total phosphorus (Raboy, 2001). Maize seeds that are homozygous for a mutation in the *lpa* allele show a 30-90% reduction in phytate concentration and an increase of inorganic phosphorus (Raboy & Gerbasi, 1996). Gutteri et al. (2004) also used mutagenesis to develop low phytic acid wheat lines. The line designated as Js-12-LPA shows a ~ 25% reduction in phytate content compared to wildtype lines which is probably due to a mutation in two or more genes (Guttieri et al., 2004).

Different genetic mutations causing low phytate content have been identified in soybean as well. Chemical mutagenesis was used to produce LR33, a low phytate soybean line, in which a one base pair mutation in the *myo*-inositol phosphate synthase 1 (*MIPS1*) gene results in ~50% reduction in phytate concentration (Sebastian et al., 2000). V99-5089, a low phytate soybean line developed at Virginia Tech also has a mutation in the *MIPS1* gene (Maupin et al., 2011). Its average phytate concentration is 9.9 ± 0.5 mg/g (Gao et al., 2008), compared to 13 and above mg/g for normal phytate seed lines.

1.2.3 Low Phytate Line CX1834-1-6

CX1834 is another low phytate soybean line that was developed at USDA/Purdue University by crossing the soybean line Athow to the low phytate soybean line M153-1-4-6-14 (from here on referred to as M153) (Wilcox et al., 2000). The low phytate phenotype in CX1834 is caused by mutations in two multidrug resistance-associated proteins (*MRP*s) (Gillman et al., 2009; Saghai Maroof et al., 2009). These genes are located on chromosome 3 (Glyma03g32500, linkage group N) and chromosome 19 (Glyma19g35230, linkage group L) (Saghai Maroof et al., 2009). For both gene loci, the wildtype allele shows complete dominance over the mutant allele (Oltmans et al., 2004). Analysis of the CX1834 line shows a low phytate phenotype with a phytate concentration of 8.6 ± 0.4 mg/g (Gao et al., 2008).

Wilcox et al. (2000) used chemical mutagenesis to develop the low phytate soybean lines, M153 and M766 (Wilcox et al., 2000). Line M153 is reported to have an 80% reduction in phytate (Wilcox et al., 2000). CX1834 is an $F_{3:5}$-derived line from a cross of M153 to 'Athow' (Gao et al., 2008). CX1834 is the highest yielding low phytate line from this cross, identified through replicated yield tests (Oltmans et al., 2004). The low phytate phenotype in CX1834 is controlled by two recessive genes (Gillman et al., 2009; Oltmans et al., 2004; Saghai Maroof et al., 2009; Walker et al., 2006). Interestingly, it was suggested that the low phytate phenotype of M153 is caused by a single gene mutation (Oltmans et al., 2004; Walker et al., 2006). The inheritance of the low phytate trait was investigated by many researchers (Gao et al., 2008; Gillman et al., 2009; Oltmans et al., 2004), but it is not known yet where the two alleles originated from. Oltmans et al. (2004) hypothesized that the normal phytate line 'Athow' that was crossed to M153 has one of the low phytate alleles, but that allele did confer a low phytate phenotype until

it was crossed to M153, which has the other mutated allele (Oltmans et al., 2004). Walker et al. (2006) created a mapping population of CX1834 and 'AGS Boggs-RR', expecting to see 25% of the progeny to have a low phytate phenotype like CX1834. But the results were surprising as they saw few low phytate plants (less than 25%) and many plants with a phytate concentration intermediate to the parents (Walker et al., 2006). Walker et al. (2006) show that two loci are needed and discovered an epistatic interaction between the loci: a locus on chromosome 19 explains 11% of variation in phytate level and a locus on chromosome 3 explains 41%. The interaction between these two, however, adds another 8-11% of variation (Walker et al., 2006). Heterozygosity at either locus gives mid-range phytate (Walker et al., 2006). The phytic acid loci on chromosomes 3 and 19 seem to be located on duplicated regions of the soybean genome and thus may share a common origin (Walker et al., 2006). The probability that the original chemical mutagenesis by Wilcox et al. (2000) produced two nonlethal mutations in independent phytate genes is low, with Walker et al. (2006) suggesting that a low phytate allele was already present in the M153 line. Gao et al. (2008) looked for associations between the low phytate QTLs and the low phytate phenotype of lines derived from a cross of CX1834 and V99-3337. They found low phytate genetic markers in high phytate lines, suggesting a linkage of high phytate genes to the low phytate QTL on chromosome 19 (Gao et al., 2008). This could be an explanation why both *MRP* genes have to be mutated to obtain the phytate phenotype. This is in agreement with the suggestion by Walker et al. (2006) that M153 already had the low phytate QTL on linkage group L before chemical mutagenesis, but phytate values were not reduced until the second mutation on linkage group N occurred.

Wilcox et al. (2000)'s chemical mutagenesis yielded not just M153 but another independent low phytate line, M766, with mutations in the *MRP* genes as well. Gillman et al. (2009) researched

the cause for low phytate in M766. They found novel SNP mutations in the same *MRP* genes on chromosome 3 and 19, but were not able to link these mutations to the low phytate phenotype because of the lack of a segregating population (Gillman et al., 2009). It was suggested that both *MRP* mutations existed in CX1515 (the line that was subjected to EMS), but this line is no longer available for testing (Gillman et al., 2009). Gillman et al. (2009) however think it unlikely, because both M766 and M153 came from the EMS treatment event of CX1515 and they have different mutations. Their theory is that all mutant alleles originated during the EMS treatment (Gillman et al., 2009).

The *MRP* genes encode ATP-Binding Cassette (ABC) transporters. These are transmembrane proteins that are present in all species (Yazaki, 2006). They use ATP to transport substrates like lipids, sugars, peptides or steroids across the membrane (Klein et al., 2006; Rea, 2007). ATP-binding cassette transporters are involved in accumulation and storage of plant secondary metabolites (Yazaki, 2006). Flavonoids, terpenoids or saponins are just a few of the plants many secondary metabolites that are suggested to be involved in protection of the plant against biotic and abiotic stressors (Yazaki, 2006). Their biosynthesis and storage are highly regulated in a temporal and spatial manner, processes in which the membrane ABC transporters play a role (Yazaki, 2006). Different groups of ABC transporters exist, with Multidrug-Resistance Associated Protein (MRP) homologs being the second largest. Various functions for MRP have been described in plants: transport of GS-conjugates, anthocyanins, to stomatal regulation (Rea, 2007). As discussed here, it was shown that *MRP* mutations cause the low phytate phenotype in the soybean line CX1834 and the low phytate maize mutants, lpa1(Shi et al., 2007). A hypothesis is that ABC transporters move phytate from the cytoplasm across the membrane of the protein

storage vacuoles (PSVs) in the seed. In lines with a mutated ABC transporter, phytate cannot enter the PSVs and stays in the cytoplasm, where it is broken down by phytases (Panzeri et al., 2011; Raboy, 2007a; Raboy, 2009a).

MRP ABC transporters are responsible for low phytate in several plant species, including maize, rice and the common bean (Gillman et al., 2009; Panzeri et al., 2011; Shi et al., 2007; Xu et al., 2009). In barley and maize the lpa1-1 mutants have a low phytate phenotype due to a mutation in one *MRP* gene, which is mapped to a single locus in barley and maize, respectively (Larson et al., 1998; Raboy et al., 2000). The soybean low phytate mutant is different from maize because here recessive mutations are required in two separate *MRP*s in order to get a low phytate phenotype (Gillman et al., 2009). Shi et al. (2007) used transposon-mediated mutagenesis to produce low phytate maize lines. One of these lines, lpa1, has a reduced phytate phenotype due to a mutation in the maize *MRP4* gene, a gene encoding for a MRP ABC transporter (Shi et al., 2007). Maize *MRP4* gene is most closely related to *MRP5* in Arabidopsis (AT1G04120) (Shi et al., 2007). They also silenced soybean *MRP* genes using part of a soybean gene that is homolog to the maize *MRP4* gene and show that this homolog has the potential to reduce phytate (Shi et al., 2007). The two soybean *MRP* gene products show a 79% overall similarity (92.6% identity without the N terminal region) to each other (Gillman et al., 2009). The soybean *MRP* gene encoded proteins (UniProt IDs: K7MYS3, I1JP84) show a similarity of 66.6% and 63.8% to the maize *MRP4* gene (UniProt ID A7KVC2) and 74.5% and 72.3% to the Arabidopsis *MRP5* gene (UniProt ID Q7GB25) (Gillman et al., 2009). *AtMRP5* is strongly expressed in guard cells and could control anion channels across the plasma membrane and thus may lead to partial drought resistance in Arabidopsis (Klein et al., 2006; Suh et al., 2007).

1.2.4 Phytate Pathway

Phytate can be synthesized by either a lipid-dependent or lipid-independent pathway in plants (Stevenson-Paulik et al., 2005). In cereals and legumes phytate is generally produced by the latter one through a series of sequential phosphorylations of inositol as shown in Figure 1.1 (Rasmussen et al., 2010). The first step – the conversion of glucose-6-phosphate to InsP3 – is catalyzed by *myo*-inositol phosphate synthase (MIPS). Inositol monophosphatase converts this inositol 3-phosphate over several steps to *myo*-inositol. This is followed by a stepwise and sequential phosphorylation of the remaining five positions of the inositol ring by a number of kinases to phytate (Raboy, 2001) (Figure 1.1). The low phytate line CX1834 has mutations in the MRP transporters that are possibly involved in moving phytate to the storage vacuole.

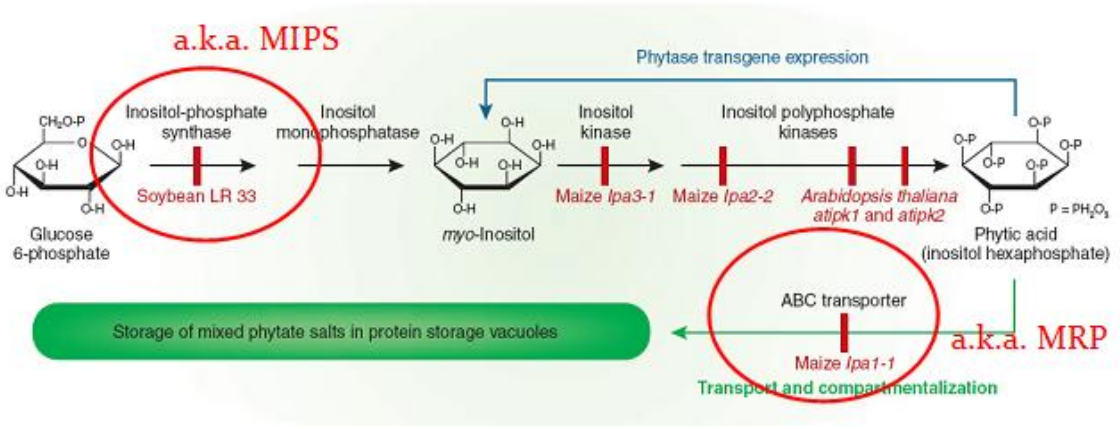


Figure 1.1. Phytate biochemical pathway (adapted by Dr. Glover from Raboy et al. 2007(Raboy, 2007a))

The *MIPS1* mutation in the soybean lines V99-5089 and LR33 disrupts phytate synthesis in that glucose-6-phosphate is not effectively converted to *myo*-inositol-3-phosphate (Hitz et al., 2002). This leads to reduction in precursor inositol levels, which in turn reduces the final phytate concentration. However, it is not only the phytate pathway that is affected, but the sucrose pathway as well (Hitz et al., 2002). Soybean seeds contain three main forms of carbohydrates:

normally 5-7% of the seed is composed of sucrose, 3-4% of stachyose and 1% of raffinose (Skoneczka et al., 2009). *Myo*-inositol is involved in the stachyose and raffinose biosynthesis and stachyose and sucrose concentrations are inversely correlated (Skoneczka et al., 2009). V99-5089 and LR33 have a low phytate, low stachyose, low raffinose, and high sucrose phenotype (Hitz et al., 2002; Skoneczka et al., 2009).

1.3 Seedling Emergence and Phytate

As important as seed germination and emergence are, these are complex traits that are not well understood (Bettey et al., 2000). Germination is the process in which the new plant emerges from the seed, which involves all the changes occurring in between the uptake of water by the seed and the formation and emergence of the embryonic axis from the protective layers of the seed (Bewley, 1997). Emergence can generally be defined as the process in which the radical of the seedling is breaking the soil. A germination rate of 80% is considered the minimum for commercial use (Smith et al., 2008). Studying seedling emergence is difficult as it is a complex genetic trait, controlled by many genes or QTLs and also heavily influenced by the environment, which includes soil moisture, temperature, soil salinity, pathogens and seed burial depth (Delouche, 1952; Wang & Shannon, 1999).

Phytate and its relationship to emergence are not only of importance in soybean, but also heavily studied in rice, barley and other important crops. In most cases a reduction in phytate also leads to reduced plant performance (e.g. yield, emergence, stress tolerance) (Bregitzer & Raboy, 2006; Raboy, 2001). Several scientists have analyzed and discussed the reasons why low phytate lines have reduced yield (Gao et al., 2008; Raboy, 2001; Zhao et al., 2008b).

Mutations in the *MIPS* and *MRP* genes in soybean not only lower the phytate content, but also impact germination and seedling emergence (Anderson & Fehr, 2008; Meis et al., 2003). Meis et al. (2003) investigated the emergence rate of low phytate lines with mutation in the *MIPS* gene and reported a reduction in emergence for those lines. The emergence of the *mips* lines is influenced by the seed source effect (Meis et al., 2003). The seed source effect describes the influence of the environment in which seeds are produced (Oltmans et al., 2005). Meis et al. (2003) reported that low phytate seeds that were produced in temperate climate have an emergence rate of 63%, if produced in subtropical climate however, the emergence rate is only 8% (Meis et al., 2003). However, the normal phytate lines have an emergence rate of 77% if produced in temperate climate and 83% if produced in subtropical climate (Meis et al., 2003).

Oltmans et al. (2005) investigated the effect of the combined *MRP* mutations on the phytate values and agronomic traits. When the low phytate line CX1834 (homozygous for both *MRP* mutations) is crossed to three soybean lines with wildtype phytate levels, the progeny lines with both mutations show a 50% reduction in phytate and ~30% increase in inorganic phosphorus compared to the normal phytate progeny lines (Oltmans et al., 2005). The emergence rate over all three populations is significantly lower in the low phytate lines (Oltmans et al., 2005). Oltmanns et al. (2005) also tested the emergence rate of the CX1834-derived populations in different environments. The low phytate lines shows lower emergence than normal phytate lines in all environments tested, however the amount of difference in emergence rates depends on the environment, suggesting an environment x genotype interaction (Oltmans et al., 2005). Apart from the emergence rate, the authors also analyzed the yield and discovered that on average the low phytate lines have a lower mean seed yield than normal phytate lines (Oltmans et al., 2005).

Yet, the difference in yield is only significant in one population and not all low phytate lines yield less than normal phytate lines, leading to the conclusion that yield might not be associated to the low phytate trait (Oltmans et al., 2005). There is no difference between the lines in maturity, lodging score, mean seed size, plant height or protein, oil or fatty acid content (Oltmans et al., 2005).

Anderson and Fehr (2008) also tested if the seed source affects the field emergence of the low phytate *MRP* mutant line CX1834. CX1834, the normal phytate line B019, and six of their low phytate backcross-derived lines (BC_3F_4) were grown in Iowa and Puerto Rico (Anderson & Fehr, 2008). The seeds from both locations were planted out in three locations in Iowa and the field emergence rate was determined. The emergence rates (25.4%) of low phytate lines whose seeds were produced in Puerto Rico (in May) are significantly lower than the emergence rates of the lines whose seeds were produced in Iowa (77%) or Puerto Rico (in January) (70.1%) (Anderson & Fehr, 2008). The emergence rates of the normal phytate parental and control lines are ~80%, no matter the seed source. Low phytate lines in this study are impacted by the environment the seeds were produced in, whereas the normal phytate lines are not impacted (Anderson & Fehr, 2008).

The difference in emergence between low and normal phytate lines is much more pronounced when the seeds were produced in tropical vs. temperate climate (Maupin & Rainey, 2011). Raboy (2009) suggests that this seed source effect is due to differences between low and normal phytate lines that are more pronounced when the seed is produced in tropical climate and further suggests that reduced heat tolerance of the low phytate seeds might be the cause (Raboy, 2009a).

Keigley and Mullen (1986) studied the effect of high temperature during the seed filling stage on the seed quality. Soybeans are subjected to different length of high temperature during seed filling and maturation and the seed germination and vigor are determined (Keigley & Mullen, 1986). If exposed to day temperatures of 27 °C, the emergence rate is 84%, if exposed to temperatures of 34 °C, the emergence rate drops to 50% and the number of shriveled, discolored and etched seeds increases more than 10 fold (Keigley & Mullen, 1986). High temperature has a significant negative impact on the seed quality and emergence rate of the next generation (Keigley & Mullen, 1986).

Egli et al. (2005) tested the influence of air temperature during seed filling on two normal phytate soybean lines. Plants are grown in Texas (37.6 °C average temperature during the seed filling state) and Kentucky (24 °C) (Egli et al., 2005). Germination is lower for the seeds grown in Texas, but the difference is only significant in one of the lines tested. This line, Hutcheson, has a germination rate of 100% when the seeds are produced in Kentucky and 85% when seeds are produced in Texas (Egli et al., 2005). Previous results have shown that high temperatures during the seed filling stage increase seed hardiness (Gibson & Mullen, 1996). Seed hardiness can lead to an increase in impermeability of the seed coat and thus delayed germination and emergence (Gibson & Mullen, 1996). Germination could be delayed for weeks after field planting (Keith & Delouche, 1998).

These results suggest that a hot and humid climate during seed production influences the emergence rate of those seeds negatively and that this is more pronounced for low phytate lines. Doria et al. (2009) suggest that phytate plays a role in fighting oxidative stress (Doria et al.,

2009) and thus low phytate lines may have a reduced stress tolerance (Raboy, 2001), which leads to reduced emergence. This seed source effect seems to have an influence on low phytate lines whose phenotype is caused by the *MIPS* and the *MRP* mutation (Meis et al., 2003; Oltmans et al., 2005), indicating that low phytate is responsible for reduced emergence and not possible pleiotrophic effects of the mutations.

1.4 Causes of the Reduced Agronomic Performance of Low Phytate Lines

Several studies were undertaken to better understand the reduced performance of low phytate lines. A study with low phytate maize mutants suggested that phytate prevents oxidative stress in seeds (Doria et al., 2009). During seed germination, the phosphorus and the mineral cations stored in the seeds are essential for seedling development (Doria et al., 2009). Phytate stores phosphorus and these mineral cations, yet, the existence of non lethal phytate mutants shows that phytate itself does not seem absolutely necessary for the plant's survival (Doria et al., 2009). However, phytate can chelate mineral cations including iron and thus it can also act as an inhibitor of reactive oxygen species (Graf et al., 1987). It is involved in the removal of Fe^{2+} and is therefore an important antioxidant (Doria et al., 2009). Doria et al. (2009) investigated the differences between a low phytate maize line (*lpa*1-241) and its wildtype isogenic line. The low phytate line has a significantly lower germination rate than the normal phytate line. Exposing both lines to an accelerated aging test (46 °C, 100% relative humidity) increases these differences. They further determined the amount of free iron and radicles in the seed. The low phytate line contains increased amount of both compared to the wildtype (Doria et al., 2009). These lines also differ in the amount of DNA and protein damage, with the low phytate line

being more affected. Yet, these differences are only apparent after the accelerated aging test (Doria et al., 2009).

Phytate is widespread in eukaryotes (Raboy, 2009a). It plays a role in numerous cellular functions, including but not limited to signal transduction, DNA repair, RNA transfer from the nucleus, ATP regeneration, control of guard cells, and phosphorus and mineral storage (Raboy, 2009b). As previously mentioned, phytate also plays a role in the regulation of K^+ and Ca^{2+} flux of guard cells to control turgor pressure and transpiration of plant cells (Lemtiri-Chlieh et al., 2003). Raboy (2011) argued that phytate-related pathways are active in most plant tissues and thus a reduction in phytate does not only influence seed chemistry but processes and pathways (e.g., ATP regeneration, mRNA export or RNA editing) in other plant tissues as well (Raboy, 2001). Reduced stress tolerance of low phytate crops may be due to these changes in the vegetative tissue (Raboy, 2009a). Since phytate derivatives function as phosphorus donors for the regeneration of ATP from ADP (Raboy, 2001), Raboy (2001) suggests that this regeneration is necessary for the advancement of seed germination until the mitochondrial membrane integrity is restored. It was also reported that low phytate seeds have a lower seed dry weight than their wildtype counterparts, which consequently reduces yield (Raboy, 2001). The increase in inorganic phosphorus and decrease in phytate may lead to reduced starch accumulation and thus a reduced dry weight (Raboy, 2001). The core structure of phytate is myo-inositol, a compound involved in several pathways tissue-wide (Raboy, 2009b). Pathways leading to cell wall polysaccharides, methyl inositols, pinitol and onitol and glycosylinositolphosphoryl ceramides utilize myo-inositol (Raboy, 2009a). As previously mentioned, a reduction in inositol does not only lead to reduced phytate but also reduced stachyose (Raboy, 2009b).

One hypothesis is that low phytate lines do not only differ in the genes responsible for phytate content but also in a linked or unlinked mutation that causes low emergence or low yield in general (Zhao et al., 2008b). Zhao et al. (2008) developed low phytate mutants in rice and studied their yield. The seed viability and the grain yield are significantly reduced in these mutants, which is most likely the cause for the overall reduction in yield (Zhao et al., 2008a). Some mutants show a germination rate similar to the controls, but have significantly lower field emergence rates. The authors further subjected their low phytate and normal phytate seeds to artificial aging test to judge the seed vigor. All lines show a reduction in seed viability after artificial aging, however, all but one low phytate line, have a significantly higher loss of viability. The authors show that low phytate rice seeds generally have a lower performance than the wildtype lines in the agronomic traits tested. The authors argued that the inferior traits of the mutants could be due to unrelated mutations and not pleiotrophy of the mutated low phytate genes. That said, progeny tests show that these traits and the low phytate phenotype do not segregate, an argument against the unrelated mutation being responsible for low yield (Zhao et al., 2008a). In the F_2 and F_3 generations, the lines without the low phytate mutation have a higher grain yield than those with the mutation, suggesting that either the low phytate phenotype or the gene responsible for low phytate causes low grain yield. However, the authors also show that it is possible for low phytate lines to outperform normal phytate lines, arguing for the hypothesis of an unrelated mutation causing the general reduction in yield and performance of low phytate lines. Zhao et al. (2008) show that some low phytate rice lines have a higher grain weight than the wildtype controls and thus they suggest that it might be possible through further breeding to increase the agronomic performance of low phytate lines (Zhao et al., 2008b). Spear and Fehr (2007) show similar results with the low phytate soybean line CX1834. The authors investigated

if it is possible to produce low phytate lines with normal emergence rates through backcrossing. CX1834 is crossed into B019, a normal phytate line with reduced palmitate content, and 36 low phytate progeny lines are obtained and analyzed (Spear & Fehr, 2007). It was determined that phytate content of these 36 lines do not differ from parent CX1834, but 18 of these lines show an emergence rate that was significantly higher than CX1834's. The authors suggest that it is possible to separate the reduced emergence of CX1834 from its low phytate phenotype and thus produce low phytate CX1834 lines with a normal emergence rate. Low emergence might be due to other loci in the genome besides the low phytate QTLs in CX1834 (Spear & Fehr, 2007). Guttieri et al. (2006) also suggest that it may be possible to breed for low phytate wheat without the deleterious effects, which was done by backcrossing the low phytate genotypes into several genetic backgrounds (Guttieri et al., 2006). At least two low phytate mutant lines, barley lpa1-1 and soybean Gm-lpa-ZC-2, exist without having negative effects on agronomic traits (Bregitzer & Raboy, 2006; Yuan et al., 2007).

As previously discussed, the reduction of phytate is generally coupled with an increase in seed's inorganic phosphorus content, to maintain the total P content of the seed, the relationship between phytate and inorganic phosphorus being inversely correlated (Saghai Maroof et al., 2009). However, a large increase of inorganic phosphorus might be toxic to the cell (Raboy, 2009a). Inorganic phosphorus for example inhibits the enzyme ADP-glucose pyrophosphorylase, that is involved in the starch synthesis in maize (Raboy, 2009a). Raboy (2009a) also suggests that inorganic phosphorus is not as effectively retained in stored low phytate seeds than in normal phytate seeds. This could lead to an increased susceptibility towards pathogens (Raboy, 2009a). It was also reported that loss of function mutations in *Arabidopsis thaliana* phytate

biosynthesis genes lead to increased susceptibility to viral, fungal or bacterial pathogens (Murphy et al., 2008).

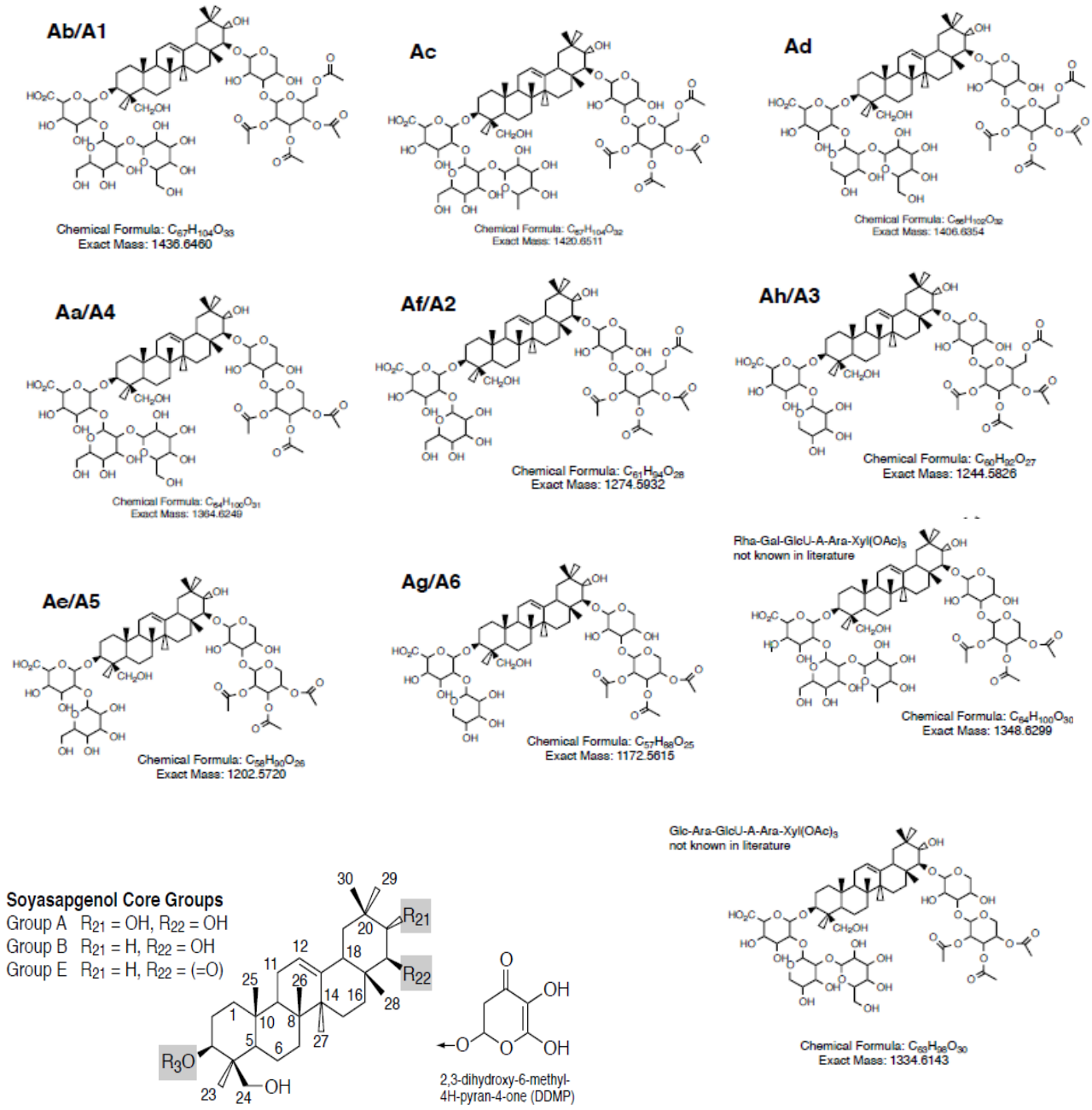
Previous research has shown that a phytate line, whose phenotype is due to a mutation in the *MIPS1* gene, does not differ in crude protein, crude oil, individual amino acids and saturated fatty acids from the normal phytate parent (Yuan et al., 2009). However, the total isoflavone content differs significantly in all environments tested. In the environment with the highest change, daidzin, glycitin and genistin concentrations are 233, 44.8 and 197.8% higher in the low phytate line, respectively (Yuan et al., 2009). Conversely, not all low phytate lines have isoflavone concentrations lower or even equal to the wildtype. Seed carbohydrates are important for desiccation tolerance and storage (Yuan et al., 2009). A typical soybean seed contains approximately 20% oil, 40% protein and 11% soluble carbohydrates (Hagely et al., 2013). Dry mature soybean seeds contain 15 to 20 different soluble carbohydrates (Tepavčević et al., 2011), with sucrose, raffinose and stachyose being the most abundant (Hagely et al., 2013). Besides these three major carbohydrates, soybean seeds contain α-galactosyl derivatives of the cyclitols *myo*-inositol, D-pinitol, trigalactosyl pinitol and galactopinitol B (Tepavčević et al., 2011). During germination soluble carbohydrates are lost (Tepavčević et al., 2011), stachyose and raffinose concentrations start to decline at hydration of the seed and monosaccharide levels increase. Proteome profiling of germinating seeds revealed that the most abundant proteins are storage related proteins and the largest changes occur in the metabolism related proteins, followed by protein biosynthetic proteins (Han et al., 2013). Of the metabolism-related proteins, the lipid and amino acid related proteins are the most abundant, like the enzyme lipoxygenase and methionine synthase (Han et al., 2013). These enzymes work on the degradation of oils and

methionine biosynthesis, respectively (Han et al., 2013). Han et al. (2013) also studied the concentration of fatty acids in germinating soybean seeds. The fatty acids C18:2 and C18:1 have the highest and second highest concentration in soybean seeds, respectively (Han et al., 2013). The total fatty acid content decreases sharply during the early stages of germination. So do the levels of minerals tested (Ca, Cr, Fe, Zn, Cu, K, Mg, Mn). The concentration of total free amino acids increase during germination, most likely due to the degradation of proteins (Han et al., 2013). During the first 3 days of germination, the carbohydrate and lipid levels decrease by 5-6%, whereas the protein level increase by 4% (Shi et al., 2010).

Apart from many lipids, carbohydrates and proteins, soybean seeds contain isoflavones, phytosterol, tocopherol, minerals and saponins. Soybeans are rich in isoflavones (Shi et al., 2010). The three major isoflavones types, genistein, daidzein and glycitein (aglycons) occur in soybean (Shi et al., 2010). These aglycons can be found in four different chemical forms: acglycon, glucosides (genistin, daidzin, glycitin), acetyl glucosides (e.g., acetyl genistin) and malonyl glucosides (for example malonyl genistin) (Shi et al., 2010). Shi et al. (2010) studied the isoflavone changes in soybean seed composition during germination (Shi et al., 2010). Isoflavones are predominantly stored as malonyl glycosides in the soybean seed. During germination the majority of malonyl daidzin (77%) and some of the malonyl genistin (30%) are converted to daidzin/daidzein and genistin/genistein, respectively. However, the total isoflavone concentration does not change (Shi et al., 2010). It was previously reported that isoflavone concentration ranges from ~ 1100 to 2500 µg/g (Eldridge & Kwolek, 1983; Hoeck et al., 2000), which is 0.05 to 0.5% of dry seed weight (Tepavčević et al., 2011). The concentration is widely dependent on the environment (Macdonald et al., 2005) and genotype (Tepavčević et al., 2011),

but daidzein and genistein and their conjugates have a similar but higher concentration than glycitein and its conjugates (Macdonald et al., 2005). Saponins are triterpenoidal or steroidal aglycones with carbohydrate moieties that can be classified into three Groups (A, B and E) (Macdonald et al., 2005). Members of Group A soyasaponins, classified as A1-A6, are generally acetylated at a terminal C22 glycoside (Figure 1.2). Members of Group B, classified with roman numerals e.g. I, can have DDMP moieties (2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyrran-4-one) (Kim et al., 2006b). Soyasaponins are synthesized through the isoprenoid metabolism pathway (Kim et al., 2006b). Their concentration depends on the seed part: Group A soyasaponins are mainly found in the hypocotyls (Kim, 2003), whereas group B's soyasaponin II is mainly detected in the cotyledon (Tsukamoto et al., 1995). Kim et al. (2006) used reverse phase HPLC to quantify the different soyasaponins. Group B's DDMP-conjugated soyasaponin βg is the major soyasaponin detected, followed by soyasaponin I and A_1 (Kim et al., 2006a). The total soyasaponin concentration lies between approximately 3620 and 4000 μg/g (approximately 0.4 – 0.5% of dry soybean), depending on seed size, genotype, degree of maturity and environment, including cultivation year and location grown (Kim et al., 2006b). A large-size seed is composed of 9.4% soyasaponins A1, 26.5% DDMP-conjugated soyasaponins, 49.9% of non-DDMP counterpart soyasaponins and 14.2% DDMP moiety (Kim et al., 2006b). Most group B soyasaponins are DDMP-conjugated in the seed and are not influenced by high temperatures during seed development (Tsukamoto et al., 1995). Large-seeded varieties have significantly higher percentage of soyasaponins II and III than small and medium size seeds, whereas the percentage of soyasaponin βg and DDMP moiety is significantly higher in medium-seed varieties. Of group A, soyasaponins A1 and A4, also known as Ab and Aa respectively, are the major components (Kim et al., 2006b). During seed development and maturation the ratio of

total isoflavone to total soyasaponins increases from 0.06 to 1.31 (Kim et al., 2006a). Protein, lipid and carbohydrates, sucrose and stachyose, concentrations increase with the developing seed. The correlation between these three components and total as well as conjugated isoflavones is significantly positive, whereas they show a negative correlation with the total soyasaponins as well as total group A and B soyasaponins (Kim et al., 2006a). Only the correlation with soyasaponins A is significant. The lipid concentration is negatively correlated with the isoflavone aglycone. Kim et al. (2006) also reported that the isoflavone and soyasaponins are negatively correlated; they suggest however that there might not be a close metabolomic interaction because these metabolites are generated by different pathways (Kim et al., 2006a).



Group A Soyasaponins: R_{21} = OH, R_{22} = OH

Names		R_3 substituent	R_{22} substituent
A1	Ab	β-D-Glc*p*-(1→2)-β-D-Gal*p*-(1→2)-β-D-GlcU*p*-(1→	2,3,4,6-tetra-*O*-acetyl-β-D-Glc*p*-(1→2)-α-L-Ara*p*-(1→
A2	Af	β-D-Gal*p*-(1→2)-β-D-GlcU*p*-(1→	2,3,4,6-tetra-*O*-acetyl-β-D-Glc*p*-(1→2)-α-L-Ara*p*-(1→
A3	Ah	α-L-Ara*p*-(1→2)-β-D-GlcU*p*-(1→	2,3,4,6-tetra-*O*-acetyl-β-D-Glc*p*-(1→2)-α-L-Ara*p*-(1→
A4	Aa	β-D-Glc*p*-(1→2)-β-D-Gal*p*-(1→2)-β-D-GlcU*p*-(1→	2,3,4-tri-*O*-acetyl-β-D-Xyl*p*-(1→3)-α-L-Ara*p*-(1→
A5	Ae	β-D-Gal*p*-(1→2)-β-D-GlcU*p*-(1→	2,3,4-tri-*O*-acetyl-β-D-Xyl*p*-(1→3)-α-L-Ara*p*-(1→
A6	Ag	α-L-Ara*p*-(1→2)-β-D-GlcU*p*-(1→	2,3,4-tri-*O*-acetyl-β-D-Xyl*p*-(1→3)-α-L-Ara*p*-(1→
	Ac	α-L-Rha*p*-(1→2)-β-D-Gal*p*-(1→2)-β-D-GlcU*p*-(1→	2,3,4,6-tetra-*O*-acetyl-β-D-Glc*p*-(1→2)-α-L-Ara*p*-(1→
	Ad	β-D-Glc*p*-(1→2)-α-L-Ara*p*-(1→2)-β-D-GlcU*p*-(1→	2,3,4,6-tetra-*O*-acetyl-β-D-Glc*p*-(1→2)-α-L-Ara*p*-(1→

Figure 1.2. Overview of group A soyasaponins (by Dr. Helm)

Frank et al. (2009) used gas chromatography to compare the metabolite profile of two low phytate soybean lines (Gm-lpa-TW75-1 and Gm-lpa-ZC-2) to the wildtype lines which highlights the power of the metabolite profiling technique. Their goal was to distinguish two different mutant lines and use the metabolites to verify previous mapping and gene cloning results. One of the mutant low phytate phenotypes is caused by a mutation in the *MIPS1* gene, but the cause of the other mutation is unknown. Even though both lines show a significant reduction in phytate compared to the wildtype line, the *MIPS1* line (Gm-lpa-ZC-2) shows no accumulation of lower inositol phosphates $InsP_3$,$InsP_4$ and $InsP_5$, whereas the other line shows an increase in these. Both low phytate lines had lower amounts of *myo*-inositol, galactinol, raffinose, stachyose and certain galactosyl cyclitols (Frank et al., 2009).

1.5 Assessing the Low Phytate Phenotype with Untargeted Metabolite Profiling

Metabolites are substrates and products of cellular biochemical reactions (Tautenhahn et al., 2010). An overview of a sample's metabolite composition can be obtained from metabolite fingerprinting techniques (Dettmer et al., 2007). Untargeted metabolomics is a metabolomic fingerprinting technique that allows an unbiased analysis of the metabolome of a cell or tissue at a certain time point (Tautenhahn et al., 2010). It does not necessarily aim for the identification and quantification of every observed metabolite (Dettmer et al., 2007). Total global metabolomics cannot fully be achieved yet, as the metabolites differ too wildly in their chemical properties and have a wide concentration range and thus it is best to separate different metabolite classes, e.g., polar from non polar components or high abundance compounds from low abundance ones (Dettmer et al., 2007).

Fiehn et al. (2000) was one of the first to use metabolite profiling for a comparison of metabolites in plants. His team extract 326 quantifiable compounds from a single *Arabidopsis* leaf and are able to identify half of them (Fiehn, 2000). Furthermore, Fiehn et al. (2000) compared four different genotypes (two ecotypes and their two corresponding mutants) and reported that each show a distinct metabolic profile. Principal component analysis classifies the data into "metabolic phenotypes". They show that metabolites can be used to distinguish between lines and to group them (Fiehn, 2000). Wu et al. (2008) used high-performance liquid chromatography mass spectrometry (HPLC-MS) analysis methods for metabolic profiling of salt-sensitive and salt-tolerant soybean lines. It shows that global profiling can distinguish between these closely related recombinant inbred lines, originating from the same cross, and thus elucidate compounds that are related to salt tolerance (Wu et al., 2008). Kim et al. (2006) also investigated how salt stress influences plant behavior. *Arabidopsis thaliana* cell cultures are subjected to salt stress and a time course metabolomic profiling experiment is performed. Producing these successive snapshots of the metabolites present is useful in determining the interacting mechanisms of pathways that are up- or down-regulated in response to stress (Kim et al., 2007). This study focuses primarily on small polar metabolites such as amino acids, organic acids and sugar alcohols. After performing principal component analysis (PCA) and batch-learning self organizing mapping analysis (BL-SOM), the authors suggest that several pathways, for example methylation cycle or the glycine betaine biosynthesis pathway, are being induced in response to salt treatment (Kim et al., 2007). Prior to this study, most researchers focused on transcriptomic and proteomic techniques to study salt tolerance. However, only with metabolomic profiling can the changes in biochemical reactions be evaluated.

1.6 Overview of Thesis Work

The relationship between seedling emergence and phytate levels is currently poorly understood. As many hypotheses for this relationship exist, it is necessary to address the problem of low emergence with a multi-disciplinary approach and a key to investigating the causes are near isogenic lines. They may help to address the complex question of the low seedling emergence in an extremely uniform genetic background. Through development of a Recombinant Inbred Line (RIL), identification of a plant heterozygous at both *MRP* loci, selfing and analyzing this plant, four soybean lines that are nearly genetically identical (originated from the same plant) except for the presence one or both of the *MRP* mutations were developed. The comparison of these four unique lines provides an excellent opportunity to compare the effect of the low phytate phenotype on the emergence rate and seed metabolite profile, while avoiding the complications resulting from different genetic backgrounds. To study the seed composition, a protocol for global metabolite profiling was developed and the near isogenic lines were compared. Changes in the metabolomic profile related to phytate content were identified. The results of this project can help to reach the long-term goal of understanding the genetic basis of low emergence, especially in relation to low phytate. In the future, these identified metabolites could be targeted for metabolic engineering or marker development.

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Chapter 2

Investigation of Seedling Emergence and Phytate Content of Near Isogenic Soybean Lines Differing in the Multidrug Resistance-Associated Protein (*MRP*) Genes

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2.1 ABSTRACT

Phytate is an important supplier of phosphorus, myo-inositol and minerals to the germinating seed and growing seedling. However, it has negative impacts on the nutrition of the seed consumer and the environment. Low phytate crops, including soybean have been successfully developed, but the reduced seedling emergence of low phytate lines is a hindering factor for the successful commercialization. The relationship between seedling emergence and phytate levels is not yet fully understood. We developed four near isogenic soybean lines that contain either one, none or both of the mutations in the multidrug resistance-associated proteins (*MRP*) of the low phytate soybean line CX1834. These four lines were compared in their phytate content, extended cold test (ECT) emergence rate, and field emergence rates. Analysis of phytate values showed that the mutations in both *MRP* genes are needed to confer a low phytate phenotype. The line with both mutations showed significantly lower phytate values than the lines with either or none of the mutations in all three years and two environments tested. The ECT showed that the emergence rate of all four NILs depended on the seed growing location, Blacksburg, VA or Mt. Holly, VA. The ECT emergence rates for the 2010-produced seeds was unacceptably low for all classes (all classes had an emergence rate below 60%), but it clearly showed that the low phytate NIL has significantly lower emergence rate than the NILs with normal phytate content. In 2011, the overall emergence rates were higher than in 2010, but the low phytate NIL did not show a significantly lower emergence rate than normal phytate NILs. The field emergence rates of the 2010-produced seeds were not influenced by seed source, the 2011-produced seeds had a higher emergence rate if they were produced in Blacksburg.

2.2 Introduction

Phytate, also known as phytic acid and myo-inositol (1,2,3,4,5,6)-hexakisphosphate, is the main storage form of phosphorus and inositol in seeds (Raboy, 2009). Phytate is degraded during germination supplying the growing plant with the needed inositol and phosphorus (Maenz et al., 1999). Because phytate has a high affinity for mineral cations such as iron and zinc and other positively charged plant molecules (Erdman Jr & Poneros-Schneier, 1989), it often forms mineral – phytate complexes, referred to as phytin (Angel et al., 2002).

These mixed salts make minerals unavailable to the end user and can lead to mineral deficiencies in humans and animals (Raboy, 2001). Furthermore, the phosphorus bound in phytate is not available to non-ruminant animals such as humans, swine, poultry, and fish, because they lack the endogenous phytase enzyme (Oatway et al., 2001). Phytate is often excreted which can lead to excessive phosphorus in the environment and thus can be considered a water and soil pollutant (Oatway et al., 2001). Because of its negative effects on the environment and nutrition, low phytate crops (including soybeans) are desirable.

A major drawback with low phytate crops, is reduced seedling emergence (Meis et al., 2003; Spear & Fehr, 2007; Yuan et al., 2007). A high emergence rate is considered to be of high economic importance for producers. Therefore, it is important to determine the causes for the low seedling emergence in low phytate plants, such as soybean.

Emergence, the process in which the seedling is breaking through the soil, is a complex trait that is not only influenced by genetics and the environment the seed is planted in, but also by the

environmental conditions the seeds were produced in (Maupin & Rainey, 2011). These conditions include soil moisture, temperature, soil salinity, pathogens and seed burial depth (Delouche, 1952; Wang & Shannon, 1999) .

Several low phytate lines that also have low seedling emergence exist (White & Broadley, 2005; Wilcox et al., 2000; Yuan et al., 2007). CX1834-1-6 (hereafter CX1834) is a low phytate soybean line that was developed at USDA/Purdue University (Wilcox et al., 2000). A low phytate line, M153-1-4, was obtained through chemical mutagenesis by Wilcox et al. (2000). CX1834 is an F3:5–derived line from a cross of M153-1-4 and normal phytate line Athow (Gao et al., 2007; Wilcox et al., 2000). The low phytate phenotype in CX1834 is caused by single base mutations in two multi drug resistance-associated proteins (*MRPs)* (Gillman et al., 2009; Saghai Maroof et al., 2009). The two mutations differ from each other but are both required for low phytate phenotype in CX1834 (Gao et al., 2007; Gillman et al., 2009; Saghai Maroof et al., 2009; Walker et al., 2006).

A low phytate phenotype can also be found in soybean lines with a mutation in the D-myo-inositol 3-phosphate synthase 1 gene (*MIPS1*), as observed in LR33 (Hitz et al., 2002) and V99-5089 (Maupin et al., 2011). These two independent lines have different mutations in the *MIPS1* gene, but do not only show reduced phytate levels but also a reduction in raffinose and stachyose and an increase in inorganic phosphorus and sucrose (Gao et al., 2008; Meis et al., 2003; Saghai Maroof & Buss, 2008; Sebastian et al., 2000).

Both the *MRP* mutant and the *MIPS* mutant lines show reduced seedling emergence (Gao et al., 2008; Maupin et al., 2011; Oltmans et al., 2005). Meis et al. (2003) studied the emergence rates of lines with the *mips* allele and found a lower emergence rate in low phytate lines than in normal phytate lines. However, the lower emergence of low phytate lines was significantly more pronounced if the seed was produced in subtropical environment and was thus heavily influenced by the environmental conditions during seed production (Meis et al., 2003). Maupin and Rainey (2011) used the extended cold germination test (ECGT) and field emergence trials to determine the emergence rates of CX1834 and V99-5089 derived lines produced in different environments. They determined that even though normal and low phytate soybean lines differed in their emergence rates, emergence of all lines is heavily influenced by the environment. In five of the 12 environments tested all lines, normal and low phytate lines, show unacceptably low emergence (Maupin & Rainey, 2011). Maupin and Rainey (2011) suggest that it is possible to develop low phytate soybean lines with normal emergence rates, as their study shows non-significant differences between low phytate soybean lines and control lines tested in certain environments. If seed from CX1834- and V99-5089-derived lines is produced in Mt. Holly, VA, the emergence rate of these seeds when planted in the Southeast and Mid-Atlantic regions is high (greater than 80%). If seeds of CX1834-derived lines are produced in Iowa though, the emergence rate of seeds replanted in Iowa is below 60% (Oltmans et al., 2005). The authors suggest that the higher soil temperatures during planting in Virginia have a positive influence on the emergence rate. Evaluating all the field emergence data of low phytate lines derived from CX1834 and V99-5089 and normal phytate lines, Maupin et al. (2011) suggest that planting environment (temperature and rainfall) has a greater influence on emergence than genotype and seed production environment (Maupin et al., 2011). This result is further underlined by their use

of the ECGT, where seeds are planted on trays, covered with soil and left in a cold room (10°C) for 21 days. The emergence rate is determined by how many seeds out of the 100 planted emerge (Trimble & Fehr, 2009). Maupin and Rainey (2011) show the emergence rates of all lines tested is significantly reduced in the ECGT, an indication that cold temperatures during planting negatively influences the emergence rate.

To the best of our knowledge, at present there have not been any studies comparing the emergence rates of near isogenic lines with the *MRP* mutations. In this study, we use four near isogenic soybean lines (NILs) that only differ in the presence or absence of either or both of the *MRP* mutations. Since these four genotypes differ only at two loci, any difference in phenotype should be because of these mutations in the *MRP* genes. Thus, using these soybean lines, we eliminate the genetic background variation. This study aims to observe the phytate and emergence levels over different environments and several years.

2.3 Materials and Methods

2.3.1 Genetic Material

Two low-phytate parental soybean lines CX1834 and V99-5089 (Gao et al., 2008) were crossed in 2002 and four near isogenic lines were developed in subsequent years. CX1834 is a low phytate soybean line (8.6 ± 0.4 mg g^{-1} (Gao et al., 2008)) due to two mutations in *MRP* genes which are located on chromosomes 19 and 3 (linkage groups L and N, respectively) (Saghai Maroof et al., 2009; Walker et al., 2006). V99-5089 shows a low phytate phenotype due to a mutation in the *MIPS1* gene (average phytate concentration of 9.9 ± 0.5 mg g^{-1} (Gao et al., 2008)), however this mutation was not retained in the progeny of this cross. From the cross of

CX1834 and V99-5089, an advanced generation recombinant inbred line (RIL) population was identified and a single plant, heterozygous for the mutation in both *MRP* genes was identified from the F8 generation. The plant was selfed and the four near isogenic lines were developed as: (I) *mrp/mrp*(3)/*mrp/mrp*(19) (homozygous for mutation in both *MRP* genes), (II) *mrp/mrp*(3)/*MRP/MRP*(19) (homozygous mutation for *MRP* gene on chromosome 3, and wildtype for *MRP* gene on chromosome 19), (III) *MRP/MRP*(3)/*mrp/mrp*(19) (wildtype for *MRP* gene on chromosome 3 and homozygous mutation for *MRP* gene on chromosome 19) and (IV) *MRP/MRP*(3)/*MRP/MRP*(19) (wildtype for both *MRP* genes). From here on, the homozygous and recessive mutations will be displayed as: (I) *mrp*(3)/*mrp*(19), (II) *mrp*(3)/*MRP*(19), (III) *MRP*(3)/*mrp*(19) and (IV) *MRP*(3)/*MRP*(19).

For each line, also referred to as class, five sublines with the same genotype were obtained. These four lines, their sublines as well as the parents CX1834 (genotype: *mrp*(3)/*mrp*(19)) and V99-5089 (genotype: *MRP*(3)/*MRP*(19)) and a control line V99-3337 (genotype: *MRP*(3)/*MRP*(19)) were used for all subsequent analyses, from here on referred to as seven classes.

2.3.2 Planting/Harvest

In 2010, F9 seed harvested in 2009 was planted at Kentland Farm, Blacksburg, Virginia and in Mt. Holly, Virginia in two replications each. Ten seeds of each class, the parents and the control were planted in each row. All five sublines of each class were planted once in Blacksburg (replication A). In replication B in Blacksburg and in both replications in Mt. Holly, only two of the five sublines were planted. All five sublines of each class were planted once in Blacksburg (Figure 2.1).

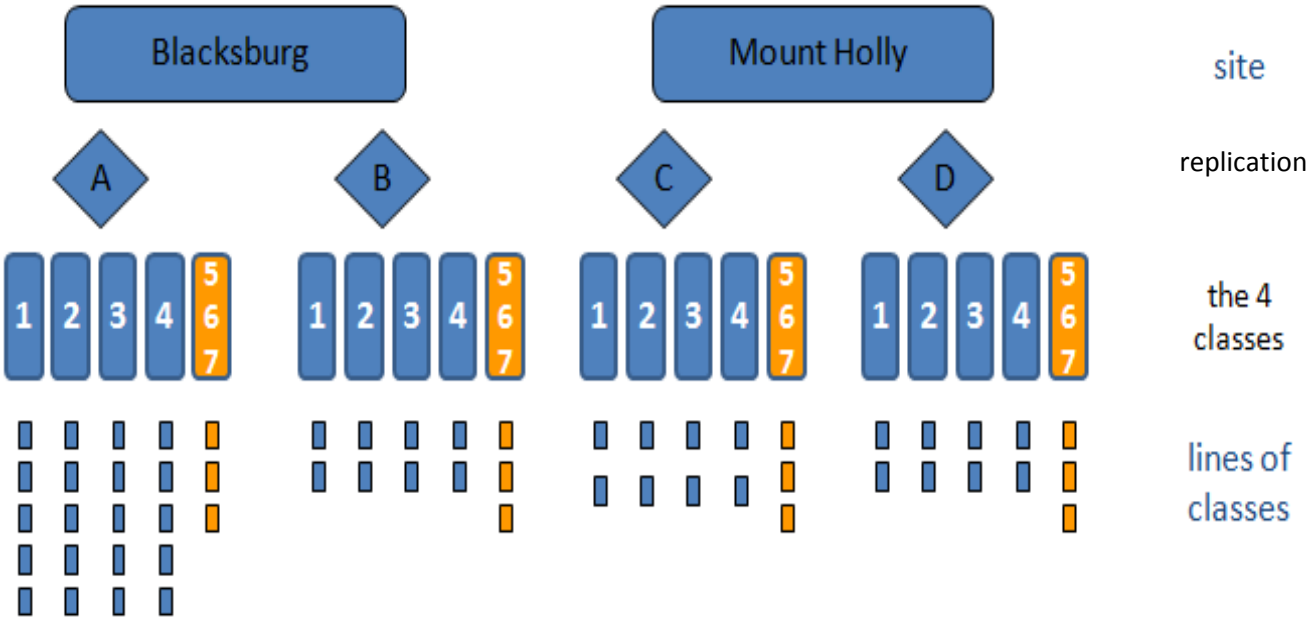

Figure 2.1. Planting layout for the 2010 field season. Numbers 1-7 are representing the different classes: 1 (*mrp(3)/mrp(19)*), 2 (*mrp(3)*/*MRP*(19)), 3 (*MRP*(3)/*mrp(19)*), 4 (*MRP*(3)/*MRP*(19)), 5 (parent CX1834), 6 (parent V99-5089), 7 (control V99-3337). The blue bars are representing the sublines, and the orange lines stand for classes 5, 6 and 7.

For the 2011 planting season, the whole 2010 experimental layout was repeated four times: in two locations in both geographical locations (Figure 2.2). F10 seed from Kentland Farm was planted at both Kentland Farm and Mt. Holly in 2011, and the F10 seed from Mt. Holly was planted at both Kentland Farm and Mt. Holly as well.

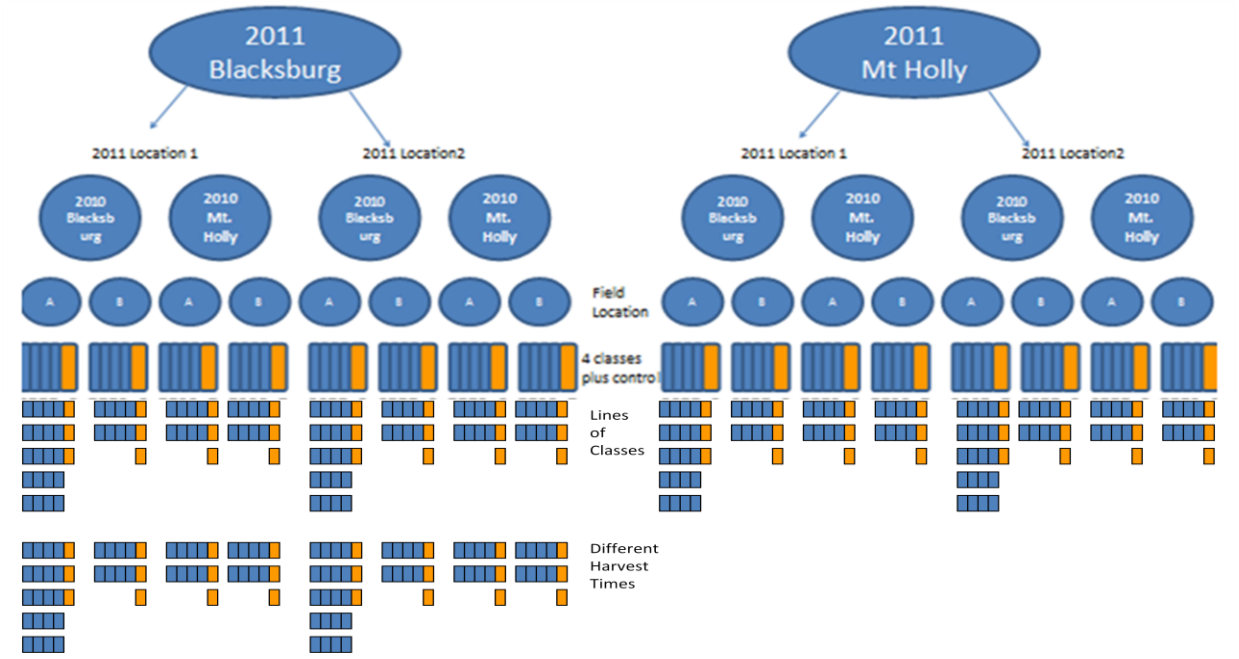

Figure 2.2. Planting layout for the 2011 field season. Blue bars are representing the different NIL classes: 1 (*mrp(3)/mrp(19)*), 2 (*mrp(3)*/*MRP*(19)), 3 (*MRP*(3)/*mrp(19)*), 4 (*MRP*(3)/*MRP*(19)), orange bars are representing the parents and the control: 5 (CX1834), 6 (V99-5089), 7 (V99-3337).

In 2012, all 2011 seeds that originated from 2010 Blacksburg replication A were planted out in two replicates in both geographical locations (Figure 2.3).

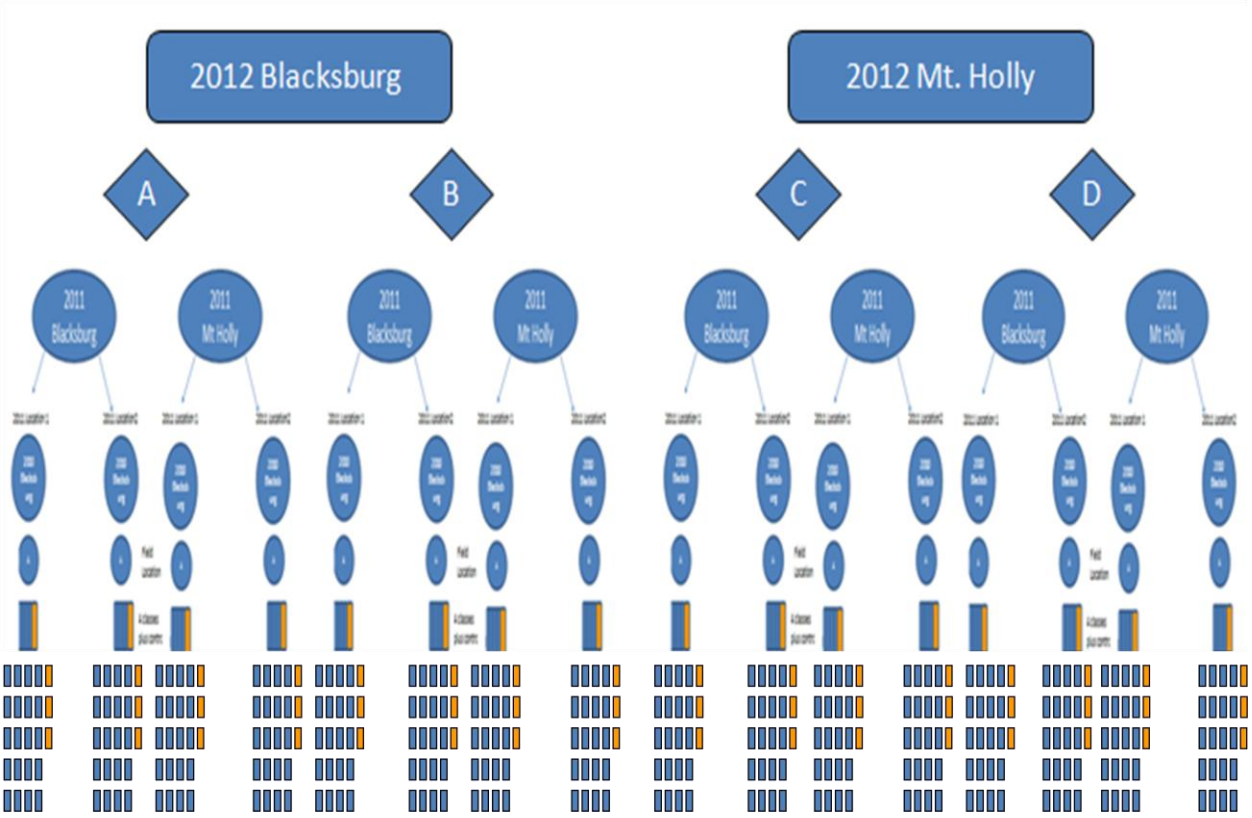

Figure 2.3. Planting layout for the 2012 field season. Blue bars are representing the different NIL classes: 1 (*mrp(3)/mrp(19)*), 2 (*mrp(3)*/*MRP*(19)), 3 (*MRP*(3)/*mrp(19)*), 4 (*MRP*(3)/*MRP*(19)), orange bars are representing the parents and the control: 5 (CX1834), 6 (V99-5089), 7 (V99-3337).

Seeds from each year were harvested and threshed in the field, dried in the drying chamber for 48 hours and stored in a cold room (10^{o} C, 15% humidity). Kentland Farm is approximately 280 miles west of Mt. Holly. The planting and harvest dates are summarized in Table 2.1.

Table 2.1. Planting and Harvest dates for all three field seasons.

	2010 Blacksburg	2010 Mt.Holly	2011 Blacksburg	2011 Mt.Holly	2012 Blacksburg	2012 Mt.Holly
Planting Date	5/28	6/11	6/6	6/30	6/2	6/8
Harvest Dates	9/6 -10/17	10/8 - 11/9	10/5 - 11/9	10/24 - 11/3	9/10 - 10/25	9/26 - 11/9

2.3.3 Confirmation of Genotype

Every year tissue was collected in bulk from each row of one replication in Blacksburg, VA and the genotype of the plants was determined. The CTAB method by Yu et al. (1994) was used to extract DNA from the freshly collected trifoliate leaves (Yu et al., 1994). The genotypes of the *MRP* mutations in CX1834 on linkage groups L and N and the *MIPS* mutation in V99-5089 on linkage group B1 were determined by SNP genotyping using the homogeneous, fluorescent Kbiosciences Competitive Allele-Specific PCR genotyping system (KASP) technology. PCR reaction was performed on 4 µl DNA (15 ng) mixed with 4 µl Reaction Mix (2x) and 0.11 µl Assay. The assay contains allele-specific primers with unlabeled tail sequences and a common reverse primer, two fluor-labeled oligos and two complementary quencher-labeled oligos. The reaction mix contains Taq polymerase enzyme and 5-carboxy-X-rhodamine (passive reference dye), succinimidyl ester, $MgCl_2$ and DMSO. After initial denaturation of 15 min at 94˚C , the PCR profile continues as follows 61˚C (60s); 1 cycle 94˚C (20s), 60.4˚C (60s) ; 1 cycle 94˚C (20s), 59.8˚C (60s); 1 cycle 94˚C (20s), 59.2˚C (60s), 1 cycle 94˚C (20s), 58.6˚C (60s), 1 cycle 94˚C (20s), 58˚C (60s), 1 cycle 94˚C (20s), 57.4˚C (60s), 1 cycle 94˚C (20s), 56.8˚C (60s), 1 cycle 94˚C (20s), 56.2˚C (60s), 1 cycle 94˚C (20s), 55.6˚C (60s), 35 cycles 94˚C (20s), 55˚C (60s).

2.3.4 Modified Colorimetric Method to Determine Phytate Content

The phytate concentrations of all lines from all locations and years were calculated. Approximately 50 seeds of each sample were ground with a Cyclone Sample Mill with a 0.5 mm mesh screen (UDY Corporation, Fort Collins, CO). Phytate concentration was determined according to the protocol by Burleson et al. (2012). In short, soybean powder (0.5 mg) from each

line was weighed into 14 mL tubes and 10 mL of 0.65 M HCl were added. The samples were vortexed, put on a shaker overnight (220 rpm at room temperature) and centrifuged at 4400 rpm at 10°C for 15 min. Then, 900 µL of the supernatant were transferred to a microcentrifuge tube and mixed with 900 µL of 20% NaCl solution. The crude extract was allowed to precipitate overnight at 4°C, and then centrifuged at 12,000 rpm for 15 min. Next, 100 µL of the supernatant were added to 400 µL of water and 1 mL of Ferric Iron (0.5 mM FeCl3•6H20 in 0.75 M HCl and 80% EtOH). The samples were incubated at room temperature for 2 hours and centrifuged at 12,000 rpm for 15 min. Then, 90 µL of the supernatant were added 270 µL of Color Reagent (1,10 phenanthroline, 0.08 mg/mL; hydroxylamine hydrochloride, 0.27%; ammonium acetate, 0.27 M) in a 96-well plate. The plate was read on a BioTek Synergy HT plate reader (BioTek Instruments, Winooski, VT) set at a wavelength of 510 nm. The phytate concentration of each sample was calculated from an average of three subsamples, using the supernatant from the ferric iron step three times. The phytate concentrations were calculated using a calibration curve consisting of eight standards (0, 1.12, 2.24, 3.36, 5.6, 7.84, 8.96, and 11.2 ppm phytate).

2.3.5 Emergence Data Collection

The emergence rate was determined at the Iowa State University's (ISU) Seed Testing Laboratory (Ames, IA) using the protocol for the extended cold test (ECT), also referred to as the extended cold germination test (ECGT) (Trimble & Fehr, 2009). Every year, the emergence rate of each line (row) was determined from the seeds field-grown in the both locations in Blacksburg and Mt. Holly. The seeds used for the ECT came from the same set of bulked seeds that were harvested from field and used for phytate analysis. Briefly, 100 seeds from each sample, coming

from a bulk of multiple plants of the same field row, were placed on fiberglass trays at ISU lab and covered with 2.54 cm of soil. The seedling emergence was calculated by counting the number of seedlings that emerged after being stored in a cold room at 10° for 21 days. In 2010 two replicates of 100 seeds each were sent for ECT, in 2011 and 2012 only one set of 100 seeds for each line.

Each year the harvested seeds were used to determine the ECT emergence rates, the phytate values and the field emergence rates. The seeds used for the field emergence test came from the same set of bulked seeds that were used for phytate analysis and the ECT. For example, seeds harvested during the 2010 field season, were ground for phytate analysis, sent to Iowa for ECT analysis and planted out in the 2011 field for field emergence rate determination. For that, the number of plants emerged from the soil in the field were counted (10 seeds were planted in each row). Counting took place on June 4th, 2010; July 4th, 2011; June 18th, 2012 in Blacksburg and June 29th, 2010; August 17th, 2011; August 14th, 2012 in Mt. Holly.

2.3.6 Statistical Analysis

SAS (version 9.3; SAS Institute Inc., Cary, NC) was used to determine the mean and standard error for the phytate values, field emergence rates and ECT emergence rates of all classes in each location and year. This data set was used to plot the histograms.

A statistical model was developed to determine statistically significant differences: generalized linear mixed model analysis to determine if there were statistically significant differences in phytate level and in percent emergence between the four lines and the controls (1) and between the growing locations (2). A binomial mixed model was used for emergence rate, and linear

mixed model was used for phytate evaluation. The model effects included year, location (Blacksburg or Mt. Holly), genotypic class and their interactions. GLIMMIX procedure in SAS was used with replications (A and B) in each location treated as random effect. These models were run in SAS and p-values of the simple effect comparison of location*class least squares means output was used to determine differences between the classes and the geographical locations (Table S2.1). A difference was considered to be significant if the p-value for that comparison is below 0.05.

The results from the model and the histogram did not completely agree. In the literature overlapping standard errors in histogram usually indicate a not statistically significant difference, whereas comparisons with non overlapping standard errors are seen as statistically different. We obtained the data (mean and standard error) for the histogram from SAS before applying the model. The model took the whole data, missing data points and experimental design into consideration and calculates the simple effect comparisons of location*class least squares means. The p-value generated by the model was used to indicate statistically significant differences between classes and growing locations. However, this p-value did not always reflect the status of the overlapping or not overlapping standard errors. We considered the results from the model (p-value) as correct, as this model best reflects the complex design and comparison of our experiment. The p values generated in the simple effect comparisons were used to determine significant differences and not the standard errors.

The locations*least squares means for phytate and emergence values for every year can be found in Table S2.2, the ANOVA tables in Table S3.3. Due to the low number of seeds planted in each row, the field emergence rate was not analyzed with the SAS model.

2.4 Results

2.4.1 2010 Results

2010 ECT Emergence

The ANOVA table (Table S2.2) for the 2010 ECT emergence rates showed that there was no significant location effect (p-value 0.1471), but a significant class (p-value <0.0001) and location*class (p-value 0.0224) effect.

The means and standard error for each class and location are displayed in a histogram in Figure 2.4. The emergence rates are low for all lines: from 1% for V99-5089 to 29.25% for V99-3337 Mt.Holly and 3.25% for CX1834 to 58.25% for V99-3337 in Blacksburg.

There is no statistically significant difference between the emergence rates of seeds harvested in Blacksburg and Mt. Holly in the classes *mrp(3)/mrp(19)*, CX1834 and V99-5989 (Table S2.1). For all other classes, the emergence rate was lower when the seeds were grown in Mt. Holly as opposed to Blacksburg.

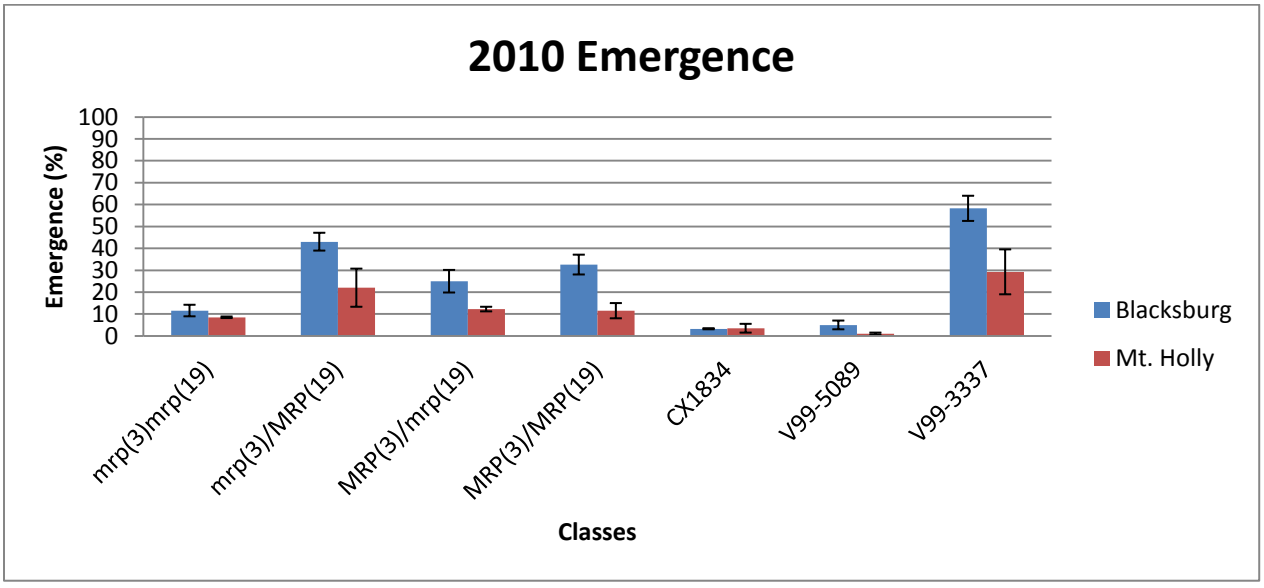

Figure 2.4. Emergence rates (%) for seeds grown in 2010.

In both locations, the parents showed the lowest emergence rate, followed by the class with both *MRP* mutations (*mrp(3)/mrp(19)*). In Blacksburg *mrp(3)/mrp(19)*'s emergence rate was significantly higher than parent CX1834, but does not differ from parent V99-5089 and was significantly lower from all other classes (Table S2.1). For the Mt. Holly grown seeds, the emergence rate of class *mrp(3)/mrp(19)* did not differ significantly from the parents or the classes *MRP*(3)/*mrp(19)* and *MRP*(3)/*MRP*(19) but is lower than *mrp(3)*/*MRP*(19) and the control V99-3337 (Table S2.1). The parents did not differ from each other in either location. In Blacksburg the three near isogenic lines and control line differed significantly from each other: V99-3337 has the highest emergence rate, followed by *mrp(3)*/*MRP*(19) and *MRP*(3)/*MRP*(19). Similar results were obtained from seeds harvested in Mt. Holly: V99-3337 has the highest emergence rate, followed by *mrp(3)*/*MRP*(19), then *MRP*(3)/*mrp(19)* and *MRP*(3)/*MRP*(19). All differences between these four classes were significant, except the difference between *MRP*(3)/*mrp(19)* and *MRP*(3)/*MRP*(19) (Table S2.1).

2010 Phytate

The ANOVA table for the 2010 phytate values showed that there was no significant location effect (p-value 0.9459) or location*class effect (p-value 0.4577). However, the class effect was significant (p-value: <0.0001) (Table S2.2).

The means and standard error for each class and location are displayed in Figure 2.5.

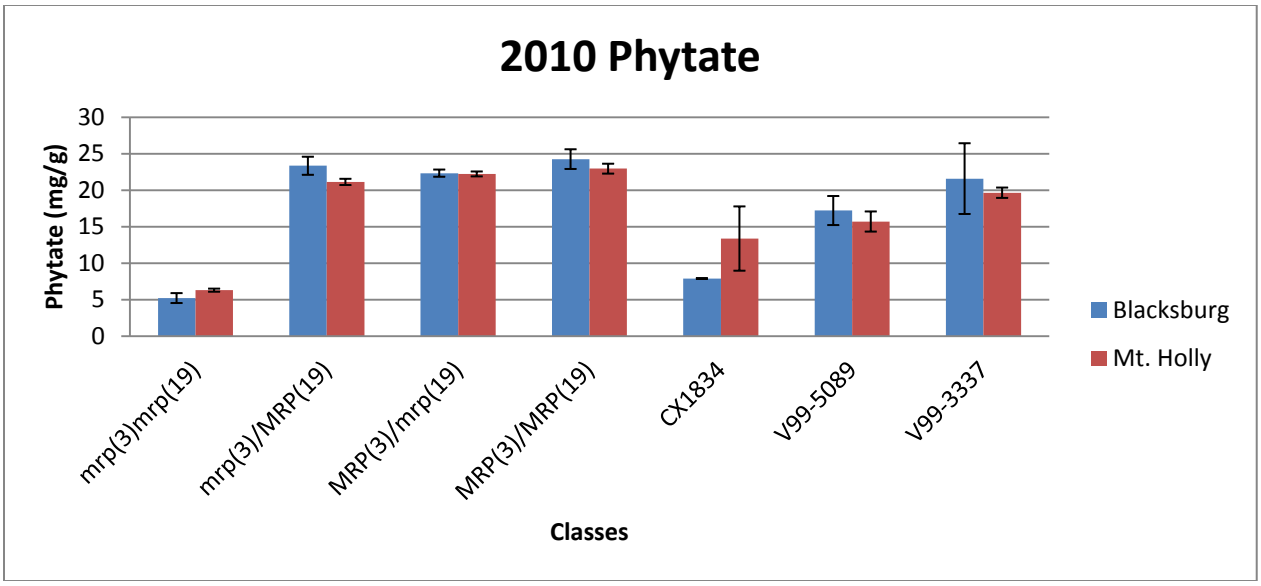

Figure 2.5. Phytate concentration (mg/g) for seeds grown in 2010.

CX1834 and V99-5089 were considered low phytate lines due to the mutations in both *MRP* genes and the *MIPS* gene, respectively. The near isogenic line mrp(3)/mrp(19) showed a low phytate phenotype (5.22 ± 0.68 mg/g). The three near isogenic lines, *mrp*(3)/*MRP*(19); *MRP*(3)/*mrp*(19) and *MRP*(3)/*MRP*(19), as well as V99-3337 showed a normal phytate phenotype with values of 20 mg/g and above (Table 2.3, Figure 2.5).

There is no statistically significant difference between the phytate concentration of seeds harvested in Blacksburg and Mt. Holly in any of the classes (Table S2.1).

In 2010 the near isogenic line with both *MRP* mutations (*mrp*(3)/*mrp*(19)) had a significantly lower phytate concentration than the three other near isogenic lines that have either one of the *MRP* mutations or none and from the parent V99-5089 and the control line V99-3337 in both Blacksburg and Mt. Holly. In Mt. Holly the phytate concentration for *mrp/mrp* was also significantly lower from CX1834. The parents CX1834 had a significantly lower phytate concentration from the near isogenic lines and V99-3337, yet not from parent V99-5089 in both

locations. The phytate concentration of parent V99-5089 was significantly higher than from class *mrp(3)/mrp(19),* but was not significantly lower from the other classes and V99-3337 (except from *MRP*(3)/*MRP*(19) class in Mt. Holly). The three near isogenic lines, *mrp(3)*/*MRP*(19), *MRP*(3)/*mrp(19)* and *MRP*(3)/*MRP*(19) and the control V99-3337 did not differ in that phytate concentration.

2010 seeds Field Emergence

The 2010 harvested seeds were planted out in field during the 2011 field season. The three low phytate lines, CX1834, V99-5089 and *mrp*(3)/*mrp*(19) had a field emergence rate of below 50% in both locations (except for *mrp*(3)/*mrp*(19) in Blacksburg (field emergence 58.75%)). NIL *mrp*(3)/*MRP*(19) planted in Blacksburg has the highest field emergence rate (73.18%) (Figure 2.6).

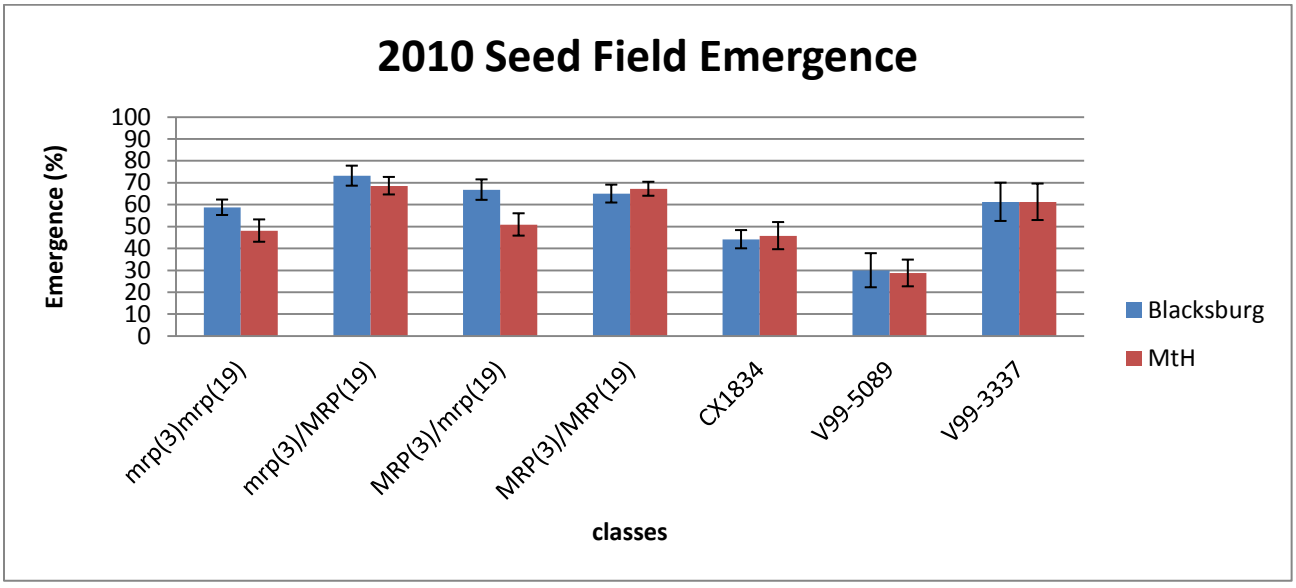

Figure 2.6. 2011 Field emergence rates (%) for seeds grown in 2010.

2.4.2 2011 Results

2011 ECT Emergence

The ANOVA table for the 2011 ECT emergence rates showed that the location effect (p-value 0.0474) as well as class (p-value <0.0001) and location*class (p-value <0.0001) effect were significant (Table S2.2).

The means and standard error for each class and location are displayed in Figure 2.7. The emergence rates were higher than the 2010 rates: from 11.58% for CX1834 to 63.14% for V99-3337 Mt. Holly and 37.88% for CX1834 to 81.06% for V99-3337 in Blacksburg.

Here the emergence rates of all classes were significantly lower when the seeds were grown in Mt. Holly as opposed to Blacksburg (Table S2.1).

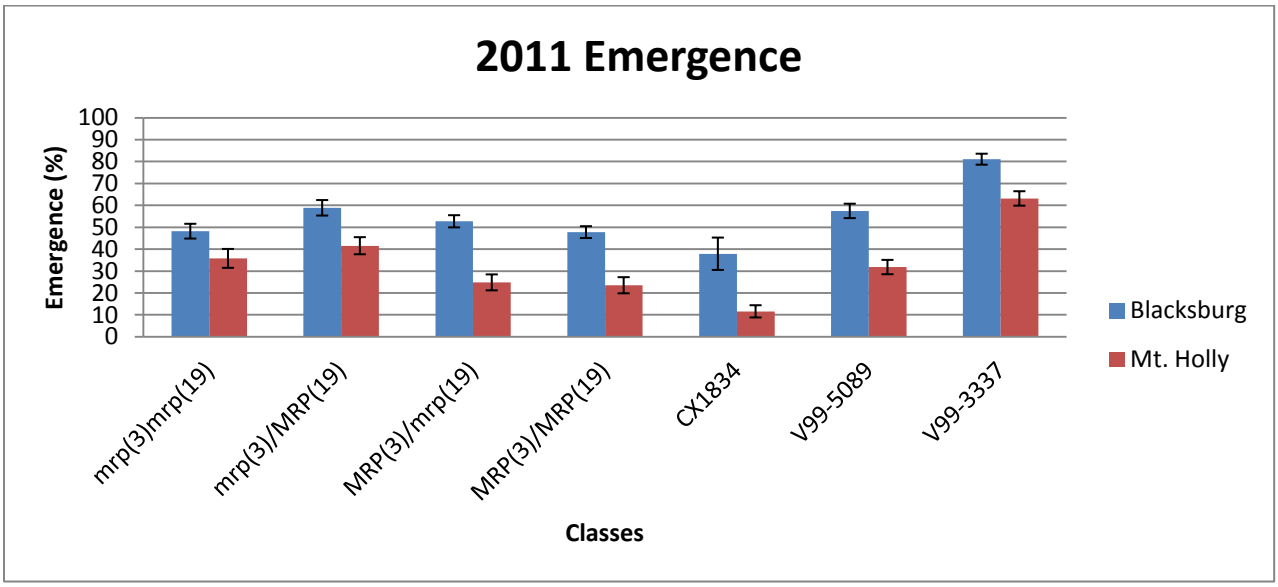

Figure 2.7. ECT emergence rates (%) for seeds grown in 2011.

CX1834 had the lowest emergence rate in both locations, followed by *mrp(3)/mrp(19)* in Blacksburg and *MRP*(3)/*MRP*(19) in Mt. Holly. For Blacksburg grown seeds, in order of highest

to lowest emergence rate: V99-3337, *mrp(3)*/*MRP*(19), V99-5089, *MRP*(3)/*mrp(19)*, *mrp(3)/mrp(19)*, *MRP*(3)/*MRP*(19) and CX1834, with only the values of *mrp(3)/mrp(19)* and *MRP*(3)/*MRP*(19) as well as *mrp(3)*/*MRP*(19) and V99-5089 being not statistically significant. In Mt. Holly the order was: V99-3337, *mrp(3)*/*MRP*(19), *mrp(3)/mrp(19)*, V99-5089, *MRP*(3)/*mrp(19)*, *MRP*(3)/*MRP*(19) and CX1834. Here all comparisons but *mrp(3)/mrp(19)* and V99-5089; and *MRP*(3)/*mrp(19)* and *MRP*(3)/*MRP*(19) were statistically significant.

2011 Phytate

The ANOVA table for the 2011 phytate values showed that there was no significant location effect (p-value 0.3184) or location*class (p-value 0.4353) effect. However, the class effect was significant (p-value <0.0001) (Table S2.2). The means and standard error for each class and location are displayed in Figure 2.8. The class with both mutations (*mrp*(3)/*mrp*(19)) as well as the parents had phytate values of 10mg/g and below. The three other NILs and the control lines phytate values were between 14.56 and 17.16 mg/g. There was no statistically significant difference between the phytate concentration of seeds harvested in Blacksburg and Mt. Holly in any of the classes (Table S2.1).

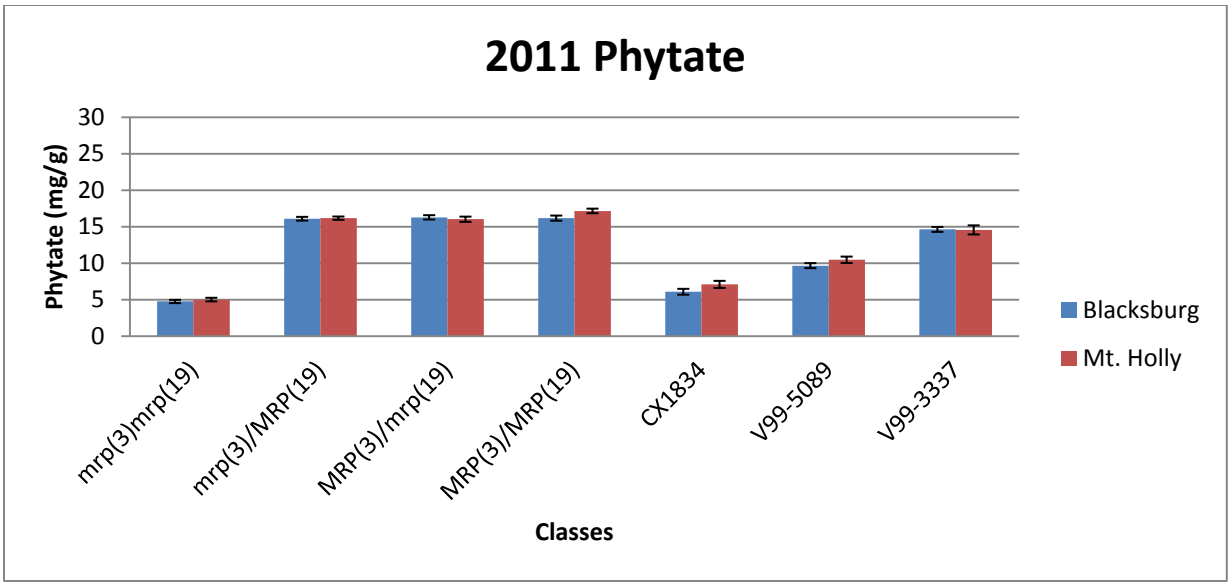

Figure 2.8. Phytate concentration (mg/g) for seeds grown in 2011.

In 2011 in both Blacksburg and Mt. Holly the phytate concentration of the class with both mutations (*mrp(3)/mrp(19)*) was significantly lower than all other classes, including parent CX1834. This is different from 2010. The parents CX1834 and V99-5089 had a significantly higher phytate concentration than *mrp(3)/mrp(19)* but significantly lower than V99-3337 and the three NILs *mrp(3)*/*MRP*(19), *MRP*(3)/*mrp(19)* and *MRP*(3)/*MRP*(19). None of these three high phytate lines differed significantly from each other in Blacksburg, but *mrp(3)*/*MRP*(19)'s phytate concentration was significantly lower than *MRP*(3)/*MRP(*19) in Mt. Holly. V99-3337 had a phytate concentration lower than the three NILS *mrp(3)*/*MRP*(19), *MRP*(3)/*mrp(19)* and *MRP*(3)/*MRP*(19), with the difference statistically significant for all comparisons except *mrp(3)*/*MRP*(19) in Blacksburg and Mt. Holly and *MRP*(3)/*mrp(19)* just in Mt. Holly.

2011 seeds Field Emergence

The 2011 harvested seeds were planted out in field during the 2012 field season. All classes had a much lower field emergence rate when planted out in Mt. Holly compared to seeds planted out in Blacksburg: from 6.25% for CX1834 to 25% for Mt. Holly and 33.75 for *MRP*(3)/*mrp*(19) to 63.75 (V99-3337), respectively. In Blacksburg the field emergence rates were lowest for the NIL with one mutation (*MRP*(3)/*mrp*(19)) and the NIL without mutation (*MRP*(3)/*MRP*(19). In Mt. Holly *MRP*(3)/*MRP*(19) had the lowest field emergence rate of the four NILs (Figure 2.9).

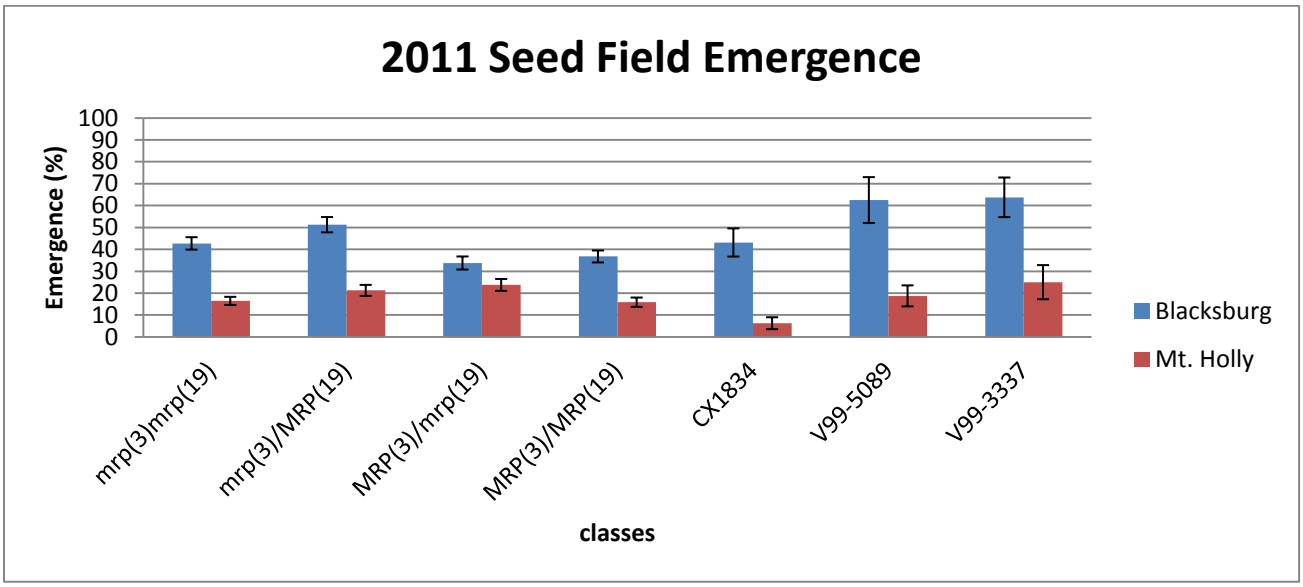

Figure 2.9. 2012 Field emergence rates (%) for seeds grown in 2011.

2.4.3 2012 Results

2012 ECT emergence

Due to problems with the ECT experimental setup and cold chamber temperatures, the 2012 ECT data had to be discarded.

2012 Phytate

The ANOVA table for the 2012 phytate values showed that only the class effect (p-value <0.0001) and not the location (p-value 0.823) or location*class (p-value 0.0781) effect was significant (Table S2.2). The means and standard error for each class and location are displayed in Figure 2.10.

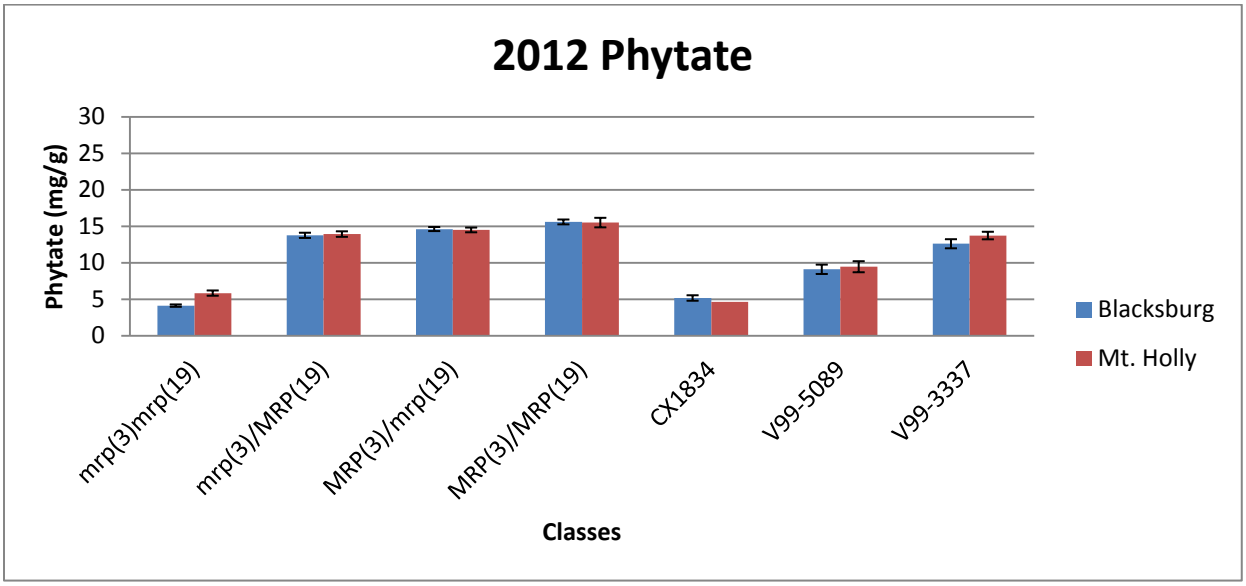

Figure 2.10. Phytate concentration (mg/g) for seeds grown in 2012.

The class with both mutations (*mrp*(3)/*mrp*(19)) as well as the parents had phytate values below 10 mg/g. The three other NILs and the control lines phytate values were between 12.6 and 15.59 mg/g. There was no statistically significant different phytate concentration between seeds grown in either location for all classes (Table S2.1).

In 2012 for the Blacksburg grown seeds, the double mutant NIL line, *mrp(3)/mrp(19)*, showed significantly lower phytate concentration than the all others classes but parent CX1834. CX1834's phytate value was significantly lower than all the other classes, except V99-5089. The

NIL lines mrp(3)/*MRP*(19) and *MRP*(3)/mrp(19) did not significantly differ from each other or the control line V99-3337. The line without *MRP* mutations, *MRP*(3)/*MRP*(19), had a phytate concentration significantly higher than all other classes, except in Mt. Holly were it did not different from V99-3337. The parents did not differ significantly from each other in either location.

2012 seeds Field Emergence

The 2012 harvested seeds were planted out in field during the 2013 field season in Blacksburg only. In Blacksburg the field emergence rates of the four NILs was between 12.27% (*mrp*(3)/*mrp*(19)) and 15.75% (*MRP*(3)/*mrp*(19)). The field emergence rates of the parents and the control line was between 20% (V99-5089) and 33.5% (V99-3337) (Figure 2.11).

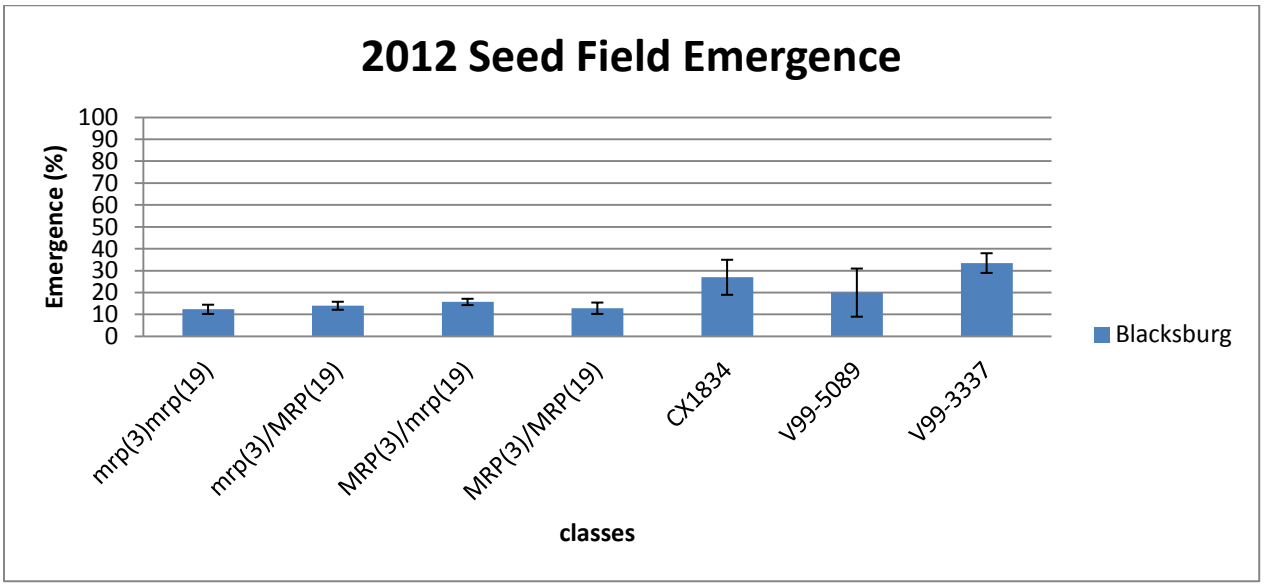

Figure 2.11. 2013 Field emergence rates (%) for seeds grown in 2012

2.5 Discussion

2.5.1 Phytate Values

Oltmans et al. (2004) show that the low phytate trait in CX1834 is controlled by two recessive genes. These two genes are mapped to two quantitative trait loci (QTL) on linkage group L (chromosome 19) and N (chromosome 3) (Walker et al., 2006). The responsible genes in these QTLs were identified by Maroof et al. (2009). A single nucleotide substitution (A to T) was discovered in a multi drug resistance-associated protein (*MRP*) on linkage group N (Glyma03g32500), changing an Arg residue to a stop codon (Saghai Maroof et al., 2009). A second single nucleotide substitution (G to A) was detected in another multi drug resistance-associated protein (*MRP*) on linkage group L (Glyma19g35230) (Glover, 2011).

Segregation studies of the progeny of reciprocal cross of CX1834 to a normal phytate line indicate that the low phytate trait in CX1834 is controlled by two recessive alleles that both have to be homozygous to obtain a low phytate phenotype (Oltmans et al., 2004). Walker et al. (2006) suggest that low phytate QTL on linkage group L in CX1834 was inherited from an ancestor of M153-1-4. The low phytate phenotype however did not appear in M153-1-4 until another mutation occurred or was crossed into the low phytate QTL on linkage group N (Walker et al., 2006). Gao et al. (2008) confirm Oltmans et al. (2004) suggestion that two homozygous alleles are needed for the low phytate phenotype. Gao et al. (2008) suggest that the low phytate QTL on linkage group L is linked to unknown high phytate genes, explaining the need for the two low phytate QTLs in CX1834 to be present for expression of a low phytate phenotype. A mutation in only one maize *lpa1* gene, a homolog to the soybeans MRP genes (Glyma03g32500 and Glyma03g35230) is responsible for the low phytate phenotype in maize (Gillman et al., 2009).

Our results showed that the phytate values were not influenced by the location the seeds were produced in, the location and the location*class effect was insignificant in all three years. However, the class effect was highly significantly, indicating a strong influence of the genotype on the phytate levels. Our results clearly demonstrated that only the NIL with both mutations (*mrp*(3)/*mrp*(19)) had a low phytate phenotype. This line had similar phytate levels as its parent CX1834. The lines with either one of the *MRP* mutations (*mrp*(3)/*MRP*(19) and *MRP*(3)/*mrp*(19)) showed phytate values similar to the line without mutation (*MRP*(3)/*MRP*(19)) and the control line V99-3337 over all three years. These four lines were considered to be normal phytate. These results are in agreement with the previous results stated above: both mutations are needed for a low phytate phenotype in CX1834.

2.5.2 Emergence Rates

Emergence is a dynamic trait which is not only influenced by the interaction between the environment and genotype during planting season but also during seed production of the previous generation. Emergence is difficult to study in the field as the slightest variation in moisture and soil condition can influence the emergence rate. Therefore, the emergence rates in this study were not only determined in the field but also by using the extended cold test, which imitates field conditions (Trimble & Fehr, 2009).

Climate

The Mt. Holly's climate is hotter with less precipitation than Blacksburg, which may cause reduced emergence of the seeds produced there. In 2010 and 2011 the temperature during seed initiation was higher in Mt. Holly than in Blacksburg (Figure 2.12). Overall the maximum

temperature in Mt. Holly was the highest in 2010 with temperatures above 100 degrees Fahrenheit in June and July. The maximum temperature also reached 100 degrees Fahrenheit in 2011 in Mt. Holly. Yet the temperature in Blacksburg did not vary much and was between 80 and 90° Fahrenheit in 2010 and 2011. There was zero precipitation (Table S2.3) during the field seasons of 2010 and 2011 in Mt. Holly, but a total of 1.86 inches of rainfall during the field season in 2010 and 4.6 inches in 2011 (Table S2.3). It seemed that precipitation together with lower temperatures during seed development positively influences seed emergence.

Anderson and Fehr (2008) report that especially the temperature during the seed filling stage, which is 30 d before physiological maturity, is an important influence on the emergence rate (Anderson & Fehr, 2008). It was previously shown that high temperatures during seed development and maturation is a form of stress that leads to a reduction in germination and seed vigor (Egli et al., 2005). Thus the location of seeds grown, depending on its temperature, rainfall and humidity, influences the emergence rate, with low phytate lines emergence rate being influenced more easily than the normal phytate lines (Anderson & Fehr, 2008).

Influence of Seed Source on ECT Emergence

We detected differences in ECT emergence rates due to genotypic and environmental effects in 2010 and 2011. The location by class affect was significant in both years, suggesting that the ECT emergence of some classes is influenced by the location the seeds were produced in.

In 2010, the ECT emergence rates of the seeds of the low phytate lines, *mrp*(3)/*mrp*(19), CX1834 and V99-5089, grown in Blacksburg were significantly higher than from the seeds of the same lines grown in Mt. Holly.

In 2011, the ECT emergence rates of all classes grown in Blacksburg were significantly higher than of the classes grown in Mt. Holly.

This suggested that the environmental conditions that were present during the seed filling and maturation stages in 2010 and 2011 in Mt. Holly were different from Blacksburg and had a negative impact on the next generation's emergence rate. This seed source effect was described previously in low phytate lines with mutations in the *MRP* genes, and in lines with low phytate phenotype due to other mutations like the *MIPS1* mutation (Anderson & Fehr, 2008; Meis et al., 2003). It was previously shown that seeds produced in hot and humid climate, like a subtropical winter nursery environment, have reduced emergence when planted out in the temperate climate (Anderson & Fehr, 2008; Meis et al., 2003). This is true for all genotypes tested but it is more pronounced for the low phytate lines (Maupin & Rainey, 2011). However, Maupin and Rainey (2011) also show that seeds produced in Mt. Holly have an emergence rate over 80% when planted out in the Mid-Atlantic region. It suggests again that low phytate lines are more strongly affected by environmental stressors than those with normal phytate content, but that the seed source affect strongly depends on the climatic conditions.

The climate during the 2010 and 2011 harvest seasons, as shown in Figure 2.12, had an effect on the location differences (Blacksburg- vs. Mt. Holly- grown seeds) and the year to year variance. Emergence rates were variable and easily influenced by the climate during seed maturation, harvest condition and time, storage length and temperature to name a few. We hand harvested the soybeans and processed by a thresher in both locations. However, different combines were used and the speed of threshing can potentially lead to visibly and invisibly cracked seed which reduces the emergence (Dr. Buss, personal communication).

Also, emergence can be negatively influenced if seeds are not harvested in a timely fashion and kept too long on the plant after seed maturation (quote from Dr. Buss, 2014). Due to the fact that Mt. Holly is a four hour drive from Blacksburg, we were not able to check field condition and seed maturation on a daily basis. It is possible that seeds were harvested after the plants already began shattering, which could explain the lower emergence rate of Mt. Holly grown seeds. It is also possible that due to the geographical differences, one field was more prone to infection by fungi or bacterial diseases.

Influence of Genotype on ECT Emergence

In all three years the class effect and thus the differences between the genotypes was significant.

It was previously shown in several crops that low phytate lines have low emergence (Raboy, 2001; Spear & Fehr, 2007; Zhao et al., 2008). Thus we expected the three low phytate lines, *mrp*(3*)/mrp*(19), CX1834 and V99-5089, to have a lower emergence rate than the normal phytate lines.

In Blacksburg 2010 the three low phytate lines also had statistically significant lower ECT emergence than the normal phytate lines, which did not differ much from each other in emergence. In Mt. Holly the low phytate lines also had a lower emergence rate than the normal phytate lines, but the difference was not always statistically significant (not if compared to *mrp(3)*/*MRP*(19) and *MRP*(3)/*MRP*(19)).

In 2011 the emergence rates did not differ significantly between all low and normal phytate lines.

In Blacksburg, the lowest emerging lines were the low phytate lines CX1834 and *mrp(3)/mrp(19)* and the normal phytate line *MRP*(3)/*MRP*(19). In Mt. Holly, the emergence rate of the low phytate line *mrp(3)/mrp(19)* was even significantly higher than the emergence rate of

the normal phytate line *MRP*(3)/*MRP*(19). We have shown here that emergence did not solely depend on genotype. In 2010 the ECT emergence rate for both the Blacksburg- and Mt. Holly-grown seeds was unacceptably low, even normally well emerging lines, like the control V99-3337 emerged poorly too, indicating an environmental problem affecting all lines. In 2011 the emergence rate was higher, but only one class reached an emergence rate of 80% or above, as is considered a must for breeders (Keith & Delouche, 1998). The highest emergence rate in 2010 was below 60% and the low phytate classes had emergence rates below 10%. In 2011, the emergence was slightly higher overall and was between 35% and 81%. It seemed that the environment during the 2010 seed producing stages was harsher than in 2011. In 2010 the emergence rate was low, the low phytate lines have a significantly lower emergence rate than normal phytate lines. It seemed that low phytate lines were not as resistant to environmental stressor as normal phytate lines are. The environmental condition during seed maturation can be found Figure 2.12 and Table S2.3.

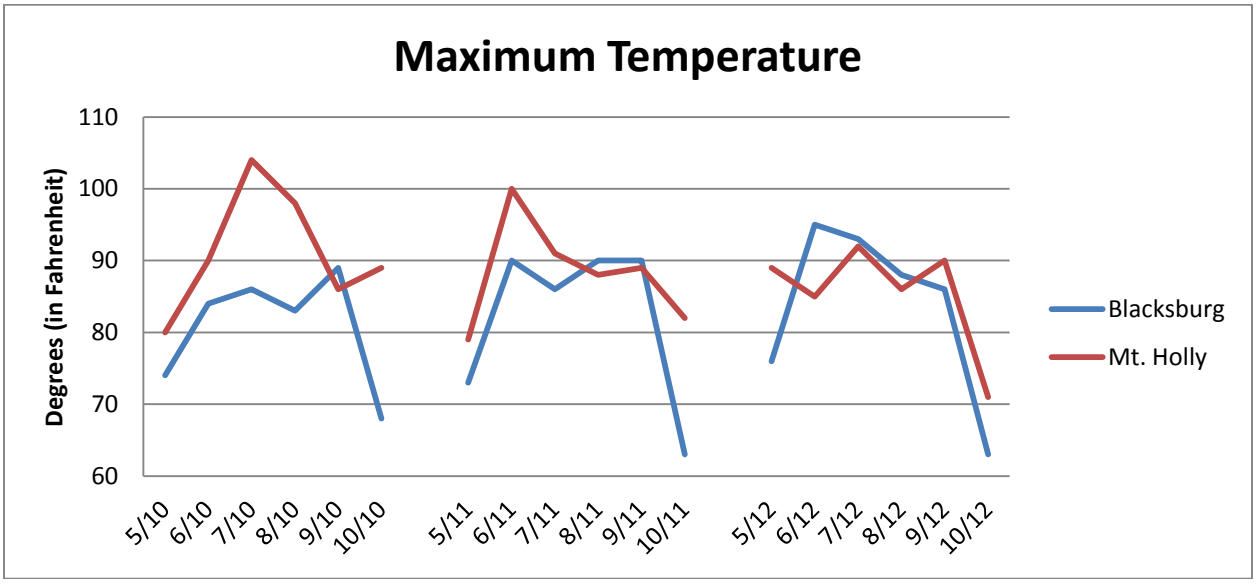

Figure 2.12. Maximum temperatures in Blacksburg and Mt. Holly during 2010, 2011 and 2012 field seasons.

Apart from the climate, the length of storage, as well as temperature and humidity during storage influence the hardness of the seed and could possibly lead to seed moldiness or dryness and thus also influences the emergence rate.

As previously mentioned, other studies show that it is possible for low and normal phytate lines to have a similar emergence rate: they can have either low or high emergence depending on the environment the seeds were grown in (Maupin et al., 2011). Previous experiments by Maupin and Rainey (2011) show that CX1834 derived seeds can have an emergence rate greater than 80% when seeds were produced and grown in a temperate climate. Maupin and Rainey (2011) suggest that the cold germination conditions of the ECT are a cause for the low emergence rates of the low phytic lines in the extended cold test and that if these seeds are grown in soil with higher temperatures the emergence might be influenced positively (Maupin & Rainey, 2011). Maupin and Rainey (2011) further suggest that the ECT is not the most suitable predictor of emergence for low phytate lines.

Influence of Field Location on Field Emergence

In 2010, we planted in Blacksburg and Mt. Holly and the seeds produced were used for the phytate analysis and the ECT emergence rate. These seeds were also planted out in the 2011 field. Seeds from all lines that originated in Blacksburg and Mt. Holly were planted out in Blacksburg and Mt. Holly the following year: 2010 Blacksburg harvested seeds were planted out in 2011 in Blacksburg and Mt. Holly, 2010 Mt. Holly harvested seeds were planted out in 2011 in Blacksburg and Mt. Holly. The 2010 Blacksburg and 2010 Mt. Holly seeds were combined in the analysis and the difference in the growing location of the next generation was analyzed.

For the 2010 produced seeds, the field emergence rates did not significantly depend on the location, except for the class *MRP*(3)/*mrp*(19), where the field emergence rate was higher in Blacksburg than in Mt. Holly.

For the 2011 produced seeds, the field emergence rates of all classes were higher in Blacksburg than in Mt. Holly. This suggested that the environmental conditions during planting or the soil characteristics in Blacksburg were more favorable than in Mt. Holly and that not only the seed source is of importance but also the conditions during planting. But this was true for all genotypic classes. In our results the low phytate phenotype was not more negatively influenced than the normal phytate lines.

The fields were prepared and managed by different people and in 2012 the field was a no till field in Mt. Holly. Crop residues from the previously planted cover crops remained on the field after soybean planting. This drastically reduced the emergence in Mt Holly. We also were not able to weed the Mt. Holly field as often as the Blacksburg field and a large number of weeds negatively influences field emergence as well.

Influence of Genotype on Field Emergence

The field emergence rates were determined for the seeds produced in 2010, 2011 and 2012. In 2010 and 2011, the seeds were planted in Blacksburg and Mt. Holly, in 2012 only in Blacksburg. There does not seem to be a correlation between genotype and field emergence. For the 2010 seeds planted in both locations the low phytate lines had a slightly lower emergence rate than the normal phytate lines. For the 2011 seeds, the low phytate lines in Blacksburg had a higher field emergence than *MRP*(3)/*mrp*(19) and *MRP*(3)/*MRP*(19). In Mt. Holly, *mrp*(3)/*mrp*(19) emerged better than *MRP*(3)/*MRP*(19). For the 2012 seeds planted in Blacksburg the low phytate lines

CX1834 and V99-5089 had the second and third highest emergence rate, respectively. There was no consistency in the results.

We only planted 10 seeds per row and thus only counted how many emerged out of the 10 seeds. This small number of seeds did not represent true field emergence and cannot be used to draw conclusions.

2.6 Conclusion

We have shown that both *MRP* mutations are needed to confer a low phytate phenotype which is in agreement with previous results.

Emergence is a trait that is still poorly understood and difficult to analyze. It is highly dynamic and strongly influenced by the environment seeds were produced in, the environment they emerge in and everything that happens in between. The results of this study showed that the choice of environment used for seed production is highly important, especially when breeding and commercially growing low phytate lines. The fact that we saw statistically significant emergence differences between the low phytate and normal phytate lines only during certain environmental conditions (year to year variation and location effect), lead us to believe that low phytate lines emerge well under optimal conditions but are often more negatively influenced by harsh environmental conditions than normal phytate lines are. The annual variation in climate and soil conditions makes it difficult to predict which environment in the US will perform best. This seed source effect suggested that the difference between low and normal phytate soybean lines lied in process of seed maturation and that this process was influenced by the climate. It was suggested that this seed source effect is due to premature aging of seeds grown in subtropical climate (Raboy, 2009). Ruby (2009) further suggest that premature aging is due to

reduced heat or desiccation tolerance which is more pronounced in low phytate lines and that phytate's role as a chelator of cations or anti-oxidant might be to blame. Breeders often make use of subtropical environments during the winter month when trying to speed up the breeding process. It is necessary to remember that when making selections in a segregating population low phytate lines are more susceptible to the environmental conditions than normal phytate lines. The frequency of low phytate lines in subsequent generations might decrease.

Additional research will be needed to determine the reasons for this seed source effect and how exactly the low phytic phenotype causes a reduction in emergence.

2.7 References

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Chapter 3

Metabolic Discrimination of Near Isogenic Low and High Phytate Soybean Seeds Reveals Differences in Soyasaponin Profiles

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3.1 Abstract

The seeds from four near isogenic soybean lines (NILs) that differ in their phytate content and emergence capabilities were subjected to untargeted metabolite profiling by liquid chromatography-mass spectrometry (LC-MS). The LC-MS analyses focused on lipid-free methanol-water extracts, with analyses relying upon orthogonal chromatographic separations (reversed-phase and hydrophilic interaction) and both positive and negative ionization modes. The low phytate phenotype is due to mutations in two multi-drug resistant proteins (*MRP*). This line was compared to three other lines having either one of the mutations or none. The double mutant exhibited significantly lower phytate values than the lines with either or none of the mutations. This low phytate line also showed a low emergence rate (below 50%), whereas the other three lines showed emergence rates above 80%. Principal component (PCA) and orthogonal projection of latent structures-discriminant analyses (OPLS-DA) of the LC-MS data allowed clear discrimination between these lines. The low phytate line separates clearly from the three normal phytate lines that group together. The major differences between these lines were in the soyasaponin group A composition. Group A soyasaponins containing C22 terminated acetylated glucosides were of lower concentration in the low phytate line, which favored soyasaponins terminated with an acetylated xyloside (soyasaponin A4, A5 and A6). The high phytate lines however, did not contain the C22 terminated acetylated xylosides. These lines had high concentration of soyasaponins A1, A2 and A3. Differences in isoflavonoids and lyso compounds concentration were also found.

3.2 Introduction

Soybean seeds, *Glycine max* (L.) Merr., are one of the most valuable crops in the world, with a worldwide economic contribution of approximately $48.6 billion (Wilson, 2008). The seeds are mainly used as a source of oil and protein (Chung et al., 2003), as a food product for human and animal consumption, and for industrial uses including biodiesel production. Over 70 million acres of soybeans were planted in 2012, making it second only to corn in total acreage sown (2012 USDA Acreage Report).

The seeds of soybeans as well as cereal grains and other legumes contain phytic acid (myo-inositol 1,2,3,4,5,6-hexakisphosphate), an acidic molecule that is generally found in its salt form. Phytic acid (or phytate) stores phosphorus, *myo*-inositol and cations in the seeds (Raboy, 2007). Phytate can chelate cations such as Ca^{+2}, Mg^{+2}, Zn^{+2}, and Fe^{+2}/Fe^{+3} and when present in high concentrations, it can limit the bioavailability of these cations to either humans or livestock (Raboy, 2009). Furthermore, monogastric animals such as swine and poultry do not possess the enzymatic activity required (phytase) to hydrolyze the alkyl phosphate bond, thus the phosphate in phytate is unavailable and excreted (Morse et al., 1992) increasing the phosphate load to receiving bodies of both land and water (Haygarth et al., 1998). While supplementation of diets with phosphate or phytase are options, they are relatively expensive (Golovan et al., 2001; Saghai Maroof et al., 2009; Walker et al., 2006).

The detrimental effects of high seed phytate levels in end use products has led to efforts to engineer low phytate crops (Hitz et al., 2002; Wilcox et al., 2000). 'LR33', a low phytate soybean line that was produced by chemical mutagenesis (Agrama et al., 2007; Sebastian et al.,

2000) contains a mutation in the gene encoding *myo*-inositol 3-phosphate synthase (*MIPS1*), essentially restricting flow through the phytic acid biosynthetic pathway. This mutation is reported to lead to 36% reduction in seed phytate levels (Hitz et al., 2002). 'V99-5089' is another low phytate soybean line that also contains a mutation in the *MIPS1* gene (Maupin et al., 2011). For both lines the phytate content is reduced and the inorganic phosphorus content is increased (Saghai Maroof et al., 2009). A third low phyate line, CX1834 was developed at USDA/Purdue University (Gillman et al., 2009; Saghai Maroof et al., 2009), and is the result of single base mutations in two multidrug resistance-associated proteins (*MRP*s) (Saghai Maroof et al., 2009). These genes are located on two separate chromosomes and encode proteins that are presently annotated as ATP-binding cassette, sub-family C, member 5 proteins (UniProt IDs: K7MYS3, I1JP84; 95% identical). This class of conserved transmembrane proteins couple ATP hydrolysis to metabolite movement across a membrane, with the closest ortholog in Arabidopsis (AtABCC5) associated with germination and root development, including the transition from primary to lateral root formation (Gaedeke et al., 2001) and drought resistance in Arabidopsis (Suh et al., 2007). While several lines of low phytate soybean seeds are available, low phytate crops typically exhibit reduced seedling emergence, resulting in lower yields per acre planted (Yuan et al., 2007). The relationship between low emergence and phytate levels has not been determined.

The advances in liquid chromatography separation technologies, improvements in mass spectrometry scan speeds and mass accuracy, along with bioinformatics have permitted the analysis of a vast array of plant isolates, allowing new insights into plant growth and development processes (Haug et al., 2013). The most complete analyses of soybean seed

metabolites involve the analysis of two related salt-sensitive and salt-tolerant soybean lines. Using high-performance liquid chromatography mass spectrometry (HPLC-MS) analysis methods for metabolic profiling, the authors show that the method can be used to discriminate between two related soybean genotypes (Wu et al., 2008). Their profiling efforts identified 1600 ions, with data processing for adduct formation reducing the number of ions to about 200 compounds. Principal component analysis shows a clear distinction between the soybean lines with genistin and Group B saponins being correlated with salt tolerance (Wu et al., 2008). Sawada and Harai (2013) describe the collection of over 40,000 MS/MS spectra from soybean seed (93 recombinant inbred lines), which were used to link with QTL data (Sawada & Hirai, 2013).

The purpose of the investigation reported here was to test the hypothesis that low phytate soybean seeds have different metabolic profiles relative to normal phytate lines. Our analyses centered on four near isogenic lines (NILs) differing only in the *MRP* genes. These lines are nearly genetically identical except for the presence of neither, either, or both of the mutant genes. Only the latter line, which contains both mutations, is low phytate. As untargeted metabolomics is sensitive to both environmental conditions and genetic differences, the near isogenic lines provide a unique opportunity to eliminate genetic background and only focus on differences caused by the mutations in the *MRP* genes. Use of orthogonal separation methods and both positive and negative ion mode data for each separation provided a rich dataset to assess the roles of the *MRP* genes in generating the low phytate phenotype.

3.3 Materials and Methods

3.3.1 Plant Material

For this study four near isogenic soybean lines were developed by crossing the low-phytate parental soybean line CX1834-1-6 (hereafter CX1834) with V99-5089. CX1834 shows a low phytate phenotype (8.6 ± 0.4 mg g^{-1} (Gao et al., 2008)) due to two mutations in *MRP* genes which are located on chromosome 19 (linkage group L, Glyma19g35230) and 3 (linkage group N, Glyma03g32500) (Saghai Maroof et al., 2009). A single plant was selected from an advanced generation recombinant inbreed line (RIL) population of this cross. This plant was heterozygous for the mutations in the two *MRP* genes. It was selfed and the four near isogenic lines were developed: (I) *mrp/mrp*(3)/*mrp/mrp*(19) (homozygous for mutation in both *MRP* genes on chromosome 3 and 19), (II) *mrp/mrp*(3)/*MRP/MRP*(19) (homozygous mutation for *MRP* gene on chromosome 3, and wildtype for *MRP* gene on chromosome 19), (III) *MRP/MRP*(3)/*mrp/mrp*(19) (wildtype for *MRP* gene on chromosome 3 and homozygous mutation for *MRP* gene on chromosome 19) and (IV) *MRP/MRP*(3)/*MRP/MRP*(19) (wildtype for both *MRP* genes). From here on, even though the mutations are homozygous and recessive, only one allele will be displayed: (I) *mrp*(3)/*mrp*(19), (II) *mrp*(3)/*MRP*(19), (III) *MRP*(3)/*mrp*(19) and (IV) *MRP*(3)/*MRP*(19).

3.3.2 Plant Harvest

Plants were grown in Blacksburg, Virginia in 2012. For each class, ten seeds from the 2011 harvest were hand planted in one row. Fully matured seeds were harvested from all plants of each row and bulked together. Seeds were stored at 4 ºC and then used for metabolomic analysis and determination of phytate values and emergence rates. In 2010 and 2011 the progenitor seeds

and in 2013 the progeny seeds were planted out in Blacksburg, Virginia as well. Phytate concentration and emergence rates were determined for these as well.

3.3.3 Confirmation of *MRP* Genotypes of Four NILs

DNA was extracted from trifoliate leaf tissue. The CTAB method as described by Yu et al. (1994) was used for DNA extraction (Yu et al., 1994). To determine the genotype of both *MRP* genes homogeneous, fluorescent Kbiosciences Competitive Allele-Specific PCR (KASP) genotyping system technology was used. Extracted DNA (4 µl, 15ng) was mixed with Reaction Mix (4µl, 2x) and an Assay solution (0.11 µl) that contained two allele-specific primers (one for each SNP allele) with an unlabelled tail sequence, one common reverse primer, two fluorescently-labeled oligos and two complimentary quencher-labeled oligos. The reaction mix contained Taq polymerase enzyme and 5-carboxy-X-rhodamine (passive reference dye), succinimidyl ester, $MgCl_2$ and DMSO. A PCR step- down profile specific for amplification of *MRP* and *MIPS1* SNPs was being used: 1 cycle 94˚C (15min), 61˚C (60s); 1 cycle 94˚C (20s), 60.4˚C (60s); 1 cycle 94˚C (20s), 59.8˚C (60s); 1 cycle 94˚C (20s), 59.2˚C (60s), 1 cycle 94˚C (20s), 58.6˚C (60s), 1 cycle 94˚C (20s), 58˚C (60s), 1 cycle 94˚C (20s), 57.4˚C (60s), 1 cycle 94˚C (20s), 56.8˚C (60s), 1 cycle 94˚C (20s), 56.2˚C (60s), 1 cycle 94˚C (20s), 55.6˚C (60s), 35 cycles 94˚C (20s), 55˚C (60s).

3.3.4 Modified Colorimetric Method to Determine Phytate Content

Phytate concentrations of seeds harvested between 2010 and 2013 were determined (parental seeds are from 2010 and 2011 and progeny seeds are from 2013). Approximately 75 seeds of each of the four classes, the parents and the control line were ground with a Cyclone Sample Mill

with a 0.5 mm mesh screen (UDY Corporation, Fort Collins, CO). Phytate concentration was determined according to the protocol by Burleson et al. (2012) in triplicate and reported in mg/g dry weight of seed (Burleson et al., 2012).

3.3.5 Emergence Data Collection

Emergence rates were determined in a laboratory setting at the Iowa State University's (ISU) Seed Testing Laboratory (Ames, IA) and in the field (Blacksburg, VA). Laboratory assessment was performed utilizing the extended cold test protocol (Trimble & Fehr, 2009) where 100 seeds of each class were placed on a tray, covered with 2.54 cm of soil and stored in a cold room at 10°C for 21 days. Seedling emergence was calculated by counting the number of seedlings out of the 100 that had emerged after 21 days. Field emergence rates were determined by the number of plants that emerged from the soil in the field vs. the number of 2012 seeds planted (10 seeds per row) and reported as a percentage.

3.3.6 Non-targeted Metabolic Profiling Analysis Using LC-MS - Sample Preparation

Solvents used were LC-MS quality (Spectrum Chemicals) with the exception of ethyl acetate (Fisher Scientific), which was HPLC-grade and dried with anhydrous $MgSO_4$ powder before use. LC-MS grade organic acids (formic and acetic) were from Sigma-Aldrich.

Only the four NILs were analyzed in biological triplicates. Seed samples (5 seeds for each triplicate and class) were randomly selected, flash-frozen in liquid nitrogen and finely ground with P14 mill (Pulverisette 14, Fritsch). The powder was transferred to pre-weighed 15 mL tubes, quickly weighed and stored in the -80 °C freezer. A subset (400 mg) of this powder was

dried overnight on a high-vacuum line. The non-polar components were extracted using 7 mL of dry ethyl acetate. The extraction procedure was repeated twice with a sequence of vortexing, sonication for 20 min and centrifugation (4000 rpm for 15 min). The supernatants were combined and concentrated to oils and stored in -80 °C. The remaining ethyl acetate was removed from the soybean powder, which was then dried on the high-vacuum line and stored at -80 °C. To analyze the polar metabolites, two 30 mg subsets of the seed powders were extracted with methanol:0.1% aqueous acetic acid (0.5 ml; 9:1, v/v). One subset of each replicate of each class was used to extract metabolites for analysis by hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-MS) and the other for the reversed-phase chromatography-mass spectrometry (RPC-MS). Internal standards were added to both extracts, with the HILIC extracts employing ^{13}C-labeled L-arginine ($^{13}C_6$, Thermo Scientific, 0.09 µg/µl final concentration before extraction) and the RPC extracts employing deuterated L-tryptophan (indole-d_5, Cambridge Isotope Labs, 0.05 µg/µl final concentration before extraction). After vortexing and sonication for 20 min, the samples were centrifuged at 4000 rpm for 15 min. This extraction was repeated twice and the extracts were pooled. The extracts were reduced in volume initially with a centrifugal concentrator under vacuum (35 °C) and subsequently taken to dryness on the high-vacuum line. The dried samples were weighed and used for HPLC-MS profiling on the HILIC and RPC columns.

3.3.7 Liquid Chromatography-Mass Spectrometry Analysis.

Analyses were performed with a UPLC-ESI-Q-TOF/MS (Waters Acquity I-class UPLC with Waters Synapt G2-S HDMS) in both positive and negative ion modes. Three analytical replicates of each sample and master mixes were injected randomly for each column and ionization mode.

After reconstitution (see below), a master mix for each class was created by combining aliquots (10 µl each) of the three biological replicates. A master mix of all classes combined was created as well by combining aliquots (10 µl each) of the four class master mixes.

Reversed-Phase Separations. Dried extracts were reconstituted with 0.1% aqueous formic acid : acetonitrile (9:1, v/v, 120 µL). Samples were briefly vortexed and sonicated for 10 min, followed by centrifugation (13k x g, 10 min, RT). An aliquot (10 µl) was transferred to an LC-MS-grade vial and diluted with 90 µl of 0.1% aqueous formic acid : acetonitrile (9:1, v/v). Sample separation was achieved with a binary solvent system of 0.1% formic acid (A) and acetonitrile (B) utilizing an ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 mm x 50 mm, Waters Corp., Milford, MA) with flow rate 200 µL/min and a 15 minute gradient. The following gradient conditions were used: isocratic at 5% B (0-1 min), followed by linear gradient to 15% B (1-2 min), to 95% B (2-11 min), isocratic at 95% B (11-12.5 min), followed by return to initial conditions (12.5-15 min). Injection volume into the column was 2 µL. The separated samples were ionized by electrospray ionization and analyzed in both positive and negative modes. The scan time was set to 0.20 sec and a mass range of 50-1800 m/z was scanned. The source parameters for positive ion mode were source temperature 125 °C, capillary voltage 3.0, cone voltage 70, source offset 80, desolvation temperature 300 °C, cone gas 50 L/h, desolvation gas 500 L/h and nebulizer gas 6.0 bar. The source parameters for negative ion mode were source temperature 125 °C, capillary voltage 2.4, cone voltage 40, source offset 80, desolvation temperature 300 °C, cone gas 50 L/h, desolvation gas 500 L/h and nebulizer gas 6.0 bar. A reference sprayer continuously infused leucine-enkephalin (200 ng/ml, Waters Corp., Milford, MA) at 5 µl/min with a scan frequency of 20 seconds.

HILIC Separations. Dried extracts were reconstituted with 0.1% aqueous formic acid : acetonitrile (1:1, v/v, 100 µL). Samples were briefly vortexed and sonicated for 10 minutes, followed by centrifugation (13k x g, 10 min, RT). An aliquot (10 µl) was transferred to an Agilent screwcap vial and diluted with 90 µl of 0.1% aqueous formic acid:acetonitrile + 0.1% (1:1, v/v). Chromatography was performed on an ACQUITY UPLC BEH Amide Column (1.7 µm, 2.1 mm x 50 mm, Waters Corp., Milford, MA) with flow rate 200 µL/min and a 10 minute gradient prepared from mobile phase A (0.1% aq. formic acid) and mobile phase B (acetonitrile). The following gradient conditions were used: isocratic at 99% B (0-50 sec), followed by linear gradient to 30% B (0.5-7 min), to 99% B (7-7.10 min), isocratic at 99% B (7.10-10 min), followed by returning to the initial conditions. Injection volume into the column was 2 µL. The separated samples were ionized by electrospray ionization in both positive and negative modes. The scan time was set to 0.20 sec and a mass range of 50-1800 m/z was scanned. The source parameters for positive ion mode were source temperature 120 °C, capillary voltage 3.0, cone voltage 30, source offset 80, desolvation temperature 500 °C, cone gas 50 L/h, desolvation gas 600 L/h and nebulizer gas 6.0 bar. The source parameters for negative ion mode were source temperature 120 °C, capillary voltage 2.2, cone voltage 30, source offset 80, desolvation temperature 500 °C, cone gas 50 L/h, desolvation gas 600 L/h and nebulizer gas 6.0 bar. A reference sprayer continuously infused leucine-enkephalin (200 ng/ml, Waters Corp., Milford, MA) at 5 µl/min with a scan frequency of 20 sec.

3.3.8 Data Processing and Analysis.

The MarkerLynx application manager software (version 4.1, Waters Corp., Milford, MA, USA) was used to process the raw UPLC/Q-TOF-MS data. The MarkerLynx parameters for the

analyses of the RPC runs were: retention time range 2.0 – 9.0 min, mass 50 – 2000 m/z, mass window 0.05, retention time window of 0.15, noise elimination of level 4, peak intensity threshold of 10000, marker intensity threshold 2400 (positive ion mode) and 4800 (negative ion mode). The MarkerLynx parameters for the analyses of the HILIC runs were: retention time range 2.0 – 13.0 min, mass 50 – 2500 m/z, mass window 0.02, retention time window of 0.15, noise elimination of level 10, peak intensity threshold of 1000, marker intensity threshold 2400 (positive ion mode) and 5000 (negative ion mode). MarkerLynx generated a data matrix consisting of all exact match-retention time pairs (EMRTs) found in the datasets alongs with their normalized peak areas. The EZinfo 2.0 software (Umetrics, Umea, Sweden) was used used to conduct principal component analyses (PCA) and orthogonal partial least squared discriminate analyses (OPLS-DA) of the datasets. The latter was visualized using a score plot. The datasets was converted to spreadsheets using Excel (Microsoft Office, 2007) and the p-value (t-test) and factor of change (peak area ratios) was determined.

Peaks were identified with the help of commercial standards, available on-line databases such as Metlin (metlin.scripps.edu) and PRIMe/MS2T (Platform for RIKEN Metabolomics, http://prime.psc.riken.jp/), from the literature, and/or manual interpretation of MS-MS fragmentation patterns. Gradient and source parameters for MS/MS runs were identical to their MS runs, except the collision energy was applied in trap and ramped from 5 to 40 eV.

3.4 RESULTS

Phytate concentrations and emergence rates of seeds from the same plants were determined, as well as of the parental and progeny seeds in the years 2010, 2011 and 2013. These four unique

lines, summarized in Table 1, gave insight to metabolomic changes as well as changes in phytate concentration and emergence rates caused by mutations in the *MRP* genes.

Table 3.1. Near Isogenic Lines (NILs) Investigated.

NIL	*mrp*(3)/*mrp*(19)	*mrp*(3)/*MRP*(19)	*MRP*(3)/*mrp*(19)	*MRP*(3)/*MRP*(19)
MRP Chr 3 mutation	yes	yes	no	no
MRP Chr 19 mutation	yes	no	yes	no
Low Phytate	yes	no	no	no

3.4.1 Determination of Phytate Concentration and Seed Emergence Rates.

The mean phytate content and laboratory-based emergence rates of seeds harvested in 2012 are shown in Figure 3.1. The phytate levels were within the normal ranges reported for low and high phytate lines (Gao et al., 2007). The phytate values for the parents of this cross were 7.62 mg/g (V99-5089) and 4.78 mg/g (CX1834); thereby both parents are considered to be low phytate lines. The class with both mutations, *mrp*(3)/*mrp*(19), exhibited the lowest phytate concentration (3.56 ± 1.22 mg/g) whereas the other three classes showed a normal phytate phenotype. Classes *mrp*(3)/*MRP*(19) (14.17 mg/g ± 0.85), *MRP*(3)/*mrp*(19) (16.49 mg/g ± 0.53) and *MRP*(3)/*MRP*(19) (14.48 mg/g ± 0.64) were not statistically different from each other (p-value > 0.02) (Figure 3.1).

The laboratory- based extended cold test (ECT) emergence rates of the 2012 seeds ranged from 47% for the double mutant to greater than 95% for the wild-type NIL (Figure 3.1). Both mutations are required to obtain low emergence, one mutation does not lead to intermediate emergence. Interestingly the field-based emergence test of the 2012 seeds showed little difference in emergence rates (Appendix B, Figure S3.1). The class with both mutations

(*mrp*(3)/*mrp*(19)) exhibited a field emergence rate of 87.5% whereas the other three classes show a field emergence rates of greater than 95%. The phytate values, ECT emergence and field emergence rates of the four classes and their parents from 2009-2013 can be found in the Supplemental Materials (Appendix B, Table S3.1 and Figure S3.2). The * denotes the four lines that were used for the metabolomic analysis.

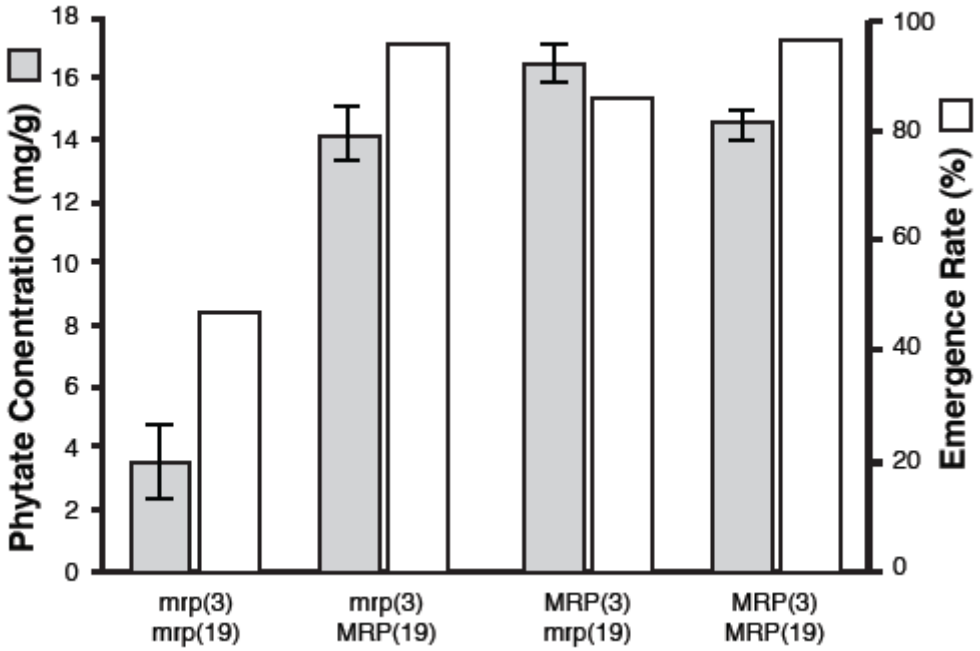

Figure 3.1. Mean seed phytate levels and emergence rates in the four NILs.

3.4.2 Metabolomic Profling.

An UPLC-ESI-MS method was developed for the detection of Methanol- soluble metabolites using freeze-dried soybean powder. Our goal was to analyze for polar and semi-polar compounds, we first removed the lipid component by extraction with ethyl acetate, with the

resulting powder subsequently extracted with MeOH:H_2O (9:1). The extracts were analyzed by two orthogonal chromatographic methods using short gradient separations to minimize LC-MS time. Triplicate random injections for all formats resulted in a total of 204 (51 per column, per ion mode) individual LC-MS runs. The datasets were then submitted to analysis with MarkerLynx, a part of the MassLynxTM Software package, in order to convert each data point into an exact mass retention time pair (EMRT). The number of EMRTs detected depended on the peak detection threshold (see Materials and Methods), which were set in a stringent manner to minimize the number of ions for downstream analysis. A total of 154 EMRTs were detected in reversed-phase positive mode and 355 EMRTs for negative mode. The HILIC-based separations resulted in 1066 EMRTs for positive-ion mode and 1221 EMRTs in negative mode (Table 3.2).

To further limit the number of mass signals to a list of ions significantly different between the NILs; factor of change (peak area ratios), *p*-values and peak heights were determined for the EMRTs in each dataset. Ions with *p*-values < 0.05 and a factor of change less than 0.5 or greater than 2 were kept for further analysis. A full listing of these EMRTs is provided in Appendix B (Table S3.2).The final ion list is tabulated in Table 3.2 and displayed graphically in Figure 3.2. Furthermore, each class was compared to the others individually, resulting in six comparisons:

1. *mrp*(3)/*mrp*(19) vs. *mrp*(3)/*MRP*(19)
2. *mrp*(3)/*mrp*(19) vs. *MRP*(3)/*mrp*(19)
3. *mrp*(3)/*mrp*(19) vs. *MRP*(3)/*MRP*(19)
4. *mrp*(3)/*MRP*(19) vs. *MRP*(3)/*mrp*(3)
5. *mrp*(3)/*MRP*(19) vs. *MRP*(3)/*MRP*(19)
6. *MRP*(3)/*mrp*(3) vs. *MRP*(3)/*MRP*(19)

The first three comparisons (1., 2. and 3.) compare the low phytate line to a normal phytate line. These three comparisons were used in the Venn Diagram (Figure 3.2). Only Ions with a peak heights > 10,000 and above that were different in all three low phytate vs. normal phytate comparisons were analyzed further. The final data set generated by separation in the reversed-phase mode resulted in less total ions (11 in positive ion mode and 35 in negative ion mode) than the one generated by the HILIC-based separation (71 different ions in positive ion mode and 67 different ion in negative ion mode).

Table 3.2. Comparison of EMRTs Across Separation Strategies and Ionization Modes.

Number of	**RPC pos**	**RPC neg**	**HILIC pos**	**HILIC neg**
EMRTs detected	154	355	1066	1221
Significantly different EMRTs[1]	32	122	240	243
Significantly Different EMRTs Between All LP vs. NP comparisons[2]	11	35	71	67

[1]Based on *p*-value (< 0.05) and factor of change (less than 0.5 or greater than 2).
[2]Based on characteristics of [1] and peak height (peak heights > 10,000 and above) (LP=low phytate, *mrp*(3)/*mrp*(19); NP=normal phytate, *mrp*(3)/*MRP*(19), *MRP*(3)/*mrp*(19) and *MRP*(3)/*MRP*(19).

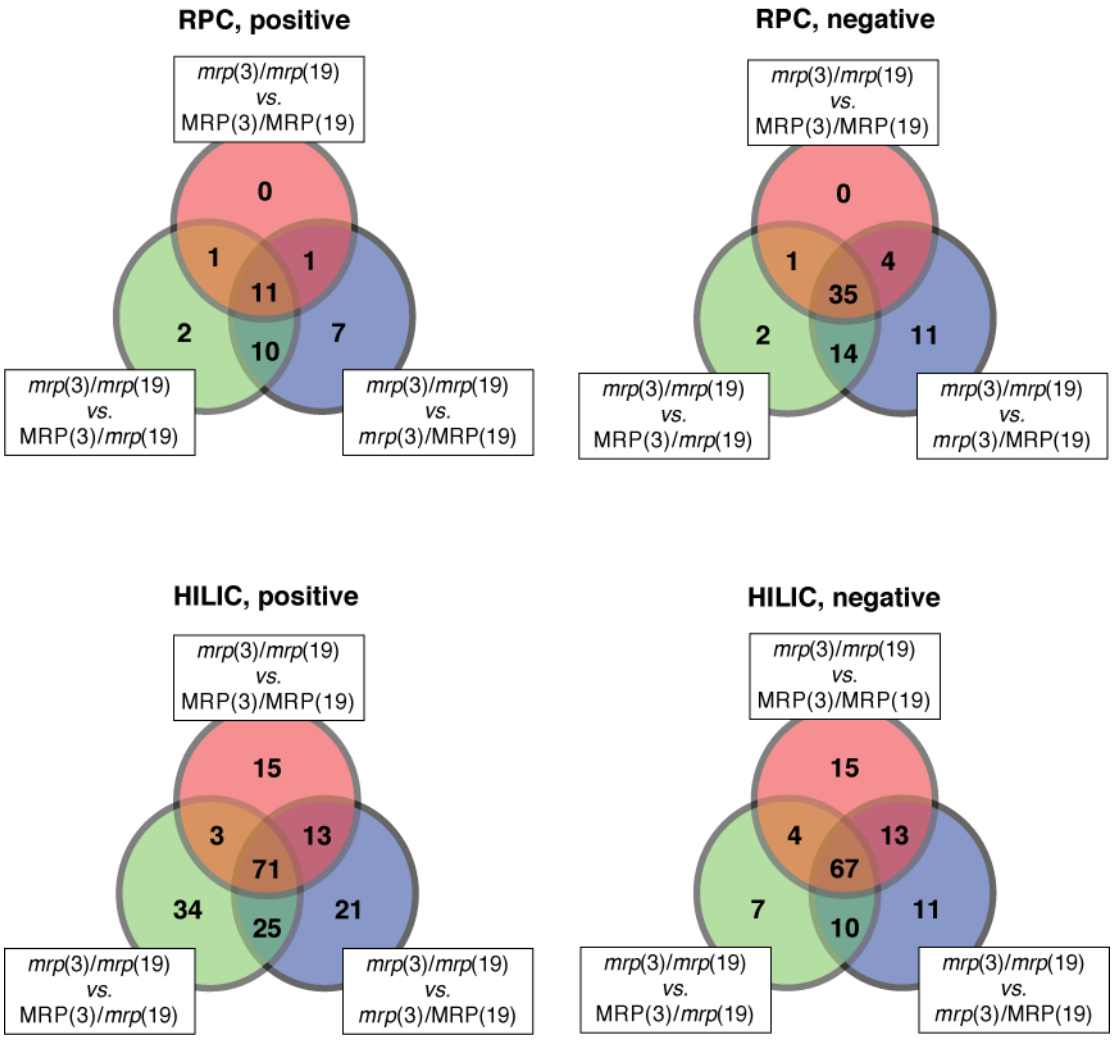

Figure 3.2. Venn diagram displaying EMRTs related to the low phytate phenotype.

The low phytate phenotype is only achieved when both mutations are present. The EMRTs present in the center of the Venn Diagram are those ions that are significantly different between the low and high phytate NILs. Our identification focused on the EMRTs since these are the ones most likely associated with the low phytate phenotype and not with an individual *MRP* mutation.

3.4.3 Principal Component and Orthogonal Partial Least Squares Discriminant Analyses

To investigate whether our method can be used to discriminate between closely related genotypes, principal component analysis (PCA) was performed. PCA is a non-biased statistical technique that dimensionally reduces the data and displays it as scores in a system of principal components (Wan et al., 2013). In our study the two-dimensional PCA score plot showed a clear separation of the three normal phytate lines from the low phytate line (Figure 3.3). With the apparent grouping of the three normal phytate classes away from the low phytate class, it is clear that the LC methods and ionization modes employed were successful in differentiating the seed extracts. PCA analysis of the three normal phytate lines without the low phytate line exhibited less overall class separation, with each mode providing different levels (Figure 3.4). The HILIC negative ion mode analyses provided the cleanest separation of classes, with the other analyses indicating that the *MRP*(3)/*MRP*(19) (Wildtype/Wildtype) line more closely resembled the *mrp*(3)/*MRP*(19) line.

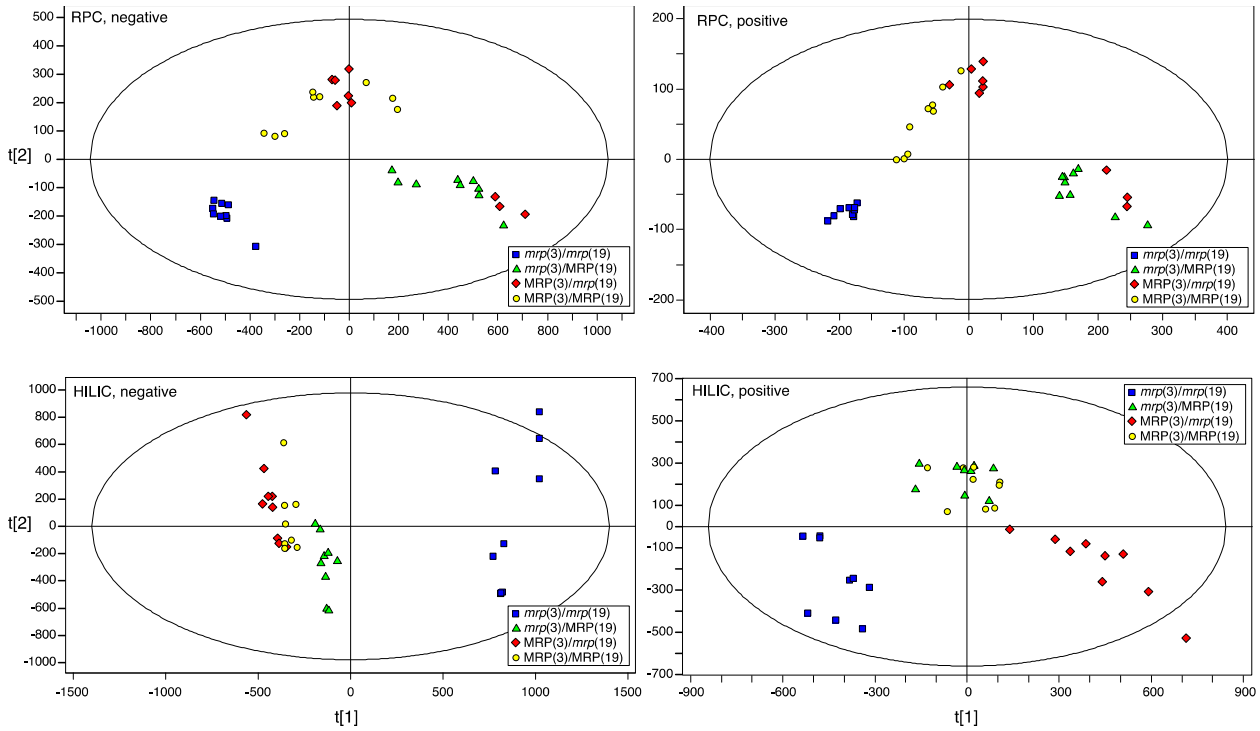

Figure 3.3. Principal Components Analysis of the Four NILs Investigated.

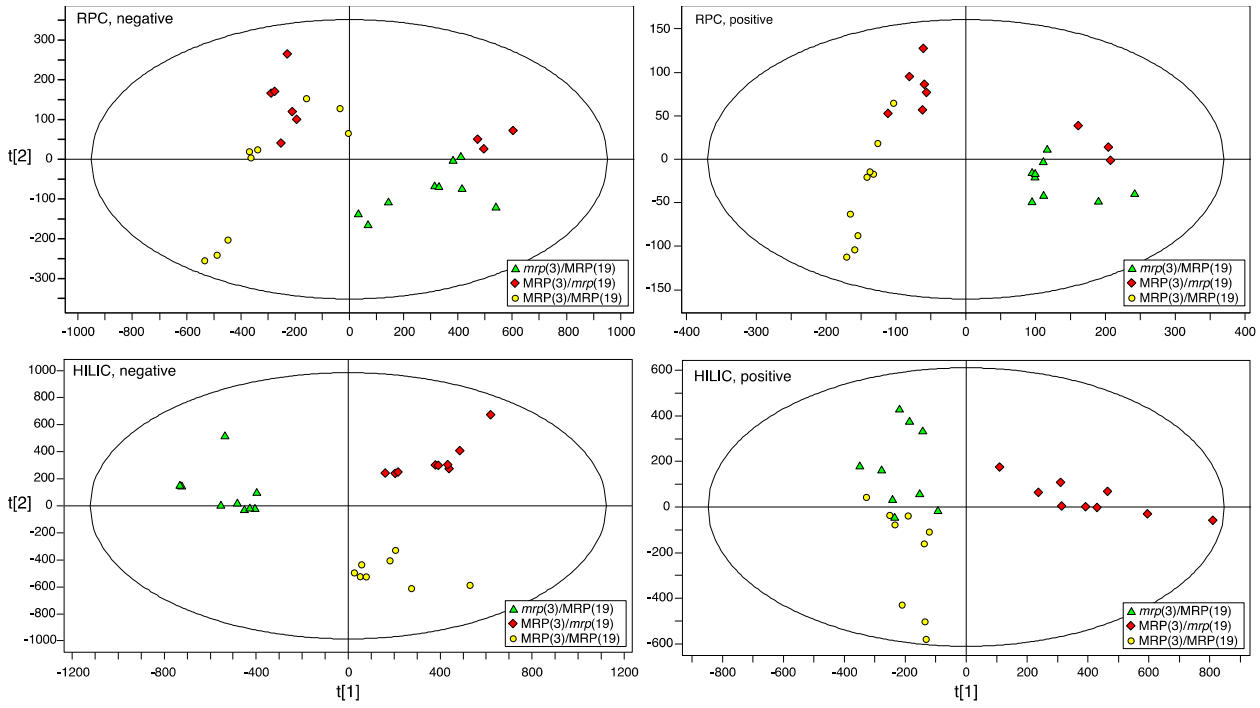

Figure 3.4. Principal Components Analysis (PCA) of the Three Normal Phytate Lines Investigated. Note: The HILIC analysis negative ion mode had an outlier (one technical replicate of one biological replicate of *MRP*(3)/*MRP*(19)). This data point was removed prior to the analysis.

Orthogonal partial least squares discriminate analysis (OPLS-DA) is often used with LC-MS dataset to further confirm the PCA results and further strengthen the discrimination (Wan et al., 2013). Orthogonal OPLS-DA is a supervised multivariate statistical analysis, as opposed to the unsupervised PCA (Wan et al., 2013), with the x-dimension providing predictive features (in this case EMRTs) and the y-axis providing non-predictive features. The OPLS-DA evaluation of the four datasets is shown in Figure 3.5. There was a clear separation between the lines differing in phytate concentration (low vs. normal phytate) in the predictive axis, with the normal phytate lines generally separated on the y- or non-predictive axis.

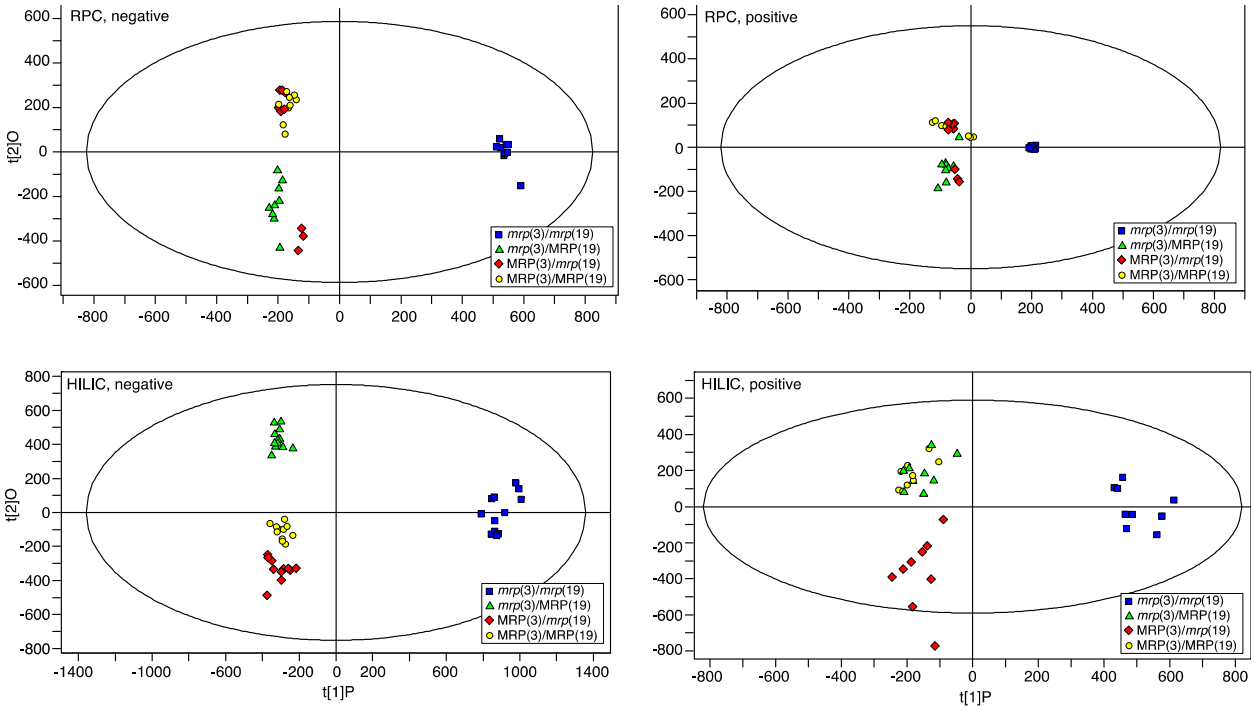

Figure 3.5. Orthogonal projection of latent structures discriminant analysis.

3.4.4 Ion Identification.

Since there were clear differences in the low and high phytate lines, the next step was to assign structures to the ions that were deemed significantly different. The identification procedures relied upon the EMRT data (mass to charge ratio and retention time), fragmentation patterns (MS/MS data), literature searches as well as publically available databases (see Materials and Methods). Table 3.3 summarizes the ions differing between the lines and a portion of the base peak ion chromatograms of the master mixes where differences are most dramatic are shown in Figure 3.6.

Table 3.3. Identified EMRTs Based on Literature or Prime ID (prime.psc.riken.jp))

LC-MS	EMRT	*mrp*(3)/ *mrp*(19)	*mrp*(3)/ *MRP*(19)	*MRP*(3)/ *mrp*(19)	*MRP*(3)/ *MRP*(19)	FOC	p-value	Identification
RPC-(+)	255.0657_3.81	283	723	840	728	0.37	1.77E-15	Malonyl Daidzin
RPC-(+)	1365.6300_5.75	463	0	0	0	5005.86	3.87E-06	Acetylsoyasaponin A4 (Aa)
RPC-(+)	1437.6510_5.88	0.09	1020	765	804	0.0001	1.72E-17	Acetylsoyasaponin A1 (Ab)
RPC-(-)	253.0500_3.81	711	2017	1875	1599	0.3886	3.56E-09	Malonyl Daidzin
RPC-(-)	1035.2040_4.35	3966	13500	13925	8974	0.3269	2.19E-11	Malonyl Genistin
RPC-(-)	698.3062_4.93	0	654	281	395	0.0018	8.16E-07	Soyasaponin Ab (2 Acetates)
RPC-(-)	719.3116_5.26	1	1433	1059	1165	0.0014	2.54E-20	Soyasaponin Ab (1 Acetate)
RPC-(-)	719.3119_5.45	6	1574	1145	1356	0.0047	8.58E-21	Soyasaponin Ab (1 Acetate)
RPC-(-)	740.3174_5.88	46	3410	2866	2964	0.0152	7.73E-29	Acetylsoyasaponin A1 (Ab)
RPC-(-)	957.5046_6.47	1288	5219	4158	2819	0.3171	4.70E-09	Soyasaponin V (Ba)
RPC-(-)	765.4420_6.86	324	1630	1665	856	0.2341	3.90E-10	Soyasaponin IV (Bc')
HILIC-(+)	439.3566_2.81	0	216	196	194	0.0000	2.85E-17	Daidzin
HILIC-(+)	1267.5700_2.82	0	371	358	336	0.0013	1.71E-37	Acetylsoyasaponin A3 (Ah) plus Na
HILIC-(+)	1275.5980_2.92	2	793	736	898	0.0031	6.10E-36	Acetylsoyasaponin A2 (Af)
HILIC-(+)	1421.6560_3.23	0	160	130	162	0.0014	9.32E-32	Acetylsoyasaponin Ac
HILIC-(+)	1403.5860_3.31	1644	1	1	1	1054.50	7.91E-12	Acetylsoyasaponin A4 (Aa) plus K
HILIC-(-)	1243.5740_2.82	5	2668	2535	2366	0.0021	1.04E-42	Acetylsoyasaponin A3 (Ah) fragment
HILIC-(-)	1273.5850_2.93	64	6426	5434	6063	0.0108	2.19E-33	Acetylsoyasaponin A2 (Af) fragment
HILIC-(-)	1435.6380_3.29	55	4719	4585	4557	0.0121	6.00E-27	Acetylsoyasaponin A1 (Ab)
HILIC-(-)	1409.6200_3.31	604	0	3	2	268.32	7.36E-17	Acetylsoyasaponin A4 (Aa) plus FA

FOC = Factor of Change or Peak Area Ratio (*mrp*(3)/*mrp*(19) peak area/average peak area of all normal phytate lines).

The identification phase of this work quickly revealed that the most significant differences between the low and normal phytate lines were within the soyasaponin composition. This can easily be seen in the chromatograms of the four lines as shown in Figure 3.6. The normalized base peak ion chromatogram revealed that there are relatively more soyasaponins in the single mutants, with overall profiles similar to that of the wild type line. The low phytate line however had a different profile with the appearance of soyasaponins A4, A5 and A6 not present in the other three lines. The low phytate lines were enriched in C22-xylose terminated Group A soyasaponins, with severely reduced levels of the C22-glucose terminated structures.

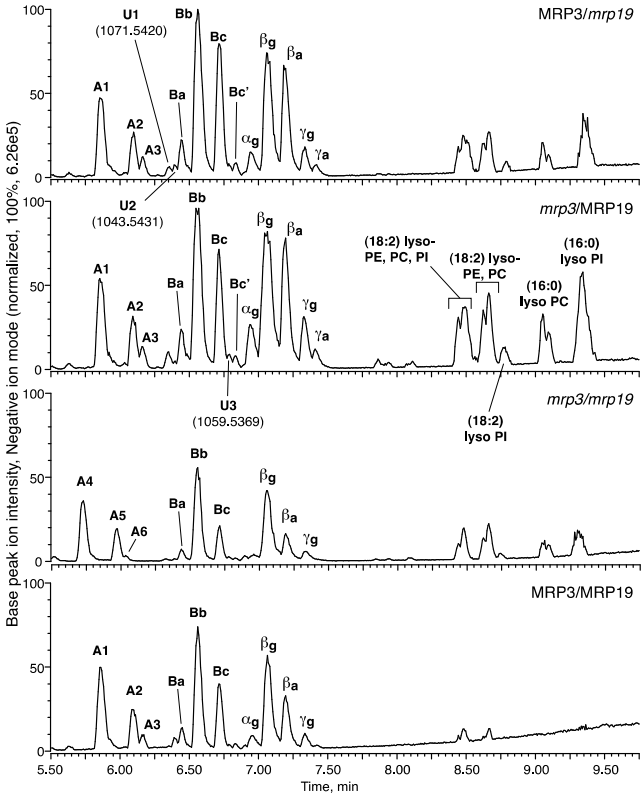

Figure 3.6. The Soyasaponin Regions of Normalized Base Peak Ion Chromatograms of the Four NILs.

As the Group A soysaponin profiles were observed to be different, the pool of Group A soysaponins was investigated in more detail by selected ion monitoring for the known masses of all Group A soysaponins with the results shown in Figure 3.7. In each set of paired traces, the *mrp3*/*mrp19* low phytate line is the top colored trace with base peak ion intensities normalized to the most intense soyasaponin in the pair. The low phytate line was clearly enriched with xylose-terminated soyasaponins. In fact, the data obtained allowed the tentative determination of two new soyasaponins in the low phytate line.

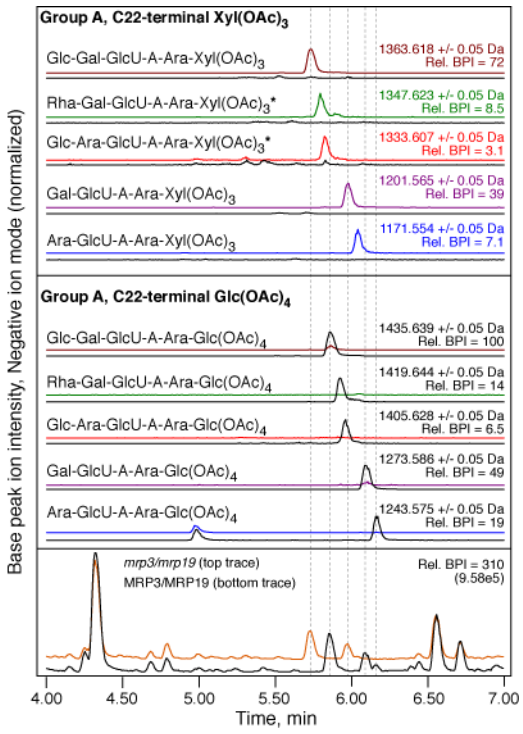

Figure 3.7. Selected Ion Intensities for the Group A Soyasaponins. In each set of paired traces, the *mrp3*/*mrp19* low phytate line is the top (colored) trace. Selected monoisotopic masses and structures are shown for each soyasaponin, with base peak ion intensities normalized to the most intense soyasaponin ion in the pair. Asterisks indicate proposed new soyasaponins in the low phytate line, and are based on relative retention times and fragmentation patterns.

3.5 DISCUSSION

The low phytate phenotype in the CX1834 NILs evaluated in this study is controlled by mutations in two ABC transporter genes located on soybean chromosomes 3 and 19 (Gao et al., 2008; Gillman et al., 2009; Oltmans et al., 2004; Saghai Maroof et al., 2009; Walker et al., 2006). We have shown here that both *MRP* mutations are needed to obtain a low phytate phenotype, which is in agreement with previous results (Gao et al., 2008; Gillman et al., 2009; Oltmans et al., 2004). The mutation of related transporters in other species has also been shown to provide the low phytate phenotype in other species including barley, maize, rice, Arabidopsis and the common bean (Gillman et al., 2009; Panzeri et al., 2011; Shi et al., 2007; Xu et al.,

2009). The cause for low phytate lines in barley and maize is mapped to a single gene mutation in a *MRP* gene (Larson et al., 2000; Walker et al., 2006; Wilcox et al., 2000). The maize *MRP4* gene is most closely related to *MRP5* in Arabidopsis (Shi et al., 2007). The soybean *MRP* genes mutated in the low phytate line encode proteins that show a similarity of 66.6% and 63.8% to the maize *MRP4* gene and 74.5% and 72.3% to the Arabidopsis *MRP5* gene (Gillman et al., 2009). In Arabidopsis, the *AtMRP5* gene encodes a putative inositol hexakisphosphate transporter (Nagy et al., 2009). It was suggested that *AtMRP5* controls anion channels across the plasma membrane in the guard cells and thus may lead to partial drought resistance in Arabidopsis (Suh et al., 2007).

Segregation studies for phytate levels in a low phytate wheat have shown that the low phytate phenotype is due to two independent loci (Guttieri et al., 2004). In soybean it was also shown that a low phytate phenotype is observed only if an individual is homozygous for the mutation of both *MRP* genes (Oltmans et al., 2004). The work of Walker et al. (2006) showed that two loci are needed to obtain a low phytate phenotype and that they are located on duplicated regions of soybean genome and may be of common origin (Walker et al., 2006).

While it is well established that the mutation of these two ATP-coupled transport proteins lead to both low phytate and low emergence (Anderson & Fehr, 2008; Gao et al., 2008; Oltmans et al., 2004; Oltmans et al., 2005) (Figure 3.1), the mechanism behind the phenotypes has yet to be established. We hypothesized that the observed phenotype may also have a seed chemotype that would provide insight into the mechanisms of both low phytate and emergence. We focused on the lipid-free metabolites employing orthogonal separation strategies and both positive and

negative ion mass spectrometer modes. Statistical analysis of the datasets by principal component analysis confirmed that the double mutant seed extracts were different from the wild type and single mutants (Figure 3.3), and when the double mutant was removed from the dataset and re-analyzed by PCA, the chemotypes appeared much more similar to one another (Figure 3.4). A list of exact mass/retention time pairs (EMRTs) (Table 3.3) identified soyasaponins containing C22 terminated acetylated glucosides as being present in much lower levels in the low phytate line, which favored soyasaponins terminated with an acetylated xyloside (Figures 3.5 and 3.6).

Soybean seeds, depending on growth location, variety and maturation, contain around 0.5% soyasaponin (Kim et al., 2006). Soyasaponin profiles vary greatly in their concentration and location in the soybean seeds (Kim et al., 2006). Soybean hypocotyls contain the largest variation and concentration of soyasaponins in developing plants, and are considered to be the main source of Group A soyasaponins (Decroos et al., 2005). Group A soyasaponins are only found in the seed hypocotyls (Sasama et al., 2010; Shiraiwa et al., 1991), whereas group B soyasaponins are found in most seed parts (Shiraiwa et al., 1991) (hypocotyls are only 2% of the seed weight (Sayama et al., 2012)). Total soyasaponin concentration decreases during seed maturation (Kim et al., 2006). Kim et al. (2006) analyzed the soyasaponin composition of different sized soybean seeds and report that almost 50% of soyasaponins found in the soybean seeds are non-DDMP conjugated soyasaponins, with 27% DDMP conjugated and 10% soyasaponin from Group A (Kim et al., 2006). Shiraiwa (1991) also show that the composition of Group A soyasaponins depends little on cultivation year and much more on variety (Shiraiwa et al., 1991).

The composition of soyasaponins in soybean seeds is regulated by five genes, *Sg-1*, *Sg-3*, *Sg-4*, *Sg-5* and *Sg-6* (on chromosome 7, 10, 1, 15, 1, respectively), controlling the soyasapogenol A or B utilization and the sequence and types of sugar chains attached to their glycosides (Krishnamurthy et al., 2014; Sasama et al., 2010; Takada et al., 2013; Takada et al., 2012; Tsukamoto et al., 1993). These five genes are differentially regulated depending on plant organ and plant variety (Tsukamoto et al., 1993). *Sg-1* controls the difference in terminal sugar at the C-22 position of soyasaponin A (Sayama et al., 2012). *Sg-3* controls the glucosylation of the second sugar at the C-3 position of soyasaponin A and B (Krishnamurthy et al., 2014).

Shiraiwa et al. (1991) investigated the Group A distribution in seed hypocotyls of 457 different soybean cultivars (Shiraiwa et al., 1991). The composition varied greatly among the different varieties with total saponin concentrations in hypocotyls ranging from 0.62 – 6.16% (Shiraiwa et al., 1991). The authors classified the varieties into seven different types based on their Group A composition. The largest grouping, which comprised 76% of the varieties contained the A1 soyasaponin, with the second largest group (17% of the varieties) only containing soyasaponin A4. Since most soybean varieties are mainly composed of either soyasaponin A1 or A4, the different varieties are classified as the glucose (A1 based) or xylose (A4) type (Tsukamoto et al., 1992).

Sayama et al. (2012) show this terminal glycosylation (xylose or glucose) is controlled by multiple alleles of a single gene (*Sg-1* locus on chromosome 7), which encodes for a UDP-sugar-dependent glycosyltransferase (Glyma07g38460). The alleles are similar to each other and lead to the glycosylation of the terminal non-acetylated sugar chain on the C-22 position. Expression

of one allele (*Sg*-1^{a}) leads to UDP-xylosyltransferase activity producing soyasaponin A4 whereas expression of the other (*Sg*-1^{b}) leads to UDP-glucosyltransferase (UGT73F2) activity producing soyasaponin A1 (Sayama et al., 2012). The two proteins have 98.3% identity (Sayama et al., 2012) with a single amino acid substitution (Ser/Gly-138) thought to be responsible for substrate specificity (Sayama et al., 2012). A third allele, *Sg*-1^{0}, is a loss of function allele of *Sg*-1 and leads to the loss of the terminal acetylated sugar (Sayama et al., 2012).

Saponins are known to have many biological activities *in planta*, ranging from antimicrobial compounds to plant growth promoters (Nagata et al., 1985; Osbourn et al., 2011; Shibuya et al., 2010). Saponins are often found in tissue subjected to bacterial or fungal attack (Eswaranandam et al., 2012; Nagata et al., 1985), and when secreted by alfalfa in the field it can result in low germination rates in nearby wheat seeds (Waller et al., 1993). In their metabolomic comparison of the salt tolerant and salt sensitive soybean lines, Wu et al. (2008) report that soyasaponins from Group B are correlated with salt tolerance, where it is suggested that biotic and abiotic stresses lead to the accumulation of soyasaponins as they serve as a reservoir of aglycones and sugars after hydrolysis and may increase nutrient and water uptake (Wu et al., 2008). The Group B soyasaponins were not found to be significantly different in the work presented here.

Membranes are marginally stable and all organisms attempt to maintain constant membrane fluidity. Saponins are known to cause membrane perturbations, hence their use as antiviral medications for humans (Augustin et al., 2011). The sugar chains are hydrophilic and are suggested to lead to the formation of pores (Augustin et al., 2011). These pores increase membrane permeability, increasing flow of both charged and neutral molecules (Augustin et al.,

2011). It was originally reported that cholesterol needs to be present in membranes in order for saponins to form pores (Augustin et al., 2011). Saponins interact with cholesterol of red blood cells forming pores that lead to cell lysis (Chwalek et al., 2006). Thus while membrane composition is important for the effect of saponins, the concentration and chemical structure of the saponin is important as well (Augustin et al., 2011). The type of aglycone as well as the numbers and types of sugar in the sugar chain are important for membrane-saponin interactions (Chwalek et al., 2006). Saponins can increase the permeability of membranes without leading to the cell's destruction and thus influence transmembrane and cytoskeletal dynamics (Chwalek et al., 2006). With respect to soyasaponin structure-activity relationshships, Chwalek et al. (2006) showed that the C22 terminal sugar (β-D-xylopyranoside or β-D-glucopyranoside), of hederagenin diglycoside saponins influence the haemolysis and cytotoxic activity of these saponins (Chwalek et al., 2006). One could hypothesize that the different soyasaponin profile of the soybean double mutant negatively influences membrane stability and/or inter-organelle communication.

Increases in temperature increase membrane instability and must be adjusted for *in vivo*. Saponin concentrations in plants are influenced by the environment as well (Augustin et al., 2011). Differences in emergence rates between low and normal phytate lines depend on the seed source and thus environment (Anderson & Fehr, 2008; Maupin & Rainey, 2011; Meis et al., 2003; Oltmans et al., 2005). The seed source effect is defined as the influence of the environment in which seeds are produced (Oltmans et al., 2005). Low phytate seeds produced and sown in temperate climate show an emergence rate of 63%, compared to 77% for a normal phytate line 77%, but if these seeds produced in subtropical climate and sown in temperate climate the

emergence rate was only 8% (83% for the normal phytate lines) (Meis et al., 2003). Oltmans et al. (2005) tested the emergence rate of the CX1834-derived lines and showed that emergence rates of low- and normal-phytate lines differ in all environments tested, but the degree of difference in emergence rates depends on the the seeds were produced and sown in (Oltmans et al., 2005). Anderson and Fehr (2008) investigated the seed source affect on CX1834. The emergence rates of seeds produced in Puerto Rico in May (25.4%) are significantly lower than the emergence rates of the lines whose seeds are produced in Iowa (77%) or Puerto Rico (in January) (70.1%) when sown in Iowa (Anderson & Fehr, 2008). These results suggest that a hot and humid climate during seed production negatively influences the emergence rate of soybean seeds. Raboy (2009a) suggests that these differences between low and normal phytate lines when produced in tropical climate is due to reduced heat tolerance of the low phytate seeds (Raboy, 2009). High temperatures during seed development and maturation are a form of stress that leads to a reduction in germination and seed vigor (Egli et al., 2005).

Reactive oxygen species may also be related to low emergence. Phytate is described as an antioxidant (Murphy et al., 2008), and it has also been suggested that soyasaponins work as antioxidants, protecting the plant from reactive oxygen species during development (Sayama et al., 2012). When developing seeds are exposed to high temperature, the isoflavone concentrations in the mature seed are reduced, decreasing the concentration of these antioxidants (Tsukamoto et al., 1995). However, the saponin concentration is reported to remain constant (Macdonald et al., 2005).

While one may propose that Group A soyasaponins are related to low emergence, a Group A deficient soybean line has been developed and its yield appears normal. Soybean line 'Kinusayaka' does not produce three lipoxygenase isozymes and Group A acetyl soyasaponins in the seeds. The breeders describe a 300kg/10a seed yield, 23.5g 100-seed weight 40.9% protein content and 19.8% oil content (Kato et al., 2007). However, in the absence of soyasaponins, soybean seedlings may invoke alternative strategies for membrane stabilization, pathogens, and/or protection from reactive oxygen species.

The analysis of the soyasaponin composition revealed that both *MRP* mutations have to be present in order to observe the xylose containing soyasaponins. The NILs with only one *MRP* mutation had a similar saponin profile as the line without mutation (wildtype). However, in regards to the lyso compounds, the single mutants were more similar to the double mutants than to the wildtype: there were less lyso compounds in the single mutants and double mutants with hardly any in the wildtype line. This may be due to increased lipase activity or issues with assembly of membrane lipids.

Our assumption at this point is that the observed phenotype is due to the redundant function of the MRP proteins, and loss of both is required to obtain the observed phenotype. Either the low phytate phenotype is responsible for the observed chemotypes or the differences are due to the pleiotropic effect of the combined *MRP* mutations. There are many different transporters in plants and the preferred combination of a substrate and a transporter is species dependent (Yazaki, 2006). For example, the flavonoid saponarin is stored in barley vacuoles via a H^+-antiporter process, however, it is stored in Arabidopsis vacuoles via an ABC transporter (Yazaki,

2006). Vacuolar ABC transporters are involved in the accumulation of secondary metabolites (Bartholomew et al., 2002). ZmMRP3 is an ABC transporter in maize that is located in the tonoplast and works in the accumulation of anthocyanin (Yazaki, 2006). MRP ABC transporters are suggested to play a role in vacuolar sequestration of glucosides as well (Bartholomew et al., 2002). Bartholomew et al. (2002) report that two phenol glucosides were transported via H^+-gradient across the vacuolar membrane of red beet, whereas glutathione conjugates were transported by an ABC transporter (Bartholomew et al., 2002). This suggests that the different sugar moieties act as tags recognized by different transporters (Yazaki, 2006). It is possible that in soybean, the MRP transporter is responsible for transport of soyasaponins into the seed storage vacuoles and the C22 terminal sugar of the Group A soyasaponins act as a tag to be recognized by different transporters. Perhaps the MRP deficient double mutant line may not capable of transporting C22 glucose terminated Group A soyasaponins across the membrane, because glucose might act as the tag recognized by MRP transporters in soybean. However, this does not explain why the normal phytate lines do not contain significant amounts of the C22 xylose-terminated Group A soyasaponins.

Soyasaponin levels are highest in the early stages of plant growth, presumably when metabolism and biomass synthesis rely upon the glyoxysome, the transient organelle that works in concert with mitochondria to avoid the two decarboxylation steps in the TCA cycle (Jyothi et al., 2007). Saponins are structurally similar to membrane sterols and thus modulate membrane permeability (Osbourn et al., 2011). Osbourn et al. (2011) suggest that the triterpenes could play a role in membrane-related processes. A saponin in oat mutants influences membrane trafficking, which can influence growth and development of plants (Osbourn et al., 2011). In the case of the low

phytate line, the profile change could influence cell development processes during emergence through processes associated with glyoxysome formation and/or associated transport processes. Ultrastructural studies involving these lines may address this question directly.

3.6 CONCLUSION

An LC-MS method was developed and employed for the metabolic discrimination of seeds from four soybean lines that are nearly genetically identical except for the presence of neither, either or both of the *MRP* mutations associated with the low phytate phenotype. These unique lines were compared at the metabolomic level with phytate concentrations and emergence rates used to confirm the low phytate phenotype of the double mutant. Assessment of the lipid-free methanol-soluble metabolites clearly separated the low phytate line from the other three, with the differences mainly in the soyasaponin profile. Our investigations showed that the low phytate double mutant contained little to no C22 glucose terminated Group A soyasaponins and almost exclusively C22 xylose terminated Group A soyasaponins (A4, A5 and A6). The normal phytate NILs had the opposite profile: containing no xylose terminated Group A soyasaponins. Further studies are needed to investigate whether low phytate leads to these changes and/or if the saponin differences directly influence emergence. Determining the function of the MRPs as well as investigating the metabolite profile of low phytate soybean lines resulting from a mutation in the *MIPS1* gene may help address these issues.

3.7 REFERENCES

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Chapter 4

Discrimination of Near Isogenic Low and High Phytate Soybean Seeds Using Non-Polar Metabolites

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4.1 Abstract

Low phytate soybean lines suffer from poor emergence rates, which limit their use. While previous efforts have evaluated the polar and semi-polar metabolites present in these seeds prior to germination and emergence, the lipid profiles of low phytate lines have received little attention. In the work reported here, the lipid fraction from four near isogenic soybean lines (NILs) differing in their phytate levels and emergence rates were subjected to liquid chromatography-mass spectrometry (LC-MS) metabolite profiling. The NILs evaluated were the progeny of the CX1834 line. Here the low phytate phenotype is due mutations in two multidrug resistant proteins (*MRP*). The four lines have neither, either or both of the mutations. The double mutant exhibited significantly lower phytate values than the lines with either or none of the mutations. This low phytate line also showed a low emergence rate (below 50%), whereas the other three lines showed emergence rates above 80%. Ethyl acetate-soluble seed extracts were separated by ultra performance liquid chromatography, with detection by electrospray ionization mass spectrometry (UPLC-ESI-MS) in both positive and negative ionization modes. Principal component (PCA) and orthogonal projection of latent structures-discriminant (OPLS-DA) analyses of the LC-MS data was used to discriminate between these lines. OPLS-DA showed clear separation of the low phytate line from the three normal phytate lines. The untargeted lipidomic analyses revealed differences in both the storage and membrane lipid profiles with the low phytate line displaying higher relative levels of tri- and diacylglycerols as well as specific phosphatidyl-choline, serine and ethanolamines.

4.2 Introduction

Phytate, also known as phytic acid and *myo*-inositol 1,2,3,4,5,6-hexakisphosphate, is found in many plant species and its main role in cereals and legumes is to store phosphorus in the seeds (Hazebroek et al., 2007). Soybean is a widely used feed source for animals (Hulke et al., 2004) and is also used for human consumption. As phytate cannot be digested by humans and monogastric animals, it is excreted, which leads to phosphorus deficiency in the animals and phosphorus runoff into rivers and lakes and thus to environmental pollution (Hazebroek et al., 2007; Hulke et al., 2004). Phytate also binds mineral cations and can lead to mineral deficiencies in the consumer (Hazebroek et al., 2007; Raboy, 2007). Use of low phytate lines can ameliorate these problems, reducing the need for supplementing feed with phytase and/or inorganic phosphorus and minerals (Hulke et al., 2004). Low phytate soybean lines, however, show reduced agronomic performance due to for example low emergence (Raboy, 2002).

CX1834 is a low phytate soybean line with a phytate concentration of 8.6 ± 0.4 mg g−1 (Gao et al., 2008). The low phytate phenotype is due to mutations in two multidrug resistance-associated proteins (MRPs) located on chromosome 3 and chromosome 19 (Gillman et al., 2009; Saghai Maroof et al., 2009). It was shown that a low phytate phenotype is observed only if an individual is homozygous for both of these recessive alleles (Oltmans et al., 2004).

Soybean seeds store oil, protein and carbohydrates as a source of energy, carbon and nitrogen during germination (Collakova et al., 2013). Inasmuch, soybean is a major commercial source of proteins and oil (Ren et al., 2009). While the protein is mainly used as animal feed, the oil is used for human consumption as well as feed and biodiesel (Clemente & Cahoon, 2009). Soybean

oil is predominantly composed of triglycerides with a fatty acid profile consisting of the 10% palmitic acid (C16:0), 4% stearic acid (C18:0), 18% oleic acid (C18:1), 55% linoleic acid (C18:2) and 13% linolenic acid (C18:3) (Clemente & Cahoon, 2009). This composition is not ideal for oil consumption, storage or use as biodiesel as it leads to low oxidative stability, off flavors, undesired saturated fat content and difficulties filtering biodiesel (Clemente & Cahoon, 2009). The most desired fatty acid profile is one with a reduced palmitate content, which provides a lower level of saturated fats (Hulke et al., 2004). Low linolenic acid soybean lines have been developed and are used for commercial production to increase the oxidative stability of the oil (Clemente & Cahoon, 2009). Soybean lines with increased oleic acid concentration have been developed as well. These lines show environmental instability and reduced yield, which is thought to be caused by a mutation unrelated to the oil content (Alt et al., 2005). The changes in seed oil content also lead to changes in the fatty acid content of the membranes, so it was suggested that the reduction in yield might be caused by the changes in membrane fluidity (Clemente & Cahoon, 2009). The membrane fluidity affects the plant's response to environmental stress (Raboy, 2002).

Fatty acids detected in soybean powder can come from the storage oils, which are mainly in the form of triacylglycerides (TGs) and from membrane lipids, which are amphipathic molecules containing two acyl chains (Collakova et al., 2013). High temperature influences seed composition, seed vigor and thus germination (Ren et al., 2009). An increase in the number of days of growth in temperatures above 30 °C led to a decrease in seed vigor. Temperature influences the oil composition of soybean seeds – with increasing temperature the oil content increases (Piper & Boote, 1999) and the individual fatty acid concentration changes (Ren et al.,

2009). Ren et al. (2009) show that a temperature increase of 10 °C during seed development increases the palmitic (by 18%), stearic (41%) and oleic acid (44%) concentration, but decreases the linoleic (3%) and linolenic acid (81%) concentrations. The protein, sugar and phytate concentrations were reported to remain the same (Ren et al., 2009). The germination rate of the seeds produced at elevated temperatures was 50% less than the germination of the control seeds (Ren et al., 2009). The authors caution that these results may not be found in all soybean cultivars as there were previous reports of increased protein and oil concentration and other studies showing an inverse relationship between protein and oil (Clemente & Cahoon, 2009; Ren et al., 2009). Davy de Virville et al. (2002) report that changes in oil composition are found in the membrane phospholipids and in the seed storage compartments. Changes in membrane lipid composition changes membrane fluidity (Davy De Virville et al., 2002).

Frank et al. (2009) analyzed the metabolomic differences between two different low phytate soybean mutant lines using a GC-MS approach. Fatty acid analyses indicated that the fatty acid profiles were not significantly different between low phytate and normal phytate lines (Frank et al., 2009). A low phytate maize line (*lpa1*) with a 65% decrease in kernel phytate concentration, also exhibited similar oil profiles to that of normal phytate kernels (Hazebroek et al., 2007). The total phosphorus concentration in kernels did not differ between the WT and *lpa1* line, but the low phytate line exhibited increased inorganic phosphate (Pi) concentrations, showing an inverse relationship between phytate and Pi concentrations (Hazebroek et al., 2007). The authors could not account for 9% of phosphorus in the WT seeds and for 32% of phosphorus in the *lpa1* line. The concentrations of the four major membrane-associated phospholipids (phosphatidylcholine, PC; phosphatidylinositol, PI; phosphatidylethanolamine, PE; phosphatidylserine, PS) did not

differ between the *lpa1* line and the WT line (Hazebroek et al., 2007). However, the TMS derivate of PI (trimethylsilyl (*TMS*) ethers is used to derivatize functional groups prior to GC analysis) showed a higher concentration in the *lpa1* line, an unidentified metabolite containing a PI fragment had a higher concentration in the WT line. Phosphate containing metabolites were more prevalent in the low phytate kernels (Hazebroek et al., 2007).

The objective of this study was to determine if there are differences in the lipid profiles of soybean seeds that can be used to distinguish between closely related lines. The study objects of this investigation were four near isogenic soybean lines (NILs) differing only in two *MRP* genes (Glyma19g35230 (on chromosome 19) and Glyma03g32500 (on chromosome 3)). These lines are nearly identical (originated from the same plant) except for the presence of neither, either, or both of the mutant genes. Only the last line, which contains both mutations, is low phytate. Since metabolite work is sensitive to environmental conditions and genetic differences of the samples compared, these near isogenic lines provide the opportunity to eliminate genetic background and only focus on the differences caused by the mutations in the *MRP* genes. In the previous chapter we showed that the polar metabolites can be used to discriminate between these near isogenic lines, the main differences residing in their Group A soyasaponins profiles. This study expands the comparisons to assess the influence of low phytate on the lipid composition of the soybean seed as well.

4.3 Materials and Methods

Information regarding the plant material, harvest, confirmation of *MRP* genotypes, methods for determining phytate levels and emergence can be found in Chapter 3. In short, seeds used for this metabolomic experiments were harvested from the 2012 field in Blacksburg. The seeds were ground and the lipids were extracted with ethyl acetate with the resulting oil submitted to LC-MS profiling.

4.3.1 Non-targeted Metabolic Profiling Analysis Using LC-MS—Sample Preparation

All solvents, except ethyl acetate (Fisher Scientific), were of LC-MS quality (Spectrum Chemicals). Ethyl acetate was HPLC-grade and was dried with anhydrous $MgSO_4$ powder. Formic and acetic acid (Sigma-Aldrich) were LC-MS grade. Three biological replicates were analyzed for all four classes. Five seeds for each triplicate and class were selected at random, flash-frozen in liquid nitrogen and finely ground with P14 mill (Pulverisette 14, Fritsch). The powder was then transferred to pre-weighed 15 mL tubes, weighed and stored in the -80 °C freezer. A subset (400 mg) of this powder was weighed out, dried overnight on a high-vacuum line and extracted with 7 mL of dry ethyl acetate. After vortexing, sonication for 20 min and centrifugation (4000 rpm for 15 min), the supernatant was collected and the extraction procedure was repeated. The supernatants were combined and concentrated. The oil containing the non-polar fraction were stored in -80 °C until separation with the high strength silica (HSS) column.

4.3.2 Liquid Chromatography-Mass Spectrometry Analysis.

The samples were analyzed using UPLC-ESI-Q-TOF/MS (Waters Acquity I-class UPLC with Waters Synapt G2-S HDMS) in both positive and negative ion mode. Each biological replicate

and master mix was injected three times in random for each ionization mode. An aliquot of each sample (10 µl) was diluted with 90 µl of dichloromethane : methanol : water (6:3:1, v/v). A master mix was created for each class by combining 10 µl of the dilution of the three biological replicates. By combining aliquots (10 µl each) of all four class master mixes, a master mix of all classes was created. Samples were separated using a binary solvent system of 60% acetonitrile (A) and 1:9 acetonitrile : isopropanol (B) in an ACQUITY UPLC HSS T3 Column (1.8 µm, 2.1 mm X 100 mm, Waters Corp., Milford, MA) with flow rate 200 µL/min and a 16 minute gradient.

The following gradient conditions were used: isocratic at 40% B, followed by linear gradient to 100% B (0-10 min), hold at 100% B (10-12 min), followed by return to initial conditions (12-13 min) and isocratic at 40% B (13-16 min). Injection volume was 5 µL. The samples were ionized by electrospray ionization and analyzed in both positive and negative modes. The scan time at 0.20 sec and a mass range of 50-1800 Da was scanned. The source parameters for positive ion mode were source temperature 125 °C, capillary voltage 3.0 kV, cone voltage 40, source offset 80, desolvation temperature 400 °C, cone gas 60 L/h, desolvation gas 600 L/h and nebulizer gas 6.0 bar. The source parameters for negative ion mode were source temperature 125 °C, capillary voltage 2.4 kV, cone voltage 40, source offset 80, desolvation temperature 400 °C, cone gas 50 L/h, desolvation gas 600 L/h and nebulizer gas 6.0 bar. A reference sprayer continuously infused leucine-enkephlan (200 ng/ml, Waters Corp., Milford, MA) at 5 µl/min with a scan frequency of 20 seconds.

4.3.3 Data Processing and Analysis.

The UPLC/Q-TOF-MS data was processed using the MarkerLynx application manager software (version 4.1, Waters Corp., Milford, MA, USA). The following MarkerLynx parameters were used: retention time range 2.0 – 13.0 min, mass 50 – 2500 m/z, mass window 0.05, retention time window of 0.15, noise elimination of level 4, peak intensity threshold of 10000, marker intensity threshold 2500. A data set was generated by MarkerLynx that consisted of all the exact match-retention time pairs (EMRTs) their normalized peak areas. The EZinfo 2.0 software (Umetrics, Umea, Sweden) was used to generate principal component analyses (PCA) and orthogonal partial least squared discriminate analyses (OPLS-DA) results. OPLS-DA was visualized using a score plot. The datasets was used to calculate p-value (T-Test) and factor of change (peak area ratios) in Excel (Microsoft Office, 2007). Ions were identified into groups based upon chain length and degree of saturation using on-line databases and literature, such as Metlin (metlin.scripps.edu), Lipid Maps (lipidmaps.org) and Castro-Perez et al. (2010) (Castro-Perez et al., 2010).

4.4 RESULTS

Phytate concentrations and field- and laboratory-emergence rates of seeds from the same plants as used for metabolomic analyses were determined. The phytate values, field and laboratory emergence rates were analyses of the parental and progeny seeds in the years 2010, 2011 and 2013. The results can be found in Chapter 3.

4.4.1 Metabolomic Profling.

The protocol developed to obtain the non-polar metabolites from freeze-dried soybean powder was based upon extraction with anhydrous ethyl acetate. The resulting extracts were dried to oils and resuspended in dichloromethane-methanol-water with injection volumes normalized to recovered oil weights. Triplicate random injections of three biological replicates of each class and the mastermixes in both ionization modes resulted in a total of 102 individual LC-MS runs. The LC-MS conditions employed were based on previously published lipid profiling work (Castro-Perez et al., 2010). The datasets were then analyzed with the MassLynx Software package to convert the datasets into an exact mass retention time pairs (EMRT) with associated peak areas. A total of 836 EMRTs were detected in HSS positive mode and 361 EMRTs for negative mode. This number depended on the peak detection threshold set (see Materials and Methods).

Example chromatograms are shown in Figure 4.1 for a low and high phytate line. The profile was dominated by the triglycerides, especially in positive ion mode.

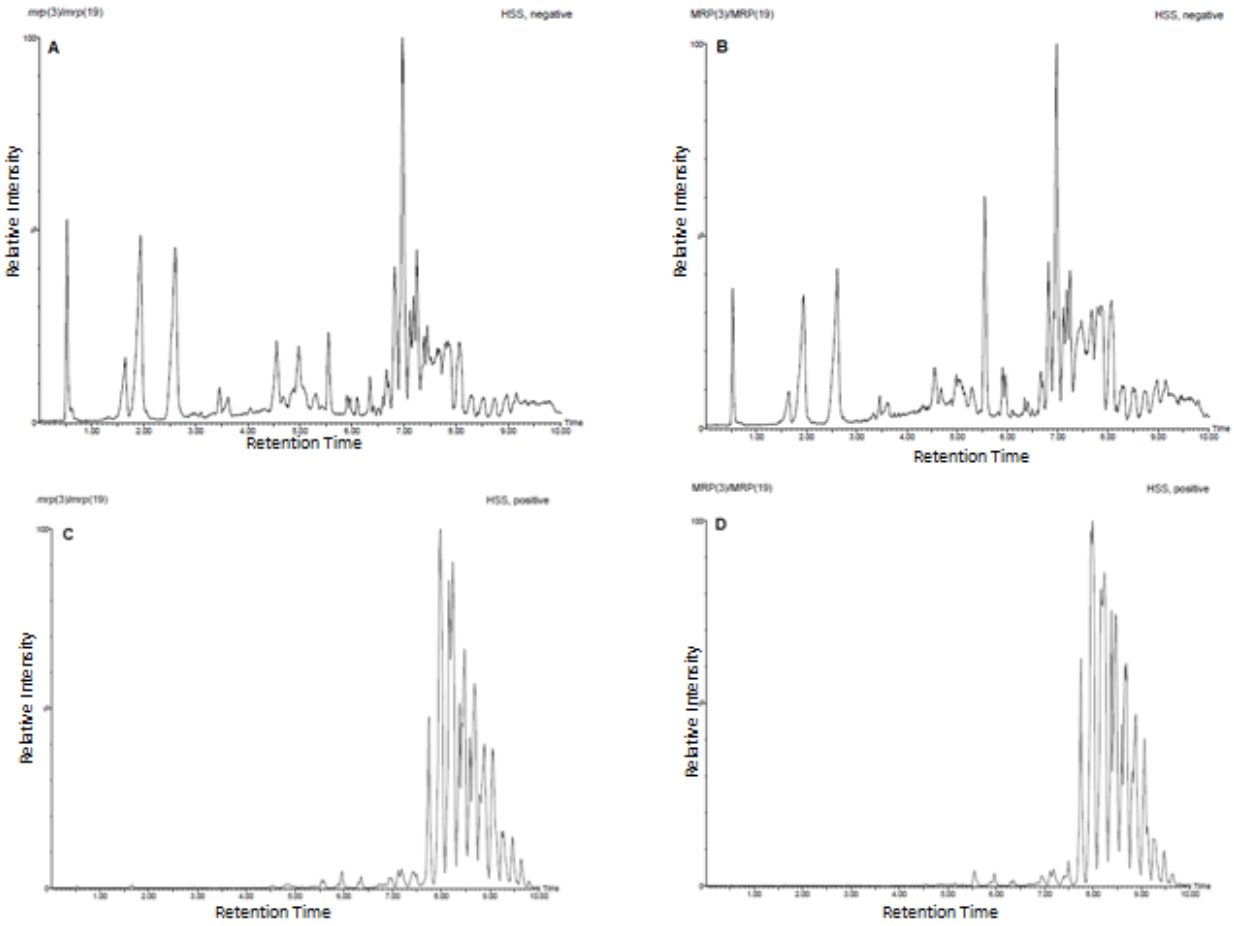

Figure 4.1. Normalized Base Peak Ion Chromatograms of the Low Phytate Line in Negative (A) and Positive Ion Mode (C) and the Wildtype Line (B (-) and D (+)).

To determine the number of statistically significant different mass signals between the classes, the factor of change (peak area ratios), *p*-values and peak heights were determined for each EMRT. Mass signals with a *p*-value < 0.05 and a factor of change less than 0.5 or greater than 2 were considered statistically significant. This led to the identification of 186 EMRTs, the full list of which can be found in Appendix (Table S4.1). The middle of the Venn Diagram represented the number of EMRTs that were focused on for identification (Figure 4.2); those are Ions that were significantly different in all three low versus normal phytate comparisons and have a peak height of 10,000 and above. The data set generated by separation in the HSS column shows 39

significantly different ions in positive ion mode and 11 ions in negative ion mode as can be seen in Table 4.1 and Figure 4.2.

Table 4.1. Comparison of EMRTs across ionization modes.

EMRT Based Upon Data Restriction	Positive	Negative
EMRTs initially detected	836	361
EMRTs with *p*-value and factor of change restrictions[1]	122	60
EMRTs with *p*-value, factor of change and peak height restrictions[2]	60	19
EMRTs Between All LP vs. NP comparisons[3]	39	11

[1]Based on *p*-value (< 0.05) and factor of change (less than 0.5 or greater than 2).
[2]Additional restriction of peak height (> 10000).
[3]*p*-value, factor of change and peak height restrictions enforced. LP = low phytate, *mrp*(3)/*mrp*(19); NP = normal phytate: *mrp*(3)/*MRP*(19), *MRP*(3)/*mrp*(19) and *MRP*(3)/*MRP*(19).

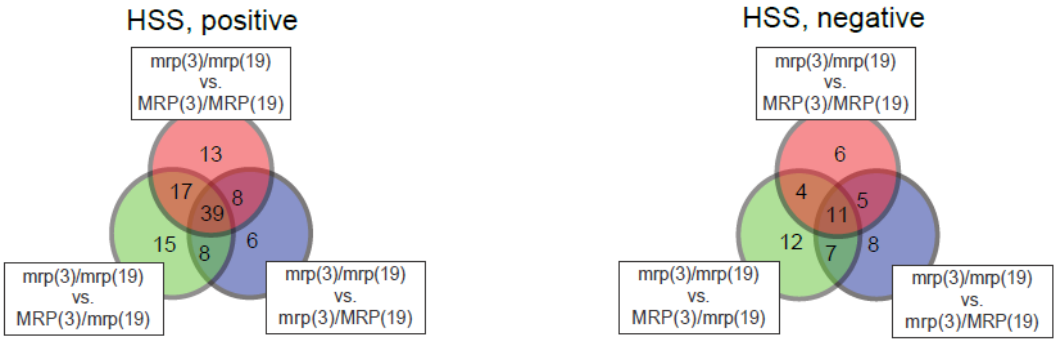

Figure 4.2. Venn Diagram Displaying EMRTs Related to the Low Phytate Phenotype.

4.4.2 Principal Component Analysis/ Orthogonal Partial Least Squares Discriminate Analysis

To investigate if our developed protocol can be used to discriminate between these closely related genotypes, Principal Components Analysis (PCA) was performed. PCA did not show a clear separation of the classes; the non polar metabolites were not sufficient to clearly separate the *mrp*(3)/*mrp*(19) from the normal phytate classes along the x-axis (Figure 4.3). In positive ion mode the low phytate line aggregated in the lower right quadrant and thus the separation is much better than in negative ion mode, where the low phytate line mingled in with the normal phytate lines.

As orthogonal partial least squares discriminate analyses (OPLS-DA) were previously used to strengthen a weak separation established by PCA (Wan et al., 2013), it was applied here to further define the two clusters differing in their non targeted metabolite profiles. The OPLS-DA plots (Figure 4.3.) showed the clear separation between the lines differing in phytate concentration with the low phytate line grouping away the normal phytate lines along the predictive x-axis (t[1]P). Since the group discrimination of the first component is forced (Wagner et al., 2007), OPLS models are more biased. They only model information that is capable of discriminating the different groups (Wagner et al., 2007), and thus can be used for with noisy LC-MS data. The preponderance of triglycerides (especially in positive ion mode: Figure 4.1, C and D, Retention time 7 – 10 min) might have made the PCA analysis ineffective, as too much of one set of compounds could make segregation difficult.

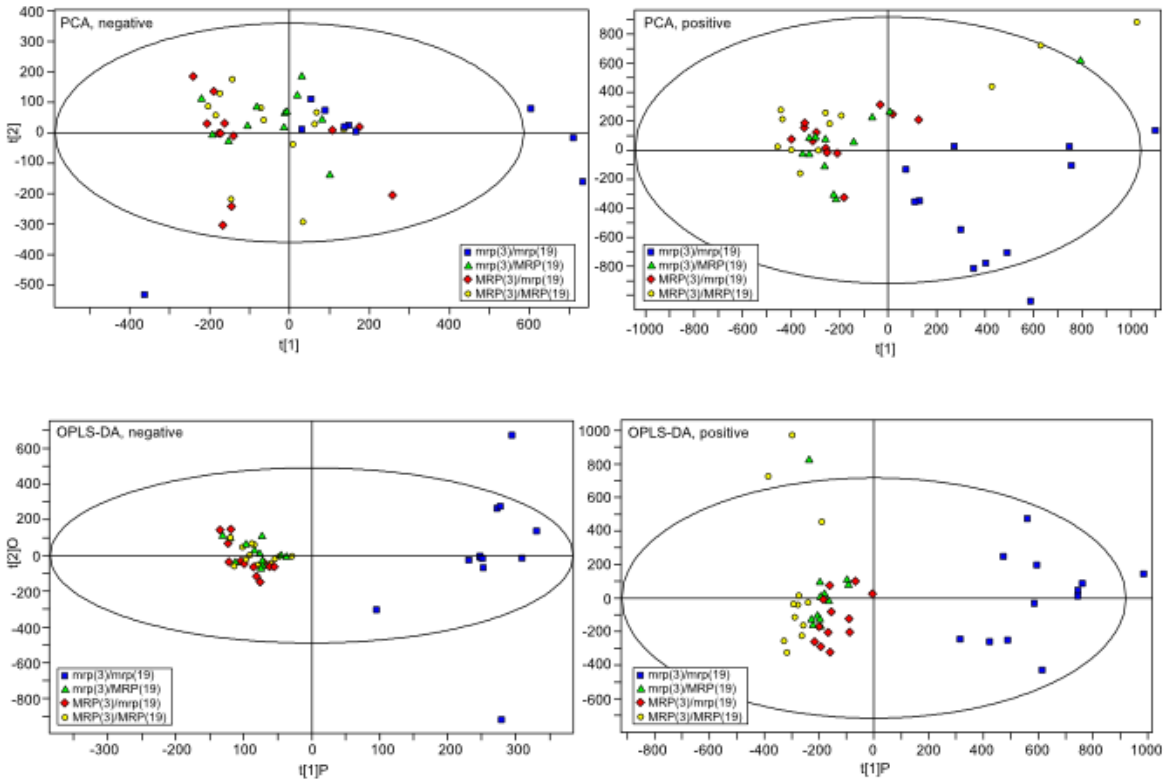

Figure 4.3. PCA and OPLS-DA Plots of the Four Near Isogenic Lines.

4.4.3 Ion Identification.

The ions were identified into classes according to their mass to charge (m/z) ratio and their Retention time (RT) based on literature review and publically available databases (see Materials and Methods). Most of the significantly different EMRTs were of higher concentration in the low phytate lines compared to the normal phytate lines. Table 4.2 summarizes the ions differing between the lines. It is clear that the low phytate line contained more storage lipids and a different phospholipid profile compared to the normal phytate lines.

Table 4.2. Identified EMRTs that are significantly different between low and normal phytate seed extracts.

			Average Peak Areas							
Ion mode	RT	m/z	*mrp*(3)/ *mrp*(19)	*mrp*(3)/ *MRP*(19)	*MRP*(3)/ *mrp*(19)	*MRP*(3)/ *MRP*(19)	Average NP lines	FOC* NP/LP	p Value	Lipid Class
(+)	5.48	577.5183	398	122	94	145	121	0.30	2.5E-03	PC(34:2)
(+)	7.55	589.4825	1438	741	667	622	676	0.47	6.2E-04	DG
(+)	7.84	591.4983	859	229	122	51	134	0.16	1.2E-08	DG
(+)	8.96	591.5342	5362	2505	2606	2300	2469	0.46	4.1E-04	DG
(+)	4.57	599.5023	5641	2202	2111	2414	2242	0.40	2.2E-03	DG
(+)	9.5	601.5188	2432	715	814	518	682	0.28	1.6E-03	DG
(+)	6.37	601.5192	1602	759	734	698	730	0.46	2.2E-06	DG
(+)	7.86	603.5339	471	132	14	7	51	0.11	1.2E-03	DG
(+)	6.77	603.5341	1097	441	462	442	448	0.41	3.1E-06	DG
(+)	8.69	603.5341	7593	26166	42229	41453	27734	3.65	5.5E-03	DG
(+)	9.48	603.5343	8650	4315	4382	3710	4137	0.48	1.6E-03	DG
(+)	5.61	604.5387	374	78	83	106	89	0.24	8.4E-04	PC(36:3)
(+)	7.69	615.4982	4309	2516	1843	1640	2000	0.46	3.2E-03	DG
(+)	8.18	617.5136	108	247	287	259	265	2.45	1.2E-03	DG
(+)	8.97	617.5498	2373	989	1008	924	974	0.41	1.3E-06	DG
(+)	8.05	631.5658	546	148	184	112	148	0.27	2.9E-05	DG
(+)	9.47	661.6123	22399	10102	9430	11077	10200	0.46	2.8E-03	DG
(+)	9.65	689.644	14529	5601	5333	5499	5478	0.38	4.7E-04	DG
(+)	9.82	717.6749	2538	639	648	515	601	0.24	4.3E-04	DG
(+)	4.88	782.5686	3646	743	914	1228	962	0.26	7.1E-03	PC(36:4)
(+)	8.81	856.7441	213	491	492	485	489	2.29	1.0E-04	TG
(+)	7.64	871.7372	524	353	198	219	257	0.49	6.1E-04	TG
(+)	9.82	990.941	878	379	373	327	360	0.41	4.7E-04	TG
(+)	9.75	1002.941	492	227	213	211	217	0.44	5.9E-04	TG
(+)	9.83	1022.916	655	264	282	258	268	0.41	8.0E-05	TG
(-)	6.95	627.5191	43	207	261	169	212	4.84	5.1E-05	PI
(-)	5.45	816.5765	295	27	55	24	35	0.12	4.5E-02	PC(28:0)
(-)	6.14	842.6703	2879	1069	1067	1036	1055	0.37	2.2E-02	PS(40:2)
(-)	4.05	861.5474	2408	764	905	794	821	0.34	4.5E-02	PI(36:2)
(-)	6.15	878.6459	649	256	240	267	255	0.39	1.7E-02	PS
(-)	6.14	952.7341	420	224	211	188	208	0.49	4.5E-02	PC

*Factor of change (peak area ratio, Average of all NP lines/Average of LP line), DG (diacylglycerol), PC (phosphatidylcholine), PS (phosphatidylserine), PI (phosphatidylinositol)

4.5 DISCUSSION

As previously shown, both *MRP* mutations are needed to obtain the low phytate and low emergence phenotypes. When this work was initiated, the mechanism by which the *MRP* mutations led to the observed phenotypes was not yet known. We hypothesized that by analyzing the metabolite differences between low and normal phytate lines, we might be able to draw

conclusions on how these mutations influence the seed composition. We have shown by analyzing methanol soluble metabolites between these low and high phytate lines that major changes lie in Group A soyasaponin levels (Chapter 3). As these metabolites have been associated with lipid membrane structure and function, we sought to determine the influence of low phytate on the lipid profiles of the same soybean lines.

PCA analysis in positive ion mode showed a separation of the low phytate line from the normal phytate lines (Figure 4.3) even though it was not as clear as it was for the methanol soluble metabolites. The lines did not separate along the x-axis, so it is a non-significant feature. In negative ion mode, there was no grouping between the classes in the PCA. If PCA does not show a clear separation, OPLS-DA plots are often used. OPLS-DA is a supervised analyses that uses class information to build algorithm to pull samples apart (Mahadevan et al., 2008). But because it is supervised, it might also lead to over fitting. In this analysis, the OPLS-DA plot showed a clear separation of the low phytate line from the normal phytate lines.

Hulke et al. (2004) crossed the low phytate line CX1834 to a line with normal phytate content and reduced palmitate content (B01769B019) and observed a reduced emergence rate in the low phytate lines of the progeny (reduced by 22.3%). Since previous results of low phytate progeny of CX1834 crossed to other lines also show a reduction in emergence rate between 20 and 30%, they suggested that the reduced palmitate content does not influence the emergence rate further (Hulke et al., 2004). Evaluation of the fatty ester content showed that the low phytate progeny has similar mean palmitate, stearate and palmitate+stearate concentrations relative to the normal phytate lines. The authors suggested that the low phytate trait does not influence the saturated

fatty acid concentration, but the concentrations were higher than those of the low palmitate parent used for making this cross (Hulke et al., 2004). This was due to increased palmitate and stearate content in parent CX1834.Hulke et al. (2002) suggested that this increased concentration could be due to genes for elevated saturated fat content in CX1834 that are linked to either or both of the *MRP* genes or that the *MRP* genes have a pleiotrophic effect on their concentration. Further study showed that the oleate concentration was significantly increased whereas the linoleate and linolenate concentrations significantly decreased in the low phytate lines (Hulke et al., 2004).

Most of the differences detected in this study are EMRTs that were of higher concentration in the low phytate line compared to the normal phytate lines (Table 4.2.). As previously mentioned, 20% of the soybean seed is oil that is used by the germinating plant as a carbon reserve. The diacylglycerols and triacylglycerols are storage lipids. The analyses conducted here indicated that low phytate seeds had an increased concentration of storage lipids than the normal phytate lines.

The phospholipids, phosphotidylethanolamines (PE), phosphatidylcholines (PC) and phosphatidylserines (PS) and phosphotidylinositol (PI), detected in the samples are components of cell membranes. The low and normal phytate lines analyzed differ in their membrane lipid profile. The low phytate line had a higher concentration of certain PI, PC and PS lipids, suggesting that the low phytate lines contained different membrane phospholipid compositions compared to normal phytate lines. The differences might make the membranes more vulnerable to environmental stressors such as temperature. It was shown that the difference in emergence

between low and normal phytate lines is much more pronounced when the seeds were produced in tropical vs. temperate climate (Maupin & Rainey, 2011). When these seeds that matured in high temperatures are planted out in colder climate, the low phytate lines have a severely reduced emergence rate compared to the normal phytate. Raboy (2009) hypothesizes that the differences between low and normal phytate lines are more pronounced when the seed was produced in tropical climate and further suggests that reduced heat tolerance of the low phytate seeds might be the cause (Raboy, 2009). The cells membrane fluidity is influenced by the phospholipid and fatty acid composition (Spector & Yorek, 1985). Membrane fluidity in turn influences many cellular processes, such as transport across the membranes or the activities of membrane bound enzymes (Spector & Yorek, 1985). Ren et al. suggest that high temperature during seed development reduces seed vigor due to the reduction in cellular membrane integrity brought about by a change in the membrane fatty acid composition (Ren et al., 2009). Dornbos et al. (1989) study the fatty acid composition of phospholipid classes and found that the composition of each class is altered when exposed to drought and high temperature stress (Dornbos et al., 1989). Increasing the temperature changes the ratio of phospholipids: increasing the concentration of phosphatidylcholine and phosphatidylinositol and decreasing the concentration of phosphatidylethanolamine (Dornbos et al., 1989). Drought and high temperature stress increase the ratios of saturated fatty acids (16:0 and 18:0) and decrease those of unsaturated fatty acids (18:2 and 18:3) in phosphatidylcholine and phosphatidylethanolamine. Similar changes are observed in the fatty acid composition of phosphatidylinositol: 18:3 concentration are increased, 16:0 decreased (Ren et al., 2009). Increased numbers of double bounds lead to an increased chilling resistance (Wada et al., 1990). The number of unsaturated fatty acids incorporated in membrane lipids increases when the cell is grown at colder temperatures (Sakamoto et al., 1997).

Thus high temperature favors less double bonds to maintain constant membrane fluidity and a seed maturing in high temps should have more saturated fatty acids. High temperature might influence membranes with a phospholipid composition of a low phytate line more negatively than that of a normal phytate line. Ren et al. (2009) explained that the stability of membranes was highly important to the developing seed when exposed to high temperatures (Ren et al., 2009). Fehr (2007) showed that soybeans with increased saturated fatty acids (palmitic and stearic acid) have decreased germination rates (Fehr, 2007). Taiz and Zeiger (1998) described the effect that high temperature has on the fatty acid composition of phospholipids. High temperature changes the composition and thus the fluidity of membrane lipids, which can lead to membrane instability, loss of function and ion leakage (Taiz & Zeiger, 1998). Dornbos et al. (1989) suggest that the changes in phospholipid class affect the seeds metabolism rates during germination.

We previously identified changes in the soyasaponin profiles between the low and normal phytate lines (Chapter 3). The low phytate line contained reduced levels of C22 glucose terminated Group A soyasaponins and higher levels of the C22 xylose terminated Group A soyasaponins, whereas the normal phytate NILs had the opposite profile. This class of compounds are present in highest concentrations in developing hypocotyls (Sasama et al., 2010) and are associated with membranes. A change in lipid membrane composition, combined with a change in the soyasaponin profile may alter the structural framework of all or specific organelle membranes hence reducing the ability of the developing seed to convert reserve protein and oil into biomass, resulting in poor emergence.

4.6 Conclusion

Low phytate lines can be discriminated from normal phytate lines according to their lipid profiles. Interestingly the low phytate line exhibited higher levels of triglycerides, similar to that observed for soybeans grown at elevated temperatures. Phospholipid profiles are also modified slightly, with a general trend of increased concentration of unsaturated phosopholipids. The combination of changes in membrane and storage lipid profiles combined with increased levels of xylose-based Group A soyasaponins may be the cause of low phytate levels. Further investigations based upon the information gained in this study should allow for a better understanding of the roles of MRPs and lipids in low phytate and emergence phenotypes.

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Chapter 5

Conclusions and Future Work

Near isogenic soybean lines differing in mutations in the two multidrug resistant protein (*MRP*) genes were developed as part of a larger effort to reduce phytate levels in soybean seeds. These lines were investigated in this dissertation in an effort to evaluate the effect of the phytate phenotype on the emergence rate and metabolite composition of the seeds. The main hypothesis tested is that the low phytate line shows a low emergence due to metabolic changes in seed composition.

The *MRP* genes encode ATP-binding cassette transporters. MRP ABC transporters are involved in accumulation of secondary metabolites and have been suggested to play a role in vacuolar sequestration of glucosides or accumulation of anthocyanin (Bartholomew et al., 2002; Yazaki, 2006). The phytate values, field emergence and extended cold test (ECT) emergence rates were determined for the four lines, their parents, CX1834 and V99-5089, as well as the control line V99-3337 for the 2010, 2011 and 2012 grown seeds. The four lines were also subjected to metabolomic profiling by analyzing the polar and non polar extract of the soybean powder.

The results presented here validated previous research efforts showing that both *MRP* mutations need to be present to obtain a low phytate phenotype. In 2010, the emergence rates of low phytate seeds grown in Blacksburg were higher than those grown in Mt. Holly. In 2011, seeds of all classes had a higher ECT emergence rate if produced in Mt. Holly. This may be due to

climatic differences, as Mt. Holly's climate was hotter, with no recorded precipitation during seed maturation and next year's planting. Analysis of the lines in two different environments showed that low phytate seeds did not always have significantly lower emergence rates relative to lines with normal phytate content. Only the low phytate lines produced in Blacksburg in 2010 showed a statistically significantly lower ECT emergence rate than the normal phytate lines. Low precipitation together with high temperatures during the seed filling and maturation stages seemed to negatively influence seed emergence of all lines, regardless of phytate content. Emergence did not solely depend on genotype. It seemed that the low phytate phenotype can be more negatively influenced than normal phytate lines but can emerge well under optimal environmental conditions during seed maturation and planting season. The low phytate phenotype might lead to structural changes in the membranes or changes in seed composition that makes the maturing seed less heat tolerant.

The assessment of the seed metabolite pools required the development of a protocol to extract and analyze the metabolites from the four near isogenic lines. Soybean seeds of each class were ground separately and extracted with ethyl acetate followed by methanol:water. The resulting non-polar and polar fractions were separated by three different orthogonal chromatographic columns (relying on reversed-phase and hydrophilic interaction) in both positive and negative ionization modes and detected by electrospray ionization mass spectrometry (UPLC-ESI-MS). The resulting datasets were then submitted to a principal component (PCA) and orthogonal projection of latent structures-discriminant (OPLS-DA) analyses to discriminate between these lines.

Analysis of the lipid-free methanol-soluble metabolites revealed differences between the low and normal phytate lines that lie mainly in the soyasaponin profile. The low phytate double mutant contained little to no C22 glucose terminated Group A soyasaponins and almost exclusively C22 xylose terminated Group A soyasaponins (A4, A5 and A6), whereas the normal phytate NILs contained no xylose terminated Group A soyasaponins. Saponins are known to cause membrane perturbations by formation of aqueous pores, which can increase membrane permeability (Augustin et al., 2011). The structure of a soyasaponin, for example the type of sugar in the sugar chain is important for the activity (Chwalek et al., 2006). It was previously shown that the C22 terminal sugar of synthesized hederagenin diglycoside saponins can influence the cytotoxic activity of the soyasaponin (Chwalek et al., 2006). Our results support the claim that the soyasaponin profile in low phytate lines negatively influences membrane stability and/or inter-organelle communication.

There are several possible explanations for the observed change in soyasaponin profiles. The low phytate levels themselves may be responsible for the observed changes or the differences are due to the pleiotropic effect of the combined *MRP* mutations. There are many different transporters in plant and the preferred combination of a substrate and a transporter is species dependent (Yazaki, 2006). Different sugar moieties on secondary metabolites act as tags recognized by different transporters (Yazaki, 2006). It is possible that the C22 terminal sugar of the Group A soyasaponins is a key determinant of MRP ABC transport processes. One can hypothesize that the terminal acetylated glucose may act as the tag recognized by MRP transporters and the MRP deficient low phytate line is thus incapable of transporting C22 glucose terminated Group A soyasaponins across the membrane. Alternatively, transport issues may

occur upstream of the final soyasaponin products (for example UDP-sugar transport), resulting in the observed profile. The net result is that the Group A profile has changed dramatically, affording C22 xylose terminated Group A soyasaponins. This change may influence seedling emergence by modulating glyoxysome formation or membrane transport processes. It has been previously shown that the terminal glycosylation (xylose or glucose) is controlled by multiple alleles of a single gene (*Sg-1* locus), which encodes for a UDP-sugar-dependent glycosyltransferase (Sayama et al., 2012). That Group A soyasaponins are found in highest concentrations in developing hypocotyls supports this possibility.

Analysis of the non-polar ethyl acetate extract showed that the low phytate line has increased amounts of storage lipids as well as a changed phospholipid profile compared to the normal phytate lines. The reasons for these differences are not known yet. It needs to be established if the changes are caused due to the *MRP* mutations, low phytate itself or if it is a secondary effect, e.g. caused by the changes in soyasaponin concentration. Nonetheless, the results described in Chapters 3 and 4 strongly support a scenario where lipid based processes are directly related to the low phytate and emergence phenotypes.

Efforts to determine the role of Group A soyasaponins on emergence could be undertaken at the metabolite and gene levels. A metabolomic analysis of other low phytate lines would provide soyasaponin levels in other lines to determine if all low phytate lines have high levels of xylose-terminated soyasaponins. Targeted transcriptional analysis of the four near isogenic lines and their parents would permit assessment of gene expression levels of the genes encoding the glycosyltransferases to determine which alleles are present in these lines and their relative

abundances. It would also be of interest to assess the soyasaponin levels in emerging seedlings. Finally, ultrastructural studies involving these lines may address possible structural changes in the seed or emerging hypocotyls that could explain the reduced emergence rates.

Emergence is difficult to study in the field as the slightest variation in temperature, moisture and soil condition can influence the emergence rate. An experimental setup utilizing growth chambers or a greenhouse where seeds are grown under controlled but different environmental conditions (heat, cold, drought), may prove informative. These seeds then need to be subsequenlty sown in the greenhouse/growth chambers under different environmental regimes. Assessment of the emergence rates, metabolite profiles and microscopic analysis of the germinated seedlings can further our understanding of soybean plant growth and development, leading to the eventual routine deployment of low phyate soybeans in the agricultural sector.

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APPENDIX A. Supplementary Figures and Tables for Chapter Two

Table S2.1. Simple effect comparisons of location*class least squares means by location (Blacksburg (A), Mt. Holly (B)) and by class (*mrp*(3)/*mrp*(19) (1), *mrp*(3)/*MRP(*19) (2), *MRP*(3)/*mrp*(19) (3), *MRP(*3)/*MRP*(19) (4), CX1834 (5), V99-5089 (6), V99-3337 (7))

2010 Emergence

Simple Effect Comparisons of Location*Class Least Squares Means By Location

Simple Effect Level	Class	_Class	Estimate	Standard Error	DF	t Value	Pr>|t|	Adj P
Location A	1	2	-1.7611	0.1410	39	-12.49	<.0001	<.0001
Location A	1	3	-0.9363	0.1472	39	-6.36	<.0001	<.0001
Location A	1	4	-1.3116	0.1434	39	-9.15	<.0001	<.0001
Location A	1	5	1.4382	0.4166	39	3.45	0.0014	0.0211
Location A	1	6	0.9887	0.3462	39	2.86	0.0068	0.0894
Location A	1	7	-2.3047	0.1867	39	-12.34	<.0001	<.0001
Location A	2	3	0.8248	0.1163	39	7.09	<.0001	<.0001
Location A	2	4	0.4496	0.1114	39	4.04	0.0002	0.0042
Location A	2	5	3.1993	0.4070	39	7.86	<.0001	<.0001
Location A	2	6	2.7498	0.3345	39	8.22	<.0001	<.0001
Location A	2	7	-0.5436	0.1639	39	-3.32	0.0020	0.0298
Location A	3	4	-0.3752	0.1192	39	-3.15	0.0031	0.0453
Location A	3	5	2.3745	0.4091	39	5.80	<.0001	<.0001
Location A	3	6	1.9250	0.3370	39	5.71	<.0001	<.0001
Location A	3	7	-1.3684	0.1691	39	-8.09	<.0001	<.0001
Location A	4	5	2.7498	0.4078	39	6.74	<.0001	<.0001
Location A	4	6	2.3002	0.3354	39	6.86	<.0001	<.0001
Location A	4	7	-0.9931	0.1658	39	-5.99	<.0001	<.0001
Location A	5	6	-0.4496	0.5144	39	-0.87	0.3875	0.9744
Location A	5	7	-3.7429	0.4242	39	-8.82	<.0001	<.0001
Location A	6	7	-3.2933	0.3552	39	-9.27	<.0001	<.0001
Location B	1	2	-1.1251	0.2175	39	-5.17	<.0001	0.0001
Location B	1	3	-0.4113	0.2365	39	-1.74	0.0899	0.5951
Location B	1	4	-0.3388	0.2392	39	-1.42	0.1647	0.7899
Location B	1	5	0.9458	0.4254	39	2.22	0.0321	0.3070
Location B	1	6	2.2267	0.7335	39	3.04	0.0043	0.0593
Location B	1	7	-1.6626	0.2775	39	-5.99	<.0001	<.0001
Location B	2	3	0.7138	0.1959	39	3.64	0.0008	0.0127
Location B	2	4	0.7863	0.1992	39	3.95	0.0003	0.0055
Location B	2	5	2.0708	0.4043	39	5.12	<.0001	0.0002
Location B	2	6	3.3518	0.7215	39	4.65	<.0001	0.0007
Location B	2	7	-0.5375	0.2452	39	-2.19	0.0344	0.3228
Location B	3	4	0.07249	0.2199	39	0.33	0.7434	0.9999
Location B	3	5	1.3570	0.4148	39	3.27	0.0022	0.0334
Location B	3	6	2.6380	0.7274	39	3.63	0.0008	0.0133
Location B	3	7	-1.2513	0.2614	39	-4.79	<.0001	0.0005
Location B	4	5	1.2846	0.4164	39	3.08	0.0037	0.0528
Location B	4	6	2.5655	0.7283	39	3.52	0.0011	0.0175
Location B	4	7	-1.3238	0.2638	39	-5.02	<.0001	0.0002
Location B	5	6	1.2809	0.8088	39	1.58	0.1213	0.6930
Location B	5	7	-2.6084	0.4393	39	-5.94	<.0001	<.0001
Location B	6	7	-3.8893	0.7415	39	-5.25	<.0001	0.0001

Simple Effect Comparisons of Location*Class Least Squares Means By Class

Simple Effect Level	Location	_Location	Estimate	Standard Error	DF	t Value	Pr>|t|	Adj P
Class 1	A	B	0.4526	0.4412	39	1.03	0.3113	0.3113
Class 2	A	B	1.0887	0.4110	39	2.65	0.0116	0.0116
Class 3	A	B	0.9776	0.4236	39	2.31	0.0264	0.0264
Class 4	A	B	1.4254	0.4239	39	3.36	0.0017	0.0017
Class 5	A	B	-0.03984	0.6752	39	-0.06	0.9533	0.9533

Class 6	A	B	1.6906	0.8714	39	1.94	0.0596	0.0596
Class 7	A	B	1.0947	0.4642	39	2.36	0.0235	0.0235

2010 Phytate

Simple Effect Comparisons of Location*Class Least Squares Means By Location

Simple Effect Level	Class	_Class	Estimate	Standard Error	DF	t Value	Pr>|t|	Adj P
Location A	1	2	-18.1257	1.3681	38	-13.25	<.0001	<.0001
Location A	1	3	-17.1129	1.3681	38	-12.51	<.0001	<.0001
Location A	1	4	-19.0357	1.3681	38	-13.91	<.0001	<.0001
Location A	1	5	-3.4507	2.7871	38	-1.24	0.2233	0.8746
Location A	1	6	-12.2262	2.0584	38	-5.94	<.0001	<.0001
Location A	1	7	-16.5962	2.0584	38	-8.06	<.0001	<.0001
Location A	2	3	1.0129	1.3681	38	0.74	0.4637	0.9890
Location A	2	4	-0.9100	1.3681	38	-0.67	0.5100	0.9938
Location A	2	5	14.6750	2.7871	38	5.27	<.0001	0.0001
Location A	2	6	5.8995	2.0584	38	2.87	0.0067	0.0881
Location A	2	7	1.5295	2.0584	38	0.74	0.4620	0.9888
Location A	3	4	-1.9229	1.3681	38	-1.41	0.1680	0.7955
Location A	3	5	13.6622	2.7871	38	4.90	<.0001	0.0003
Location A	3	6	4.8866	2.0584	38	2.37	0.0228	0.2375
Location A	3	7	0.5166	2.0584	38	0.25	0.8032	1.0000
Location A	4	5	15.5850	2.7871	38	5.59	<.0001	<.0001
Location A	4	6	6.8095	2.0584	38	3.31	0.0021	0.0309
Location A	4	7	2.4395	2.0584	38	1.19	0.2433	0.8954
Location A	5	6	-8.7755	3.1567	38	-2.78	0.0084	0.1063
Location A	5	7	-13.1455	3.1567	38	-4.16	0.0002	0.0030
Location A	6	7	-4.3700	2.5596	38	-1.71	0.0959	0.6155
Location B	1	2	-14.8375	1.8099	38	-8.20	<.0001	<.0001
Location B	1	3	-15.9250	1.8099	38	-8.80	<.0001	<.0001
Location B	1	4	-16.6500	1.8099	38	-9.20	<.0001	<.0001
Location B	1	5	-7.0750	2.2167	38	-3.19	0.0028	0.0412
Location B	1	6	-9.4100	2.2167	38	-4.25	0.0001	0.0024
Location B	1	7	-12.8414	2.8896	38	-4.44	<.0001	0.0013
Location B	2	3	-1.0875	1.8099	38	-0.60	0.5515	0.9964
Location B	2	4	-1.8125	1.8099	38	-1.00	0.3229	0.9506
Location B	2	5	7.7625	2.2167	38	3.50	0.0012	0.0188
Location B	2	6	5.4275	2.2167	38	2.45	0.0191	0.2072
Location B	2	7	1.9961	2.8896	38	0.69	0.4939	0.9924
Location B	3	4	-0.7250	1.8099	38	-0.40	0.6910	0.9996
Location B	3	5	8.8500	2.2167	38	3.99	0.0003	0.0049
Location B	3	6	6.5150	2.2167	38	2.94	0.0056	0.0748
Location B	3	7	3.0836	2.8896	38	1.07	0.2926	0.9338
Location B	4	5	9.5750	2.2167	38	4.32	0.0001	0.0019
Location B	4	6	7.2400	2.2167	38	3.27	0.0023	0.0343
Location B	4	7	3.8086	2.8896	38	1.32	0.1954	0.8393
Location B	5	6	-2.3350	2.5596	38	-0.91	0.3674	0.9683
Location B	5	7	-5.7664	3.1603	38	-1.82	0.0759	0.5404
Location B	6	7	-3.4314	3.1603	38	-1.09	0.2844	0.9285

Simple Effect Comparisons of Location*Class Least Squares Means By Class

Simple Effect Level	Location	_Location	Estimate	Standard Error	DF	t Value	Pr>|t|	Adj P
Class 1	A	B	-1.3112	1.7968	38	-0.73	0.4700	0.4700
Class 2	A	B	1.9770	1.7968	38	1.10	0.2781	0.2781
Class 3	A	B	-0.1234	1.7968	38	-0.07	0.9456	0.9456
Class 4	A	B	1.0745	1.7968	38	0.60	0.5534	0.5534
Class 5	A	B	-4.9355	3.2549	38	-1.52	0.1377	0.1377
Class 6	A	B	1.5050	2.6797	38	0.56	0.5777	0.5777

Class 7	A	B	2.4436	3.2584	38	0.75	0.4579	0.4579

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Simple Effect Comparisons of Location*Class Least Squares Means By Location

Simple Effect Level	Class	_Class	Estimate	Standard Error	DF	t Value	Pr>|t|	Adj P
Location A	1	2	-0.4319	0.04759	265	-9.08	<.0001	<.0001
Location A	1	3	-0.1809	0.04724	265	-3.83	0.0002	0.0030
Location A	1	4	0.01729	0.04723	265	0.37	0.7146	0.9998
Location A	1	5	0.4232	0.06829	265	6.20	<.0001	<.0001
Location A	1	6	-0.3733	0.05626	265	-6.64	<.0001	<.0001
Location A	1	7	-1.5295	0.08095	265	-18.90	<.0001	<.0001
Location A	2	3	0.2510	0.04761	265	5.27	<.0001	<.0001
Location A	2	4	0.4492	0.04760	265	9.44	<.0001	<.0001
Location A	2	5	0.8551	0.06855	265	12.47	<.0001	<.0001
Location A	2	6	0.05858	0.05656	265	1.04	0.3013	0.9453
Location A	2	7	-1.0976	0.08116	265	-13.52	<.0001	<.0001
Location A	3	4	0.1982	0.04725	265	4.19	<.0001	0.0007
Location A	3	5	0.6040	0.06831	265	8.84	<.0001	<.0001
Location A	3	6	-0.1924	0.05627	265	-3.42	0.0007	0.0127
Location A	3	7	-1.3486	0.08096	265	-16.66	<.0001	<.0001
Location A	4	5	0.4059	0.06830	265	5.94	<.0001	<.0001
Location A	4	6	-0.3906	0.05626	265	-6.94	<.0001	<.0001
Location A	4	7	-1.5468	0.08095	265	-19.11	<.0001	<.0001
Location A	5	6	-0.7965	0.07483	265	-10.64	<.0001	<.0001
Location A	5	7	-1.9527	0.09480	265	-20.60	<.0001	<.0001
Location A	6	7	-1.1562	0.08653	265	-13.36	<.0001	<.0001
Location B	1	2	-0.2594	0.07001	265	-3.71	0.0003	0.0047
Location B	1	3	0.5415	0.07726	265	7.01	<.0001	<.0001
Location B	1	4	0.5877	0.07550	265	7.78	<.0001	<.0001
Location B	1	5	1.4742	0.1289	265	11.44	<.0001	<.0001
Location B	1	6	0.2084	0.08515	265	2.45	0.0150	0.1832
Location B	1	7	-1.1181	0.09374	265	-11.93	<.0001	<.0001
Location B	2	3	0.8009	0.07555	265	10.60	<.0001	<.0001
Location B	2	4	0.8471	0.07368	265	11.50	<.0001	<.0001
Location B	2	5	1.7336	0.1279	265	13.56	<.0001	<.0001
Location B	2	6	0.4678	0.08363	265	5.59	<.0001	<.0001
Location B	2	7	-0.8587	0.09227	265	-9.31	<.0001	<.0001
Location B	3	4	0.04623	0.08061	265	0.57	0.5668	0.9975
Location B	3	5	0.9327	0.1318	265	7.07	<.0001	<.0001
Location B	3	6	-0.3330	0.08960	265	-3.72	0.0002	0.0046
Location B	3	7	-1.6596	0.09791	265	-16.95	<.0001	<.0001
Location B	4	5	0.8865	0.1309	265	6.77	<.0001	<.0001
Location B	4	6	-0.3793	0.08821	265	-4.30	<.0001	0.0005
Location B	4	7	-1.7058	0.09652	265	-17.67	<.0001	<.0001
Location B	5	6	-1.2658	0.1366	265	-9.27	<.0001	<.0001
Location B	5	7	-2.5923	0.1422	265	-18.23	<.0001	<.0001
Location B	6	7	-1.3265	0.1043	265	-12.72	<.0001	<.0001

Simple Effect Comparisons of Location*Class Least Squares Means By Class

Simple Effect Level	Location	_Location	Estimate	Standard Error	DF	t Value	Pr>|t|	Adj P
Class 1	A	B	0.5317	0.2388	265	2.23	0.0268	0.0268
Class 2	A	B	0.7042	0.2382	265	2.96	0.0034	0.0034
Class 3	A	B	1.2541	0.2405	265	5.22	<.0001	<.0001
Class 4	A	B	1.1022	0.2399	265	4.59	<.0001	<.0001
Class 5	A	B	1.5828	0.2663	265	5.94	<.0001	<.0001

Class 6	A	B	1.1135	0.2450	265	4.54	<.0001	<.0001
Class 7	A	B	0.9431	0.2548	265	3.70	0.0003	0.0003

2011 Phytate

Simple Effect Comparisons of Location*Class Least Squares Means By Location

Simple Effect Level	Class	_Class	Estimate	Standard Error	DF	t Value	Pr>|t|	Adj P
Location A	1	2	-11.3342	0.3487	316	-32.50	<.0001	<.0001
Location A	1	3	-11.5268	0.3509	316	-32.85	<.0001	<.0001
Location A	1	4	-11.4129	0.3606	316	-31.65	<.0001	<.0001
Location A	1	5	-1.3346	0.4269	316	-3.13	0.0019	0.0315
Location A	1	6	-4.9020	0.4270	316	-11.48	<.0001	<.0001
Location A	1	7	-9.8733	0.5031	316	-19.62	<.0001	<.0001
Location A	2	3	-0.1926	0.3585	316	-0.54	0.5915	0.9983
Location A	2	4	-0.07867	0.3680	316	-0.21	0.8308	1.0000
Location A	2	5	9.9996	0.4332	316	23.08	<.0001	<.0001
Location A	2	6	6.4322	0.4333	316	14.85	<.0001	<.0001
Location A	2	7	1.4609	0.5085	316	2.87	0.0043	0.0648
Location A	3	4	0.1139	0.3701	316	0.31	0.7584	0.9999
Location A	3	5	10.1922	0.4349	316	23.44	<.0001	<.0001
Location A	3	6	6.6248	0.4351	316	15.23	<.0001	<.0001
Location A	3	7	1.6535	0.5100	316	3.24	0.0013	0.0221
Location A	4	5	10.0782	0.4429	316	22.76	<.0001	<.0001
Location A	4	6	6.5109	0.4427	316	14.71	<.0001	<.0001
Location A	4	7	1.5396	0.5166	316	2.98	0.0031	0.0483
Location A	5	6	-3.5674	0.4985	316	-7.16	<.0001	<.0001
Location A	5	7	-8.5386	0.5650	316	-15.11	<.0001	<.0001
Location A	6	7	-4.9712	0.5650	316	-8.80	<.0001	<.0001
Location B	1	2	-11.1468	0.5294	316	-21.06	<.0001	<.0001
Location B	1	3	-11.0158	0.5436	316	-20.27	<.0001	<.0001
Location B	1	4	-12.1348	0.5294	316	-22.92	<.0001	<.0001
Location B	1	5	-2.0704	0.6687	316	-3.10	0.0021	0.0345
Location B	1	6	-5.4874	0.6462	316	-8.49	<.0001	<.0001
Location B	1	7	-9.5509	0.6965	316	-13.71	<.0001	<.0001
Location B	2	3	0.1310	0.5370	316	0.24	0.8074	1.0000
Location B	2	4	-0.9880	0.5226	316	-1.89	0.0596	0.4880
Location B	2	5	9.0764	0.6633	316	13.68	<.0001	<.0001
Location B	2	6	5.6595	0.6409	316	8.83	<.0001	<.0001
Location B	2	7	1.5959	0.6914	316	2.31	0.0216	0.2432
Location B	3	4	-1.1190	0.5370	316	-2.08	0.0380	0.3648
Location B	3	5	8.9454	0.6747	316	13.26	<.0001	<.0001
Location B	3	6	5.5284	0.6521	316	8.48	<.0001	<.0001
Location B	3	7	1.4649	0.7022	316	2.09	0.0378	0.3634
Location B	4	5	10.0644	0.6633	316	15.17	<.0001	<.0001
Location B	4	6	6.6475	0.6409	316	10.37	<.0001	<.0001
Location B	4	7	2.5839	0.6914	316	3.74	0.0002	0.0041
Location B	5	6	-3.4170	0.7600	316	-4.50	<.0001	0.0002
Location B	5	7	-7.4805	0.8030	316	-9.32	<.0001	<.0001
Location B	6	7	-4.0636	0.7841	316	-5.18	<.0001	<.0001

Simple Effect Comparisons of Location*Class Least Squares Means By Class

Simple Effect Level	Location	_Location	Estimate	Standard Error	DF	t Value	Pr>|t|	Adj P
Class 1	A	B	-0.2500	0.4976	316	-0.50	0.6157	0.6157
Class 2	A	B	-0.06263	0.4959	316	-0.13	0.8996	0.8996
Class 3	A	B	0.2610	0.5123	316	0.51	0.6108	0.6108

Class 4	A	B	-0.9720	0.5042	316	-1.93	0.0548	0.0548
Class 5	A	B	-0.9858	0.6881	316	-1.43	0.1529	0.1529
Class 6	A	B	-0.8354	0.6659	316	-1.25	0.2106	0.2106
Class 7	A	B	0.07232	0.7629	316	0.09	0.9245	0.9245

2012 Emergence

Simple Effect Comparisons of Location*Class Least Squares Means By Location

Simple Effect Level	Class	_Class	Estimate	Standard Error	DF	t Value	Pr>|t|	Adj P
Location A	1	2	-0.4571	0.09171	108	-4.98	<.0001	<.0001
Location A	1	3	0.5436	0.08256	108	6.58	<.0001	<.0001
Location A	1	4	-0.00817	0.08631	108	-0.09	0.9248	1.0000
Location A	1	5	0.4725	0.1352	108	3.50	0.0007	0.0119
Location A	1	6	-0.6195	0.2936	108	-2.11	0.0371	0.3542
Location A	1	7	0.2882	0.1238	108	2.33	0.0217	0.2401
Location A	2	3	1.0007	0.09485	108	10.55	<.0001	<.0001
Location A	2	4	0.4490	0.09813	108	4.58	<.0001	0.0003
Location A	2	5	0.9296	0.1430	108	6.50	<.0001	<.0001
Location A	2	6	-0.1624	0.2972	108	-0.55	0.5860	0.9980
Location A	2	7	0.7454	0.1323	108	5.63	<.0001	<.0001
Location A	3	4	-0.5518	0.08963	108	-6.16	<.0001	<.0001
Location A	3	5	-0.07113	0.1373	108	-0.52	0.6055	0.9985
Location A	3	6	-1.1631	0.2945	108	-3.95	0.0001	0.0026
Location A	3	7	-0.2554	0.1261	108	-2.03	0.0453	0.4052
Location A	4	5	0.4807	0.1396	108	3.44	0.0008	0.0140
Location A	4	6	-0.6113	0.2956	108	-2.07	0.0410	0.3791
Location A	4	7	0.2964	0.1286	108	2.30	0.0231	0.2513
Location A	5	6	-1.0920	0.3134	108	-3.48	0.0007	0.0123
Location A	5	7	-0.1843	0.1654	108	-1.11	0.2678	0.9226
Location A	6	7	0.9077	0.3086	108	2.94	0.0040	0.0592
Location B	1	2	-0.4501	0.07920	108	-5.68	<.0001	<.0001
Location B	1	3	0.1950	0.07581	108	2.57	0.0115	0.0829
Location B	1	4	0.5806	0.09708	108	5.98	<.0001	<.0001
Location B	1	7	-0.8227	0.1378	108	-5.97	<.0001	<.0001
Location B	2	3	0.6451	0.08069	108	7.99	<.0001	<.0001
Location B	2	4	1.0307	0.1009	108	10.21	<.0001	<.0001
Location B	2	7	-0.3726	0.1406	108	-2.65	0.0092	0.0684
Location B	3	4	0.3856	0.09830	108	3.92	0.0002	0.0014
Location B	3	7	-1.0177	0.1387	108	-7.34	<.0001	<.0001
Location B	4	7	-1.4033	0.1514	108	-9.27	<.0001	<.0001

Simple Effect Comparisons of Location*Class Least Squares Means By Class

Simple Effect Level	Location	_Location	Estimate	Standard Error	DF	t Value	Pr>|t|	Adj P
Class 1	A	B	0.5685	0.07662	108	7.42	<.0001	<.0001
Class 2	A	B	0.5755	0.09388	108	6.13	<.0001	<.0001

Class 3	A	B	0.2198	0.08181	108	2.69	0.0083	0.0083
Class 4	A	B	1.1573	0.1049	108	11.03	<.0001	<.0001
Class 7	A	B	-0.5425	0.1687	108	-3.22	0.0017	0.0017

2012 Phytate

Simple Effect Comparisons of Location*Class Least Squares Means By Location

Simple Effect Level	Class	_Class	Estimate	Standard Error	DF	t Value	Pr>|t|	Adj P
Location A	1	2	-9.6258	0.4094	285	-23.51	<.0001	<.0001
Location A	1	3	-10.4691	0.4123	285	-25.39	<.0001	<.0001
Location A	1	4	-11.4909	0.4094	285	-28.07	<.0001	<.0001
Location A	1	5	-1.2123	1.1216	285	-1.08	0.2807	0.9333
Location A	1	6	-4.9305	0.7633	285	-6.46	<.0001	<.0001
Location A	1	7	-8.4641	0.7204	285	-11.75	<.0001	<.0001
Location A	2	3	-0.8433	0.4288	285	-1.97	0.0502	0.4384
Location A	2	4	-1.8651	0.4259	285	-4.38	<.0001	0.0003
Location A	2	5	8.4135	1.1279	285	7.46	<.0001	<.0001
Location A	2	6	4.6953	0.7724	285	6.08	<.0001	<.0001
Location A	2	7	1.1617	0.7300	285	1.59	0.1126	0.6880
Location A	3	4	-1.0218	0.4288	285	-2.38	0.0178	0.2097
Location A	3	5	9.2568	1.1285	285	8.20	<.0001	<.0001
Location A	3	6	5.5386	0.7737	285	7.16	<.0001	<.0001
Location A	3	7	2.0050	0.7316	285	2.74	0.0065	0.0922
Location A	4	5	10.2787	1.1279	285	9.11	<.0001	<.0001
Location A	4	6	6.5605	0.7724	285	8.49	<.0001	<.0001
Location A	4	7	3.0268	0.7300	285	4.15	<.0001	0.0009
Location A	5	6	-3.7182	1.2981	285	-2.86	0.0045	0.0667
Location A	5	7	-7.2518	1.2741	285	-5.69	<.0001	<.0001
Location A	6	7	-3.5336	0.9736	285	-3.63	0.0003	0.0061
Location B	1	2	-8.1512	0.4824	285	-16.90	<.0001	<.0001
Location B	1	3	-8.7697	0.4747	285	-18.48	<.0001	<.0001
Location B	1	4	-9.7506	0.5365	285	-18.17	<.0001	<.0001
Location B	1	5	1.8381	1.9153	285	0.96	0.3380	0.9620
Location B	1	6	-3.7051	1.3710	285	-2.70	0.0073	0.1013
Location B	1	7	-7.9768	0.8370	285	-9.53	<.0001	<.0001
Location B	2	3	-0.6185	0.4860	285	-1.27	0.2042	0.8639
Location B	2	4	-1.5994	0.5467	285	-2.93	0.0037	0.0565
Location B	2	5	9.9893	1.9195	285	5.20	<.0001	<.0001
Location B	2	6	4.4461	1.3750	285	3.23	0.0014	0.0229
Location B	2	7	0.1744	0.8435	285	0.21	0.8363	1.0000
Location B	3	4	-0.9809	0.5394	285	-1.82	0.0701	0.5368
Location B	3	5	10.6078	1.9184	285	5.53	<.0001	<.0001
Location B	3	6	5.0646	1.3721	285	3.69	0.0003	0.0049
Location B	3	7	0.7930	0.8388	285	0.95	0.3453	0.9648
Location B	4	5	11.5887	1.9342	285	5.99	<.0001	<.0001
Location B	4	6	6.0455	1.3948	285	4.33	<.0001	0.0004
Location B	4	7	1.7738	0.8754	285	2.03	0.0437	0.4004
Location B	5	6	-5.5432	2.3093	285	-2.40	0.0170	0.2022
Location B	5	7	-9.8149	2.0381	285	-4.82	<.0001	<.0001
Location B	6	7	-4.2717	1.5356	285	-2.78	0.0058	0.0829

Simple Effect Comparisons of Location*Class Least Squares Means By Class

Simple

Effect Level	Location_Location		Estimate	Standard Error	DF	t Value	Pr>|t|	Adj P
Class 1	A	B	-1.6140	0.9132	285	-1.77	0.0782	0.0782
Class 2	A	B	-0.1394	0.9268	285	-0.15	0.8805	0.8805
Class 3	A	B	0.08535	0.9238	285	0.09	0.9265	0.9265
Class 4	A	B	0.1263	0.9559	285	0.13	0.8950	0.8950
Class 5	A	B	1.4364	2.3220	285	0.62	0.5367	0.5367
Class 6	A	B	-0.3886	1.7089	285	-0.23	0.8203	0.8203
Class 7	A	B	-1.1267	1.2953	285	-0.87	0.3851	0.3851

Table S2.2. ANOVA tables (Type III Test of Fixed Effects)

2010 Emergence ANOVA Table **2010 Phytate ANOVA Table**

Type III Test of Fixed Effects

Effect	Num DF	Den DF	F Value	PR > F
Location	1	2	5.34	0.1471
Class	6	39	50.8	<0.0001
Location*Class	6	39	2.82	0.0224

Type III Test of Fixed Effects

Effect	Num DF	Den DF	F Value	PR > F
Location	1	2	0.01	0.9459
Class	6	38	60.17	<0.0001
Location*Class	6	38	0.97	0.4577

2011 Emergence ANOVA Table

Type III Test of Fixed Effects

Effect	Num DF	Den DF	F Value	PR > F
Location	1	2	19.63	0.0474
Class	6	265	175.44	<0.0001
Location*Class	6	265	19.49	<0.0001

2011 Phytate ANOVA Table

Type III Test of Fixed Effects

Effect	Num DF	Den DF	F Value	PR > F
Location	1	2	1.74	0.3184
Class	6	316	401.57	<0.0001
Location*Class	6	316	0.99	0.4353

2012 Phytate ANOVA Table

Type III Test of Fixed Effects

Effect	Num DF	Den DF	F Value	PR > F
Location	1	2	0.06	0.823
Class	6	285	249.67	<0.0001
Location*Class	6	285	1.92	0.0781

Table S2.3. Weather History for Blacksburg, VA and Tappahannock, VA

Blacksburg			Max Temper-ature	Mean Temper-ature	Min Temper-ature	Heating Degree Days (base 65)	Cooling Degree Days (base 65)	Growing Degree Days (base 50)	Dew Point	Preci-pitation	Sea Level Pressure
2010	**May**	**Avg**	74 °F	63 °F	51 °F	4	2	15	59 °F	0.18 in	30.10 in
2010	**June**	**Avg**	84 °F	72 °F	61 °F	0	7	25	69 °F	0.04 in	30.09 in
2010	**July**	**Avg**	86 °F	74 °F	62 °F	0	9	26	69 °F	0.15 in	30.14 in
2010	**August**	**Avg**	83 °F	73 °F	63 °F	0	8	26	70 °F	0.19 in	30.11 in
2010	**September**	**Avg**	89 °F	73 °F	62 °F	13	8	25	72 °F	1.20 in	30.34 in
2010	**October**	**Avg**	68 °F	55 °F	42 °F	10	0	6	45 °F	0.10 in	30.05 in
2011	**May**	**Avg**	73 °F	62 °F	51 °F	5	2	12	55 °F	0.28 in	30.04 in
2011	**June**	**Avg**	90 °F	77 °F	64 °F	4	12	28	77 °F	1.38 in	30.34 in
2011	**July**	**Avg**	86 °F	75 °F	64 °F	0	10	26	69 °F	0.15 in	30.10 in
2011	**August**	**Avg**	90 °F	79 °F	70 °F	1	14	30	79 °F	0.51 in	30.30 in
2011	**September**	**Avg**	90 °F	75 °F	63 °F	18	10	28	73 °F	2.17 in	30.41 in
2011	**October**	**Avg**	63 °F	52 °F	41 °F	13	0	6	46 °F	0.11 in	30.08 in
2012	**May**	**Avg**	76 °F	65 °F	53 °F	2	2	16	60 °F	0.14 in	30.09 in
2012	**June**	**Avg**	95 °F	80 °F	66 °F	9	15	34	73 °F	1.22 in	30.38 in
2012	**July**	**Avg**	93 °F	80 °F	68 °F	0	15	30	75 °F	1.29 in	30.32 in
2012	**August**	**Avg**	88 °F	76 °F	66 °F	0	11	28	75 °F	1.11 in	30.32 in
2012	**September**	**Avg**	86 °F	76 °F	68 °F	15	11	27	75 °F	1.20 in	30.46 in
2012	**October**	**Avg**	63 °F	52 °F	41 °F	13	0	5	48 °F	0.06 in	30.04 in

Mt. Holly			Max Temper-ature	Mean Temper-ature	Min Temper-ature	Heating Degree Days (base 65)	Cooling Degree Days (base 65)	Growing Degree Days (base 50)	Dew Point	Preci-pitation	Sea Level Pressure
2010	**May**	**Avg**	80 °F	69 °F	58 °F	2	6	19	57 °F	0.00 in	30.02 in
2010	**June**	**Avg**	90 °F	79 °F	68 °F	0	14	29	66 °F	0.00 in	29.94 in
2010	**July**	**Avg**	104 °F	91 °F	78 °F	0	26	41	79 °F	0.00 in	30.27 in
2010	**August**	**Avg**	98 °F	86 °F	77 °F	0	20	36	79 °F	0.00 in	30.40 in
2010	**September**	**Avg**	86 °F	74 °F	62 °F	0	9	24	60 °F	0.00 in	29.98 in
2010	**October**	**Avg**	89 °F	76 °F	71 °F	19	12	26	75 °F	0.00 in	30.30 in
2011	**May**	**Avg**	79 °F	69 °F	58 °F	2	5	19	61 °F	0.00 in	29.96 in
2011	**June**	**Avg**	100 °F	84 °F	75 °F	0	18	34	81 °F	0.00 in	30.19 in
2011	**July**	**Avg**	91 °F	81 °F	71 °F	0	16	31	73 °F	0.00 in	29.95 in
2011	**August**	**Avg**	88 °F	78 °F	68 °F	0	13	28	71 °F	0.00 in	29.91 in
2011	**September**	**Avg**	89 °F	79 °F	71 °F	7	14	29	81 °F	0.00 in	30.37 in
2011	**October**	**Avg**	82 °F	75 °F	68 °F	24	10	25	75 °F	0.00 in	30.53 in
2012	**May**	**Avg**	89 °F	80 °F	71 °F	6	14	30	75 °F	0.00 in	30.33 in
2012	**June**	**Avg**	85 °F	73 °F	60 °F	0	8	23	62 °F	0.00 in	29.96 in
2012	**July**	**Avg**	92 °F	81 °F	71 °F	0	17	31	71 °F	0.00 in	29.96 in
2012	**August**	**Avg**	86 °F	77 °F	68 °F	0	12	27	71 °F	0.00 in	30.00 in
2012	**September**	**Avg**	90 °F	81 °F	74 °F	8	16	31	79 °F	0.00 in	30.39 in
2012	**October**	**Avg**	71 °F	60 °F	50 °F	6	2	11	55 °F	0.00 in	29.97 in

APPENDIX B. Supplementary Figures and Tables for Chapter Three

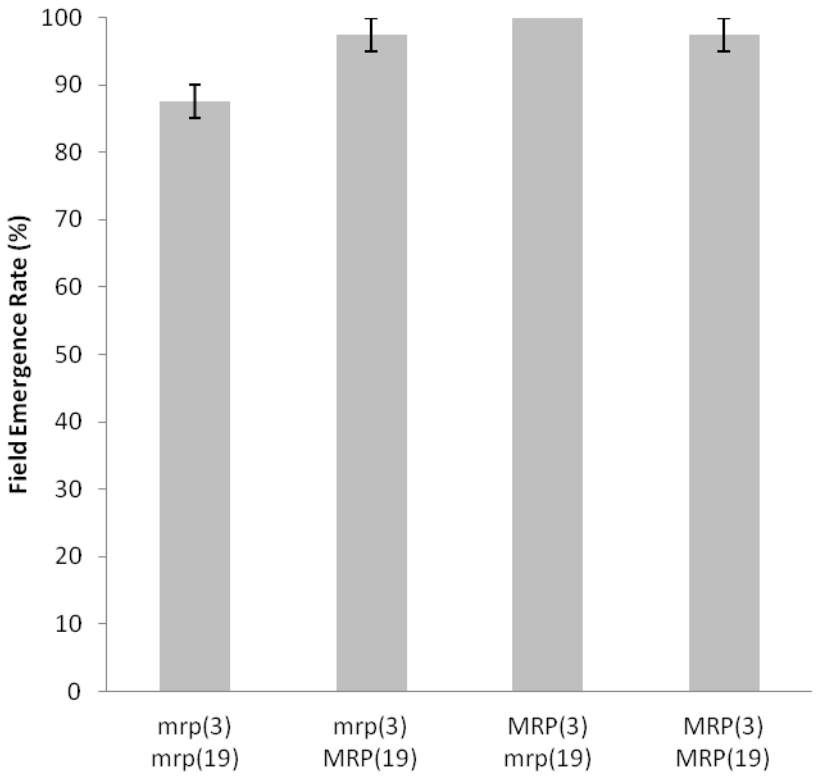


Figure S3.1. Mean field emergence rates in the four NILs of the 2012 harvested seeds.

Table S3.1. Phytate values, ECT emergence and field emergence rates of the four classes and their parents from 2009-2013. *was used for Metabolomics, **about 4 weeks after planting or when available, ^came from 2012 Blacksburg, ^^came from 2012 Mt. Holly

	row #	Phytate				ECT Emergence			Field Emergence**
		rep 1	rep 2	rep 3	average	rep 1	rep 2	average	
2012 Blacksburg									
mrp(3)/*mrp*(19)	715*	5.83	1.63	3.23	**3.56**	47	N/A	**47**	**50**
mrp(3)/*MRP*(19)	720*	13.62	15.85	13.05	**14.17**	96	N/A	**96**	**60**
MRP(3)/*MRP*(19)	731*	13.99	15.53	13.92	**14.48**	97	N/A	**97**	**60**
MRP(3)/*mrp*(19)	733*	15.60	16.98	16.88	**16.49**	86	N/A	**86**	**30**
CX1834	704	7.65	7.37	5.46	**6.83**	N/A	N/A	N/A	**80**
V995089	714	8.17	7.21	10.29	**8.55**	N/A	N/A	N/A	**70**
V993337	717	13.86	14.37	11.76	**13.33**	N/A	N/A	N/A	**70**
2013 Blacksburg (Field A^)									
mrp(3)/*mrp*(19)	2862	N/A	N/A	N/A	N/A	10	2	**6**	**90**
mrp(3)/*MRP*(19)	2863	N/A	N/A	N/A	N/A	37	54	**45.5**	**100**
MRP(3)/*MRP*(19)	2869	N/A	N/A	N/A	N/A	37	22	**29.5**	**100**
MRP(3)/*mrp*(19)	2859	N/A	N/A	N/A	N/A	65	78	**71.5**	**100**
CX1834	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
V995089	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
V993337	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2013 Blacksburg (Field B^)									
mrp(3)/*mrp*(19)	2963	7.68	7.78	10.89	**8.79**	5	16	**10.5**	**85**
mrp(3)/*MRP*(19)	2959	15.51	19.12	16.74	**17.13**	44	60	**52**	**95**
MRP(3)/*MRP*(19)	2953	17.90	20.43	18.18	**18.83**	41	50	**45.5**	**100**
MRP(3)/*mrp*(19)	2950	17.01	18.40	17.45	**17.62**	64	64	**64**	**95**

CX1834	2961	12.70	9.63	9.21	**10.51**	N/A	N/A	N/A	**87.5**
V995089	2957	10.94	12.68	15.89	**13.17**	N/A	N/A	N/A	**77.5**
V993337	2951	17.31	17.85	16.79	**17.32**	N/A	N/A	N/A	**72.5**
2012 Mt. Holly (corresponding to 2012 Blacksburg)									
mrp(3)/*mrp*(19)	41	5.89	3.55	5.13	**4.86**	N/A	N/A	N/A	**30**
mrp(3)/*MRP*(19)	44	15.78	13.80	14.33	**14.64**	50	N/A	**50**	**20**
MRP(3)/*MRP*(19)	52	16.24	19.46	18.64	**18.11**	N/A	N/A	N/A	**10**
MRP(3)/*mrp*(19)	59	15.68	12.23	14.54	**14.15**	57	N/A	**57**	**50**
CX1834	103	4.65	N/A	N/A	**4.65**	N/A	N/A	N/A	**40**
V995089	99	10.21	N/A	N/A	**10.21**	N/A	N/A	N/A	**30**
V993337	34	12.51	12.11	11.24	**11.96**	N/A	N/A	N/A	**10**
2013 Blacksburg (Field A^^)									
mrp(3)/*mrp*(19)	2870	N/A	N/A	N/A	N/A	N/A	N/A	N/A	**75**
mrp(3)/*MRP*(19)	2867	N/A	N/A	N/A	N/A	N/A	N/A	N/A	**100**
MRP(3)/*MRP*(19)	2876	N/A	N/A	N/A	N/A	N/A	N/A	N/A	**90**
MRP(3)/*mrp*(19)	2877	N/A	N/A	N/A	N/A	N/A	N/A	N/A	**80**
CX1834	2866	N/A	N/A	N/A	N/A	N/A	N/A	N/A	**95**
V995089	2861	N/A	N/A	N/A	N/A	N/A	N/A	N/A	**90**
V993337	2874	N/A	N/A	N/A	N/A	N/A	N/A	N/A	**95**
2013 Blacksburg (Field B^^)									
mrp(3)/*mrp*(19)	2955	7.21	9.21	7.61	**8.01**	N/A	N/A	N/A	N/A
mrp(3)/*MRP*(19)	2952	17.16	18.36	17.16	**17.56**	N/A	N/A	N/A	N/A
MRP(3)/*MRP*(19)	2947	18.53	20.09	16.51	**18.38**	N/A	N/A	N/A	N/A
MRP(3)/*mrp*(19)	2946	16.43	16.70	16.68	**16.61**	N/A	N/A	N/A	N/A
CX1834	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
V995089	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
V993337	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2012 Blacksburg (Field B)									
mrp(3)/*mrp*(19)	2066	2.89	4.45	3.48	**3.61**	N/A	N/A	N/A	**20**
mrp(3)/*MRP*(19)	2086	13.02	19.18	14.90	**15.70**	82	N/A	**82**	**70**

MRP(3)/*MRP*(19)	2091	13.75	15.93	15.26	**14.98**	N/A	N/A	N/A	**30**
MRP(3)/*mrp*(19)	2094	12.59	10.73	14.03	**12.45**	54	N/A	**54**	**50**
CX1834	2053	5.65	5.63	7.78	**6.35**	N/A	N/A	N/A	**30**
V995089	2067	7.14	5.42	8.66	**7.07**	N/A	N/A	N/A	**90**
V993337	2078	12.56	13.70	13.42	**13.23**	N/A	N/A	N/A	**70**
2012 Mt. Holly (Field B)									
mrp(3)/*mrp*(19)	166	N/A	N/A	N/A	N/A	N/A	N/A	N/A	**30**
mrp(3)/*MRP*(19)	170	N/A	N/A	N/A	N/A	85	N/A	**85**	**20**
MRP(3)/*MRP*(19)	160	14.73	N/A	N/A	**14.73**	N/A	N/A	N/A	**20**
MRP(3)/*mrp*(19)	153	12.37	16.99	N/A	**14.68**	45	N/A	**45**	**40**
CX1834	103	4.65	N/A	N/A	**4.65**	N/A	N/A	N/A	**10**
V995089	154	8.36	9.04	N/A	**8.70**	N/A	N/A	N/A	**10**
V993337	228	5.22	6.37	4.45	**5.35**	N/A	N/A	N/A	**20**
2011 Blacksburg									
mrp(3)/*mrp*(19)	2069	2.33	2.65	2.30	**2.43**	47	28	**37.5**	**40**
mrp(3)/*MRP*(19)	2075	10.63	11.03	11.25	**10.97**	34	35	**34.5**	**60**
MRP(3)/*MRP*(19)	2081	13.64	12.38	13.26	**13.09**	38	51	**44.5**	**80**
MRP(3)/*mrp*(19)	2073	12.31	11.50	12.37	**12.06**	40	55	**47.5**	**90**
CX1834	2086	6.37	7.26	6.06	**6.56**	35	7	**21**	**60**
V995089	2087	11.20	10.58	9.85	**10.54**	44	57	**50.5**	**60**
V993337	2085	14.80	14.34	14.23	**14.46**	37	70	**53.5**	**80**
2010 Blacksburg									
mrp(3)/*mrp*(19)	343	4.02	7.02	3.70	**4.91**	3	1	**2**	**40**
mrp(3)/*MRP*(19)	349	18.95	19.44	14.54	**17.65**	58.5	57	**57.75**	**50**
MRP(3)/*MRP*(19)	355	20.43	21.44	19.48	**20.45**	52.5	32	**42.25**	**40**
MRP(3)/*mrp*(19)	347	14.77	15.74	24.03	**18.18**	27	33	**30**	**60**
CX1834	360	N/A	N/A	N/A	N/A	5	2	**3.5**	**10**
V995089	361	13.27	12.31	14.91	**13.50**	12	2	**7**	**10**
V993337	359	19.46	19.17	19.98	**19.54**	76	52	**64**	**90**
2009 Blacksburg									

mrp(3)/*mrp*(19)	919-28-36	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
mrp(3)/*MRP*(19)	919-28-38	11.21	10.55	13.34	**11.70**	81.33	N/A	**81.33**	N/A
MRP(3)/*MRP*(19)	919-28-29	15.43	14.75	16.99	**15.73**	77.03	N/A	**77.03**	N/A
MRP(3)/*mrp*(19)	919-28-30	15.26	13.04	18.29	**15.53**	N/A	N/A	N/A	N/A
CX1834	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
V995089	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
V993337	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

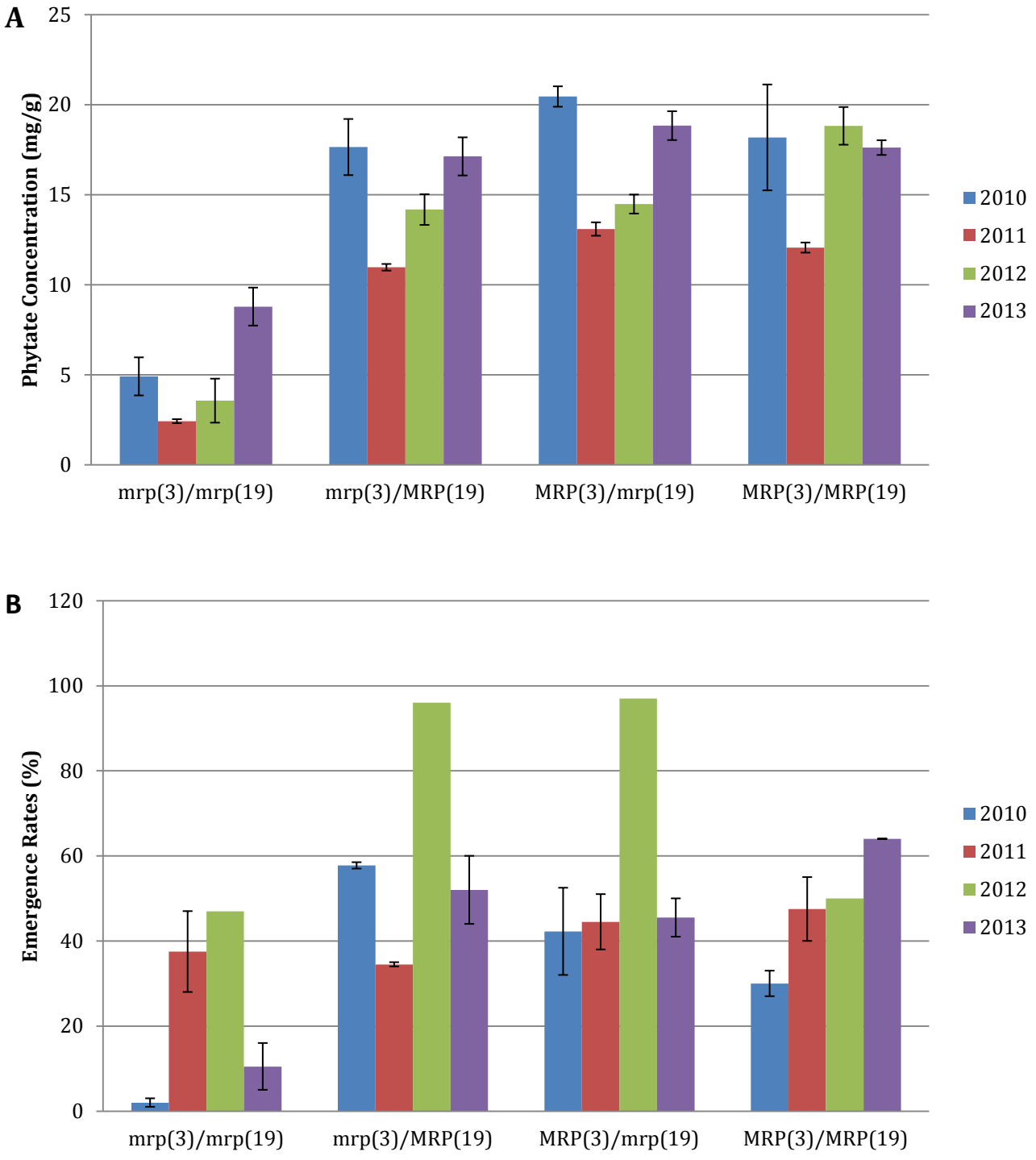


Figure S3.2. Mean seed phytate levels (A) and ECT emergence rates (B) in the four NILs from 2009-2013.

Table S3.2. EMRTs with a *p*-value < 0.05 and a factor of change less than 0.5 or greater than 2 in all the comparison between the low phytate line and all normal phytate lines for both columns and ionization modes. The averages of the classes represent the peak areas.

	Column	Ion mode	RT	m/z	average *mrp*(3)/ *mrp*(19)	average *mrp*(3)/ *MRP*(19)	average *MRP*(3)/ *mrp*(19)	average *MRP*(3)/ *MRP*(19)	average all normal phytate (NP) lines	factor of change NP/LP	p Value
1	RPC	positive	3.81	255.0657	283.20	723.89	840.21	728.11	764.07	2.70	1.77E-15
2	RPC	positive	3.93	503.1187	1796.53	3769.87	4154.35	3045.40	3656.54	2.04	2.97E-17
3	RPC	positive	4.17	689.2790	126.60	303.74	272.93	189.60	255.43	2.02	2.87E-07
4	RPC	positive	4.35	271.0607	7621.02	16800.52	16803.08	12151.98	15251.86	2.00	8.33E-15
5	RPC	positive	4.35	519.1135	3721.60	8219.39	8315.46	6035.67	7523.51	2.02	2.47E-16
6	RPC	positive	4.42	1392.3780	0.06	175.70	362.62	318.19	285.51	4475.06	2.50E-06
7	RPC	positive	4.42	1392.7120	0.03	262.64	553.10	493.83	436.52	13631.72	1.93E-07
8	RPC	positive	5.2	973.6720	40.04	1375.51	602.36	489.70	822.53	20.54	4.57E-08
9	RPC	positive	5.21	973.4221	162.77	745.74	360.78	393.12	499.88	3.07	9.67E-04
10	RPC	positive	5.75	1107.5560	506.99	0.68	0.80	0.16	0.54	0.00	2.18E-08
11	RPC	positive	5.75	1365.6300	463.88	0.12	0.11	0.05	0.09	0.00	3.87E-06
12	RPC	positive	5.88	975.5144	1.22	1173.73	936.64	965.80	1025.39	842.66	7.09E-23
13	RPC	positive	5.88	1107.5570	0.00	305.40	260.99	231.27	265.88	n/a	8.55E-22
14	RPC	positive	5.88	1437.6510	0.09	1020.30	765.24	804.34	863.29	9434.25	1.72E-17
15	RPC	positive	6.11	813.4628	0.18	271.48	232.66	238.69	247.61	1387.09	1.80E-19
16	RPC	positive	6.11	1275.5990	0.00	157.28	118.87	120.33	132.16	n/a	7.35E-12
17	RPC	positive	6.46	959.5198	177.78	744.58	560.85	370.99	558.81	3.14	2.23E-08
18	RPC	positive	6.58	423.3620	390.52	1197.23	938.29	605.99	913.83	2.34	2.38E-05
19	RPC	positive	6.58	797.4673	120.78	474.04	415.83	245.69	378.52	3.13	3.41E-07
20	RPC	positive	6.58	943.5246	1854.76	5534.71	5379.34	3329.78	4747.94	2.56	4.55E-08
21	RPC	positive	6.74	423.3619	238.60	1009.64	1007.75	509.86	842.42	3.53	9.47E-09
22	RPC	positive	6.74	441.3726	148.02	619.63	659.60	325.44	534.89	3.61	2.64E-09
23	RPC	positive	6.74	599.3941	98.37	435.55	430.22	203.24	356.34	3.62	1.03E-09
24	RPC	positive	6.74	913.5144	71.00	329.12	362.90	173.82	288.62	4.07	7.17E-11
25	RPC	positive	6.75	797.4673	124.12	475.69	419.89	245.44	380.34	3.06	3.43E-07
26	RPC	positive	6.81	1061.5500	81.80	394.98	306.20	127.57	276.25	3.38	8.05E-07
27	RPC	positive	6.97	1085.5500	0.00	577.54	205.68	59.70	280.97	2.00	1.73E-04
28	RPC	positive	7.08	423.3621	123.10	914.85	354.83	112.40	460.69	3.74	2.42E-03
29	RPC	positive	7.08	1069.5560	1211.64	6794.85	4182.01	1892.72	4289.86	3.54	3.00E-05
30	RPC	positive	7.09	1091.5380	130.17	577.93	363.41	197.27	379.54	2.92	9.24E-04
31	RPC	positive	7.21	423.3620	16.04	540.53	478.56	95.46	371.52	23.16	7.66E-06
32	RPC	positive	7.35	923.4985	26.61	674.82	295.62	100.95	357.13	13.42	2.56E-05

1	RPC	negative	3.81	253.0500	711.60	2017.77	1875.65	1599.98	1831.14	0.39	3.56E-09
2	RPC	negative	9.1	255.2321	295.44	1108.01	641.83	168.22	639.35	0.46	4.17E-02
3	RPC	negative	8.59	265.1472	88.85	961.10	468.89	49.37	493.12	0.18	4.14E-04
4	RPC	negative	8.8	295.2263	23.93	497.24	50.15	21.82	189.74	0.13	4.95E-04
5	RPC	negative	8.61	297.1519	153.23	993.30	222.04	108.71	441.35	0.35	2.23E-02
6	RPC	negative	8.82	297.1520	250.23	1074.12	383.45	147.89	535.16	0.47	4.72E-02
7	RPC	negative	8.52	315.0479	77.68	740.83	469.06	72.57	427.49	0.18	4.95E-05
8	RPC	negative	9.36	315.0479	0.00	650.04	334.09	47.57	343.90	0.00	6.07E-06
9	RPC	negative	5.94	329.2320	9.46	498.79	6.33	10.89	172.00	0.06	7.41E-04
10	RPC	negative	2.67	355.0662	230.15	842.15	682.92	644.52	723.20	0.32	1.20E-07
11	RPC	negative	2.91	355.0663	73.48	444.66	302.82	203.34	316.94	0.23	5.36E-07
12	RPC	negative	2.44	355.0664	133.30	534.38	430.29	356.46	440.38	0.30	6.10E-07
13	RPC	negative	2.29	359.0975	567.35	232.97	299.23	298.56	276.92	2.05	1.63E-05
14	RPC	negative	2.59	389.0715	268.25	404.13	664.60	582.08	550.27	0.49	3.05E-09
15	RPC	negative	2.06	389.0716	203.33	469.72	831.68	735.52	678.97	0.30	2.36E-08
16	RPC	negative	9.36	391.2244	0.00	713.03	349.81	82.79	381.88	0.00	8.82E-07
17	RPC	negative	2.86	395.1548	553.54	96.70	212.37	152.08	153.72	3.60	5.44E-03
18	RPC	negative	3.6	403.1601	326.81	786.74	644.35	566.69	665.93	0.49	2.49E-07
19	RPC	negative	4.71	421.2070	804.47	2008.38	1495.61	1438.86	1647.61	0.49	5.15E-08
20	RPC	negative	2.92	433.1130	573.91	1195.14	1525.06	1067.61	1262.60	0.45	2.54E-11
21	RPC	negative	3.21	435.1295	107.69	241.69	234.54	217.33	231.18	0.47	1.46E-09
22	RPC	negative	2.13	443.1903	9.45	150.96	77.76	76.64	101.79	0.09	2.02E-03
23	RPC	negative	2.44	447.1137	293.13	628.74	691.07	920.18	746.66	0.39	1.09E-13
24	RPC	negative	3.64	459.1289	136.09	323.45	239.62	256.44	273.17	0.50	5.28E-08
25	RPC	negative	4.35	473.1078	278.77	614.95	651.49	489.26	585.23	0.48	3.14E-11
26	RPC	negative	3.77	473.1079	464.06	1001.42	1094.19	850.64	982.08	0.47	9.67E-10
27	RPC	negative	8.1	474.2617	140.66	605.24	327.19	141.75	358.06	0.39	1.96E-04
28	RPC	negative	7.93	474.2618	133.01	628.86	347.84	165.35	380.68	0.35	1.08E-04
29	RPC	negative	8.64	476.2773	2016.14	6827.07	3964.09	1311.96	4034.38	0.50	4.39E-03
30	RPC	negative	3.31	485.2017	188.16	785.22	299.94	345.69	476.95	0.39	1.34E-06
31	RPC	negative	4.24	489.1024	112.00	361.23	376.57	278.48	338.76	0.33	1.15E-14
32	RPC	negative	3.52	491.1186	358.68	783.75	642.66	764.13	730.18	0.49	1.75E-08
33	RPC	negative	3.79	491.1762	234.54	551.04	448.36	528.79	509.39	0.46	5.08E-07
34	RPC	negative	7.96	502.2926	34.83	261.72	139.08	58.60	153.13	0.23	6.72E-05
35	RPC	negative	8.13	502.2930	65.56	479.29	225.88	71.04	258.74	0.25	2.77E-04
36	RPC	negative	9.19	507.2719	398.26	1472.50	890.69	205.58	856.26	0.47	1.32E-02
37	RPC	negative	4.16	513.1969	99.76	260.51	213.56	189.51	221.19	0.45	9.13E-07
38	RPC	negative	4.71	525.1968	161.15	377.39	398.82	269.52	348.58	0.46	1.65E-06
39	RPC	negative	5.65	527.1546	2.13	4.73	335.88	5.92	115.51	0.02	1.39E-02

40	RPC	negative	3.81	535.1085	197.66	474.19	1699.49	1127.75	1100.48	0.18	1.47E-09
41	RPC	negative	3.65	535.1086	162.24	415.70	896.24	661.86	657.93	0.25	4.97E-09
42	RPC	negative	4.03	535.1086	87.96	175.96	949.12	801.71	642.26	0.14	6.66E-09
43	RPC	negative	7.96	562.3138	219.05	850.07	452.14	243.15	515.12	0.43	7.52E-04
44	RPC	negative	8.13	562.3142	257.73	1126.62	493.64	251.61	623.96	0.41	1.15E-03
45	RPC	negative	4	565.1189	0.00	289.26	966.15	1024.54	759.98	0.00	4.06E-11
46	RPC	negative	3.76	565.1191	638.64	1485.51	2685.70	2067.59	2079.60	0.31	7.57E-14
47	RPC	negative	3.19	569.1140	169.62	511.08	287.72	325.51	374.77	0.45	2.78E-06
48	RPC	negative	9	571.2877	22.09	766.35	443.47	39.62	416.48	0.05	1.03E-06
49	RPC	negative	9.36	571.2882	4636.64	19167.55	11445.51	4661.85	11758.30	0.39	3.46E-04
50	RPC	negative	4.2	577.1188	513.29	416.89	0.31	0.76	139.32	3.68	2.46E-07
51	RPC	negative	4.42	577.1189	1057.00	1446.94	4.80	3.29	485.01	2.18	3.91E-04
52	RPC	negative	4.6	577.1194	332.29	282.19	1.19	1.00	94.79	3.51	4.00E-06
53	RPC	negative	3.44	587.2337	116.73	528.58	389.45	424.21	447.41	0.26	7.05E-17
54	RPC	negative	3.76	593.1497	143.40	412.11	546.41	218.02	392.18	0.37	1.94E-08
55	RPC	negative	7.88	593.2726	209.72	1156.22	652.75	231.46	680.14	0.31	1.19E-04
56	RPC	negative	8.12	593.2733	30.83	200.70	186.62	26.75	138.02	0.22	7.51E-04
57	RPC	negative	3.37	595.1289	33.65	35.44	168.05	165.33	122.94	0.27	4.58E-06
58	RPC	negative	3.98	595.1297	563.44	958.81	1492.32	1316.77	1255.97	0.45	3.95E-08
59	RPC	negative	3.55	595.1299	716.43	1244.96	1823.04	1355.20	1474.40	0.49	8.47E-09
60	RPC	negative	3.69	595.1300	925.24	2375.09	4962.78	3911.78	3749.88	0.25	2.47E-11
61	RPC	negative	8.79	595.2878	1028.40	3725.53	2414.17	820.60	2320.10	0.44	9.15E-04
62	RPC	negative	8.52	595.2884	3101.15	10165.72	6653.56	2551.91	6457.06	0.48	5.97E-04
63	RPC	negative	4.13	607.1296	671.64	701.31	3.33	0.62	235.09	2.86	2.13E-04
64	RPC	negative	4.37	607.1299	1208.63	1322.07	8.82	11.71	447.53	2.70	7.45E-07
65	RPC	negative	4.56	607.1300	655.75	383.84	1.78	3.34	129.65	5.06	1.16E-04
66	RPC	negative	5.65	615.2827	0.74	420.47	274.84	304.37	333.23	0.00	1.44E-15
67	RPC	negative	6.59	615.3889	73.45	407.77	314.54	147.12	289.81	0.25	5.46E-06
68	RPC	negative	3.7	617.1124	84.20	212.98	342.90	287.33	281.07	0.30	1.67E-12
69	RPC	negative	5.1	630.3059	188.81	715.05	705.02	634.63	684.90	0.28	2.37E-13
70	RPC	negative	7.31	633.3994	44.51	153.42	294.79	94.61	180.94	0.25	9.98E-06
71	RPC	negative	4.51	637.1397	1159.51	1111.25	0.32	1.37	370.98	3.13	1.69E-05
72	RPC	negative	4.08	637.1403	858.74	944.01	0.99	1.29	315.43	2.72	2.59E-05
73	RPC	negative	5.65	638.2856	0.59	382.59	249.97	278.61	303.72	0.00	1.32E-12
74	RPC	negative	9.35	639.2755	0.00	478.88	273.11	56.26	269.42	0.00	5.97E-06
75	RPC	negative	5.03	645.3111	671.64	1802.66	1285.68	1347.06	1478.46	0.45	2.95E-07
76	RPC	negative	4.95	649.2872	78.74	426.45	319.44	207.95	317.95	0.25	2.81E-07
77	RPC	negative	5.1	653.3084	53.26	296.78	254.98	194.69	248.81	0.21	1.68E-07
78	RPC	negative	4.08	656.2958	0.24	249.53	162.81	211.96	208.10	0.00	4.37E-16

79	RPC	negative	6.12	659.2911	1.59	427.02	309.62	325.61	354.08	0.00	1.19E-21
80	RPC	negative	5.03	668.3136	216.88	665.63	460.21	449.39	525.08	0.41	8.31E-06
81	RPC	negative	4.94	675.3033	2.87	488.71	314.36	405.94	403.00	0.01	4.36E-16
82	RPC	negative	5.45	696.3092	2.43	1066.90	727.78	821.61	872.10	0.00	3.26E-18
83	RPC	negative	4.75	698.3050	1.40	54.16	11.60	53.16	39.64	0.04	1.82E-02
84	RPC	negative	4.93	698.3062	0.78	654.55	281.24	395.68	443.82	0.00	8.16E-07
85	RPC	negative	5.31	711.3136	0.12	314.77	208.83	224.29	249.29	0.00	1.93E-13
86	RPC	negative	5.5	711.3136	0.62	403.04	276.31	248.81	309.39	0.00	3.82E-14
87	RPC	negative	6.05	717.3145	0.21	322.13	252.44	185.21	253.26	0.00	3.68E-17
88	RPC	negative	5.26	719.3116	1.71	1433.52	1059.74	1165.33	1219.53	0.00	2.54E-20
89	RPC	negative	5.45	719.3119	6.40	1574.28	1145.68	1325.69	1348.55	0.00	8.58E-21
90	RPC	negative	5.95	732.3197	1.80	1056.59	844.19	849.89	916.89	0.00	7.07E-24
91	RPC	negative	5.53	740.3173	0.46	455.99	348.17	372.26	392.14	0.00	1.52E-18
92	RPC	negative	5.88	740.3174	46.87	3410.09	2866.62	2964.77	3080.49	0.02	7.73E-29
93	RPC	negative	5.49	748.3146	0.84	706.80	672.71	655.88	678.46	0.00	8.94E-22
94	RPC	negative	6.86	765.4420	324.11	1630.30	1665.95	856.75	1384.33	0.23	3.90E-10
95	RPC	negative	6.76	795.4524	1314.84	3914.07	2946.73	2021.64	2960.82	0.44	1.79E-06
96	RPC	negative	7.44	891.4733	208.28	2950.79	1797.17	771.35	1839.77	0.11	4.19E-07
97	RPC	negative	6.66	897.4838	72.48	444.45	351.26	225.10	340.27	0.21	4.26E-09
98	RPC	negative	7.24	909.4842	251.01	602.43	869.13	339.57	603.71	0.42	6.55E-04
99	RPC	negative	6.74	911.5000	3739.74	17022.30	18991.09	9280.22	15097.87	0.25	4.20E-11
100	RPC	negative	7.36	921.4840	1017.25	8154.82	4209.50	2422.55	4928.96	0.21	2.51E-06
101	RPC	negative	6.52	925.4790	17.35	382.77	252.23	122.74	252.58	0.07	9.79E-09
102	RPC	negative	6.81	925.5152	218.11	532.69	548.78	251.58	444.35	0.49	1.91E-04
103	RPC	negative	6.54	927.4937	5.17	257.22	345.83	116.93	239.99	0.02	2.35E-06
104	RPC	negative	3.92	933.2085	416.65	1214.04	1223.13	1033.08	1156.75	0.36	3.11E-11
105	RPC	negative	6.59	941.5105	12675.36	34112.06	29508.17	19731.11	27783.78	0.46	2.25E-07
106	RPC	negative	8.46	953.5635	5.82	142.33	71.27	8.57	74.06	0.08	3.64E-04
107	RPC	negative	6.93	955.4886	70.75	232.55	212.60	118.47	187.87	0.38	5.54E-04
108	RPC	negative	6.47	957.5046	1288.25	5219.21	4158.40	2810.99	4062.86	0.32	4.70E-09
109	RPC	negative	6.74	957.5055	878.37	3738.88	4210.81	2103.71	3351.13	0.26	3.22E-11
110	RPC	negative	6.74	979.4871	309.65	1090.82	1151.90	676.06	972.92	0.32	2.10E-12
111	RPC	negative	6.58	987.5158	6581.12	16784.37	15164.83	10368.05	14105.75	0.47	1.37E-07
112	RPC	negative	7.36	989.4713	46.48	484.19	223.77	133.81	280.59	0.17	5.46E-06
113	RPC	negative	4.68	992.4154	67.93	279.51	150.60	193.10	207.74	0.33	7.56E-11
114	RPC	negative	6.69	1013.5310	78.48	904.17	924.67	297.84	708.90	0.11	2.96E-08
115	RPC	negative	6.47	1025.4930	103.17	395.06	311.88	218.49	308.48	0.33	1.12E-08
116	RPC	negative	6.25	1027.5110	52.33	150.61	206.79	107.64	155.01	0.34	5.44E-05
117	RPC	negative	7.01	1029.5260	119.35	662.03	719.04	257.10	546.06	0.22	8.29E-09

118	RPC	negative	3.95	1033.2250	495.34	1455.92	1325.13	1156.25	1312.44	0.38	3.08E-09
119	RPC	negative	4.35	1035.2040	3966.68	13500.03	13925.37	8974.51	12133.30	0.33	2.19E-11
120	RPC	negative	9.11	1035.6620	30.82	197.27	94.35	10.52	100.71	0.31	4.82E-03
121	RPC	negative	7.22	1037.5310	2956.01	21741.71	15824.57	7834.67	15133.65	0.20	2.41E-08
122	RPC	negative	6.56	1041.5270	19.45	436.08	376.22	149.69	320.66	0.06	8.27E-09
1	HILIC	positive	4.75	70.0656	372.64	99.38	73.03	115.10	95.84	3.89	5.36E-17
2	HILIC	positive	3.88	98.9849	769.20	0.00	0.00	0.00	0.00	n/a	1.17E-09
3	HILIC	positive	4.82	110.0719	213.71	21.66	8.76	22.58	17.67	12.09	3.50E-04
4	HILIC	positive	4.75	116.0717	927.23	361.87	369.82	432.05	387.91	2.39	8.60E-10
5	HILIC	positive	3.83	124.0399	194.77	0.00	0.00	0.00	0.00	n/a	1.39E-05
6	HILIC	positive	4.75	130.0981	608.01	201.39	208.91	243.33	217.88	2.79	4.27E-11
7	HILIC	positive	4.23	134.0453	167.11	408.27	341.09	324.60	357.99	0.47	1.40E-05
8	HILIC	positive	4.82	136.0624	278.37	86.36	147.99	142.11	125.49	2.22	2.48E-05
9	HILIC	positive	5.66	136.9317	176.64	67.33	37.18	38.50	47.67	3.71	1.72E-02
10	HILIC	positive	2.4	145.0289	679.85	516.97	5.82	7.16	176.65	3.85	1.18E-14
11	HILIC	positive	2.41	151.0395	228.11	60.95	0.00	0.00	20.32	11.23	2.81E-07
12	HILIC	positive	2.96	151.0396	65.47	150.25	170.33	214.71	178.43	0.37	4.82E-04
13	HILIC	positive	3.97	151.6261	253.03	1194.52	974.15	1201.10	1123.26	0.23	5.03E-19
14	HILIC	positive	4.11	151.6261	16.32	174.00	163.67	133.78	157.15	0.10	2.81E-17
15	HILIC	positive	4.74	157.1088	204.04	15.49	21.02	54.95	30.49	6.69	1.76E-06
16	HILIC	positive	4.75	158.0930	1346.93	529.51	487.43	607.00	541.31	2.49	1.71E-09
17	HILIC	positive	4.28	159.0765	194.06	43.58	60.55	67.28	57.14	3.40	4.07E-12
18	HILIC	positive	2.4	175.0395	957.47	814.05	6.08	0.62	273.58	3.50	1.27E-12
19	HILIC	positive	4.75	175.1195	11762.38	4546.25	4368.65	5074.05	4662.99	2.52	1.16E-08
20	HILIC	positive	2.4	177.0552	2081.77	1583.98	25.17	31.13	546.76	3.81	1.13E-14
21	HILIC	positive	2.39	181.0501	1104.44	807.08	97.00	215.59	373.23	2.96	3.99E-16
22	HILIC	positive	4.68	184.0740	504.05	81.73	91.75	88.48	87.32	5.77	1.60E-04
23	HILIC	positive	2.4	186.0555	296.05	224.57	1.79	2.58	76.32	3.88	8.95E-15
24	HILIC	positive	3.94	197.0933	19.05	136.46	147.26	144.91	142.88	0.13	2.47E-09
25	HILIC	positive	3.89	198.1857	280.99	3.37	13.53	9.39	8.76	32.06	4.84E-07
26	HILIC	positive	3.33	202.8916	125.47	269.33	342.29	257.82	289.81	0.43	5.31E-04
27	HILIC	positive	3.19	203.0824	97.58	346.89	175.98	250.66	257.84	0.38	4.61E-03
28	HILIC	positive	2.4	207.0657	3290.41	2933.54	11.97	15.95	987.16	3.33	8.77E-12
29	HILIC	positive	2.92	209.1541	15.45	93.81	81.92	62.54	79.42	0.19	1.77E-06
30	HILIC	positive	3.85	211.1057	0.53	436.20	193.20	229.81	286.41	0.00	5.76E-17
31	HILIC	positive	3.85	227.0797	0.49	476.39	253.48	287.60	339.16	0.00	1.16E-20
32	HILIC	positive	5.65	241.9795	38.21	117.03	130.39	145.93	131.12	0.29	1.90E-03
33	HILIC	positive	4.19	243.1702	2.91	83.93	27.26	93.18	68.13	0.04	5.31E-03
34	HILIC	positive	3.96	243.1707	152.22	3837.28	2372.44	3631.29	3280.34	0.05	6.88E-19

35	HILIC	positive	4.47	243.1708	34.44	167.92	135.08	181.02	161.34	0.21	6.13E-09
36	HILIC	positive	3.81	258.1314	42.92	242.10	94.86	98.46	145.14	0.30	9.91E-07
37	HILIC	positive	4.31	260.1973	77.60	256.29	257.26	297.64	270.40	0.29	5.00E-08
38	HILIC	positive	3.75	266.1596	0.00	190.95	203.45	237.52	210.64	0.00	6.87E-29
39	HILIC	positive	3.16	268.9465	222.52	169.29	56.83	87.78	104.63	2.13	4.75E-02
40	HILIC	positive	4.62	272.1722	1429.94	434.69	432.32	416.03	427.68	3.34	2.61E-08
41	HILIC	positive	2.36	273.0786	0.00	17.76	36.54	0.00	18.10	0.00	2.94E-02
42	HILIC	positive	3.81	274.1053	43.52	194.21	98.47	112.61	135.10	0.32	8.38E-08
43	HILIC	positive	4.87	286.1406	160.82	58.86	56.29	62.78	59.31	2.71	7.81E-07
44	HILIC	positive	3.13	287.0553	158.89	332.68	439.01	260.77	344.15	0.46	3.94E-16
45	HILIC	positive	4.09	288.2282	8.92	369.58	248.36	292.02	303.32	0.03	1.65E-10
46	HILIC	positive	4.05	290.1347	135.48	4.59	13.04	9.49	9.04	14.98	1.76E-06
47	HILIC	positive	3.83	291.1300	223.12	13.66	75.43	75.37	54.82	4.07	1.98E-12
48	HILIC	positive	3.96	302.2440	63.38	3306.44	2039.35	3060.60	2802.13	0.02	4.71E-20
49	HILIC	positive	4.19	302.2442	0.00	105.92	119.15	231.39	152.16	0.00	1.45E-06
50	HILIC	positive	5.31	305.0718	46.38	132.44	171.53	169.79	157.92	0.29	9.10E-04
51	HILIC	positive	3.43	307.0790	225.75	74.25	106.91	99.87	93.68	2.41	7.06E-16
52	HILIC	positive	3.86	336.1229	192.13	54.62	60.95	59.09	58.22	3.30	9.42E-12
53	HILIC	positive	4.03	337.1129	12.79	16.33	217.66	17.53	83.84	0.15	1.19E-04
54	HILIC	positive	3.65	345.1998	35.61	145.13	41.64	46.16	77.64	0.46	1.91E-05
55	HILIC	positive	4.54	347.0254	137.24	0.00	0.00	0.00	0.00	n/a	8.63E-04
56	HILIC	positive	3.8	371.0616	243.69	38.01	0.00	61.82	33.28	7.32	7.07E-15
57	HILIC	positive	4.63	375.2242	259.06	84.15	103.14	105.04	97.44	2.66	6.07E-05
58	HILIC	positive	3.86	376.1715	264.62	62.37	76.85	96.18	78.47	3.37	9.97E-19
59	HILIC	positive	2.96	383.0583	9.68	70.49	78.62	135.46	94.86	0.10	5.51E-06
60	HILIC	positive	2.8	383.0950	456.41	146.04	196.54	215.80	186.13	2.45	4.45E-15
61	HILIC	positive	3.09	393.0793	827.64	366.19	291.01	284.40	313.86	2.64	4.75E-11
62	HILIC	positive	2.97	399.0328	20.75	156.07	142.62	218.07	172.25	0.12	5.64E-09
63	HILIC	positive	2.81	399.0691	210.29	58.99	92.52	73.83	75.11	2.80	4.77E-07
64	HILIC	positive	3.85	399.2212	0.00	441.17	73.36	121.12	211.88	0.00	8.19E-09
65	HILIC	positive	4.01	400.2111	860.66	2342.86	1763.35	1961.27	2022.49	0.43	5.05E-07
66	HILIC	positive	2.77	405.3515	68.17	152.53	207.76	227.01	195.77	0.35	5.21E-06
67	HILIC	positive	2.4	415.0874	454.85	373.77	4.16	2.41	126.78	3.59	2.83E-13
68	HILIC	positive	2.85	419.1522	832.30	208.18	334.14	247.59	263.31	3.16	2.15E-10
69	HILIC	positive	2.99	421.1107	84.53	185.24	170.91	199.67	185.27	0.46	2.28E-12
70	HILIC	positive	3.27	421.3465	0.00	217.80	369.44	407.86	331.70	0.00	5.75E-18
71	HILIC	positive	3.08	421.3466	39.02	256.04	97.39	90.80	148.08	0.26	7.91E-05
72	HILIC	positive	2.88	435.1263	1661.67	330.79	686.50	404.01	473.77	3.51	2.08E-10
73	HILIC	positive	3.08	439.3562	34.30	323.14	121.40	55.79	166.78	0.21	8.14E-04

74	HILIC	positive	2.81	439.3566	0.00	216.72	196.39	194.72	202.61	0.00	2.85E-17
75	HILIC	positive	3.27	439.3570	0.00	309.63	483.22	524.20	439.02	0.00	4.86E-19
76	HILIC	positive	5.16	445.0716	271.68	15.25	16.01	16.94	16.06	16.91	5.80E-07
77	HILIC	positive	2.82	457.3676	2.29	105.67	112.78	100.37	106.27	0.02	9.98E-13
78	HILIC	positive	3.28	457.3676	0.00	249.59	226.28	259.83	245.23	0.00	3.28E-32
79	HILIC	positive	2.41	479.1890	18.17	19.92	68.19	179.35	89.16	0.20	2.97E-06
80	HILIC	positive	2.37	484.3225	0.00	2.09	171.27	182.15	118.50	0.00	7.18E-10
81	HILIC	positive	2.67	484.7841	110.30	233.90	249.07	192.19	225.05	0.49	5.16E-04
82	HILIC	positive	4.38	515.2133	497.86	221.58	284.54	172.68	226.27	2.20	2.33E-06
83	HILIC	positive	2.93	516.2486	220.09	18.67	37.00	5.82	20.50	10.74	1.72E-06
84	HILIC	positive	4.7	527.1583	126.01	577.57	804.77	365.14	582.49	0.22	2.13E-07
85	HILIC	positive	2.44	541.0956	65.60	221.31	487.85	384.61	364.59	0.18	5.82E-17
86	HILIC	positive	4.35	541.1742	64.95	115.77	168.38	120.50	134.88	0.48	1.45E-04
87	HILIC	positive	2.86	554.2609	48.25	158.80	88.17	108.33	118.43	0.41	1.84E-15
88	HILIC	positive	2.72	557.0898	51.95	138.09	128.33	84.51	116.98	0.44	1.48E-07
89	HILIC	positive	2.4	561.1241	259.24	189.41	0.81	0.38	63.53	4.08	2.34E-15
90	HILIC	positive	2.24	561.3858	106.18	292.54	142.35	562.52	332.47	0.32	3.54E-03
91	HILIC	positive	2.77	567.4039	47.81	78.81	113.05	141.70	111.19	0.43	5.42E-06
92	HILIC	positive	2.77	581.3837	58.47	47.46	157.07	150.37	118.30	0.49	6.53E-05
93	HILIC	positive	2.4	591.1345	594.88	428.71	6.63	3.40	146.24	4.07	8.87E-16
94	HILIC	positive	3.11	597.3786	0.00	127.06	0.00	0.00	42.35	0.00	1.76E-02
95	HILIC	positive	2.85	599.3940	48.85	14.87	165.14	201.22	127.08	0.38	1.60E-02
96	HILIC	positive	2.42	601.1167	1342.55	1162.65	14.45	9.24	395.45	3.40	2.71E-12
97	HILIC	positive	2.87	611.2315	11.63	148.60	65.50	91.43	101.84	0.11	8.17E-10
98	HILIC	positive	3.28	615.3898	0.00	149.92	152.34	163.57	155.28	0.00	1.71E-28
99	HILIC	positive	2.46	616.3839	0.00	27.80	177.36	282.63	162.60	0.00	2.36E-09
100	HILIC	positive	2.42	617.0913	270.58	226.63	1.68	1.66	76.66	3.53	1.39E-11
101	HILIC	positive	4.24	620.2038	79.81	195.38	169.59	155.05	173.34	0.46	2.88E-06
102	HILIC	positive	2.39	621.1452	556.28	443.15	1.98	1.62	148.92	3.74	8.29E-14
103	HILIC	positive	2.4	626.1719	215.61	194.54	2.22	2.00	66.25	3.25	1.45E-11
104	HILIC	positive	2.85	627.2051	150.63	520.20	403.36	385.60	436.38	0.35	6.52E-13
105	HILIC	positive	3.69	629.2418	270.71	835.34	758.95	714.61	769.63	0.35	1.27E-35
106	HILIC	positive	2.4	631.1275	2428.13	1972.05	52.93	45.75	690.24	3.52	1.81E-13
107	HILIC	positive	4.67	636.2586	67.69	187.47	104.58	138.55	143.53	0.47	2.04E-12
108	HILIC	positive	3.69	645.2154	158.02	392.89	369.08	328.72	363.56	0.43	1.53E-20
109	HILIC	positive	2.4	647.1008	482.73	403.18	8.97	7.15	139.76	3.45	2.15E-12
110	HILIC	positive	2.4	656.1824	881.44	949.90	2.23	2.08	318.07	2.77	2.37E-08
111	HILIC	positive	2.4	661.1377	3706.64	3494.44	25.60	14.55	1178.20	3.15	7.98E-11
112	HILIC	positive	2.41	673.2843	87.82	219.38	299.95	343.48	287.60	0.31	4.21E-05

113	HILIC	positive	2.4	677.1113	727.35	695.97	4.41	2.23	234.20	3.11	3.11E-10
114	HILIC	positive	2.39	684.2137	210.45	182.75	0.39	0.02	61.05	3.45	4.03E-03
115	HILIC	positive	2.38	697.1376	0.00	0.00	69.15	50.09	39.75	0.00	3.55E-03
116	HILIC	positive	3.41	700.2702	355.41	3.66	4.07	3.50	3.74	94.91	6.11E-12
117	HILIC	positive	2.34	714.5434	65.77	156.50	360.32	175.72	230.85	0.28	1.30E-03
118	HILIC	positive	3.13	719.1225	22.95	90.72	103.74	36.16	76.87	0.30	5.46E-05
119	HILIC	positive	3.31	721.2754	848.97	0.00	0.00	0.62	0.21	4086.91	1.08E-14
120	HILIC	positive	3.37	736.2806	6.16	629.87	547.90	643.86	607.21	0.01	1.45E-34
121	HILIC	positive	2.57	736.4908	185.10	352.50	386.42	392.47	377.13	0.49	2.87E-04
122	HILIC	positive	3.28	738.3078	2.64	279.63	302.14	332.10	304.62	0.01	4.07E-38
123	HILIC	positive	3.32	740.2527	407.58	0.26	2.17	0.96	1.13	359.81	7.12E-15
124	HILIC	positive	3.28	749.2989	1.69	211.30	203.49	215.70	210.17	0.01	1.83E-38
125	HILIC	positive	3.36	755.2590	1.43	228.31	203.90	228.02	220.08	0.01	2.51E-34
126	HILIC	positive	3.29	757.2854	22.52	1787.13	1902.64	2167.64	1952.47	0.01	4.03E-36
127	HILIC	positive	3.41	765.2829	0.44	177.32	176.12	196.37	183.27	0.00	3.68E-37
128	HILIC	positive	3.3	776.2632	4.59	556.46	585.60	664.10	602.06	0.01	1.12E-34
129	HILIC	positive	2.96	782.5687	0.00	0.00	52.18	37.39	29.86	0.00	4.40E-02
130	HILIC	positive	2.42	785.3335	284.31	238.12	1.31	0.28	79.90	3.56	2.86E-12
131	HILIC	positive	3.23	795.4513	54.30	215.58	91.93	72.66	126.73	0.43	4.89E-04
132	HILIC	positive	2.89	795.4517	80.23	607.52	289.03	301.21	399.26	0.20	1.00E-06
133	HILIC	positive	2.51	810.6007	33.04	62.05	100.37	56.19	72.87	0.45	3.44E-02
134	HILIC	positive	3.3	813.2281	1.91	126.72	156.85	166.89	150.15	0.01	7.94E-28
135	HILIC	positive	3	813.4625	0.00	0.00	71.18	64.84	45.34	0.00	4.76E-05
136	HILIC	positive	2.92	813.4627	324.38	1537.51	1759.80	2125.55	1807.62	0.18	2.21E-30
137	HILIC	positive	2.4	815.3439	510.36	424.35	18.01	18.04	153.47	3.33	3.91E-13
138	HILIC	positive	2.89	817.4332	15.78	92.31	29.47	24.19	48.66	0.32	2.06E-03
139	HILIC	positive	2.89	833.4069	31.67	151.78	48.69	38.64	79.70	0.40	4.51E-03
140	HILIC	positive	3.28	840.3520	1.25	141.64	149.59	133.62	141.62	0.01	9.55E-19
141	HILIC	positive	4.84	844.3169	194.01	95.74	85.01	98.92	93.22	2.08	5.11E-08
142	HILIC	positive	2.4	845.3539	739.54	711.36	8.85	6.15	242.12	3.05	1.72E-10
143	HILIC	positive	2.61	846.5475	0.59	57.91	3.23	0.86	20.67	0.03	1.86E-02
144	HILIC	positive	3.29	872.2764	1.22	171.74	196.16	227.86	198.59	0.01	5.33E-32
145	HILIC	positive	3.3	891.2538	0.79	162.97	185.40	211.62	186.66	0.00	1.33E-32
146	HILIC	positive	3.08	911.4986	29.63	156.60	68.32	53.87	92.93	0.32	3.90E-05
147	HILIC	positive	3.11	913.5136	100.78	249.11	241.04	162.11	217.42	0.46	1.71E-12
148	HILIC	positive	3.09	933.4805	17.82	138.34	33.27	29.80	67.14	0.27	1.87E-03
149	HILIC	positive	3.12	935.4961	86.39	214.78	201.15	132.26	182.73	0.47	5.66E-10
150	HILIC	positive	3.23	941.5090	359.71	1570.56	568.92	476.29	871.92	0.41	1.23E-03
151	HILIC	positive	3.09	949.4546	104.86	598.83	206.43	149.80	318.36	0.33	1.48E-03

152	HILIC	positive	3.12	951.4694	448.60	1160.13	1031.34	638.35	943.27	0.48	1.84E-08
153	HILIC	positive	2.44	953.7152	84.35	71.36	297.74	144.31	171.14	0.49	1.32E-02
154	HILIC	positive	2.92	955.4886	24.23	83.71	38.55	40.30	54.18	0.45	1.36E-04
155	HILIC	positive	3.29	957.5039	70.51	199.52	124.92	126.80	150.41	0.47	3.59E-08
156	HILIC	positive	2.87	958.4083	55.56	198.70	105.12	140.99	148.27	0.37	1.32E-15
157	HILIC	positive	3.23	959.5195	0.38	123.59	130.05	152.43	135.36	0.00	7.80E-19
158	HILIC	positive	3.23	963.4898	21.69	136.03	26.11	20.05	60.73	0.36	2.41E-02
159	HILIC	positive	3.33	973.4996	3.49	103.28	30.82	11.27	48.46	0.07	3.82E-04
160	HILIC	positive	3.29	975.5145	161.23	1440.97	1338.24	1549.78	1443.00	0.11	2.91E-35
161	HILIC	positive	2.87	976.4180	43.08	147.92	79.70	94.12	107.25	0.40	1.23E-12
162	HILIC	positive	3.23	979.4648	379.53	1499.22	472.89	397.15	789.75	0.48	8.34E-03
163	HILIC	positive	3	994.4292	138.84	400.02	235.34	339.50	324.96	0.43	1.05E-16
164	HILIC	positive	3.3	995.4605	28.69	188.92	118.18	100.73	135.95	0.21	1.06E-06
165	HILIC	positive	3.34	1011.4550	3.13	243.53	93.70	60.60	132.61	0.02	9.36E-06
166	HILIC	positive	3.3	1013.4700	0.00	431.56	427.70	478.52	445.93	0.00	7.38E-31
167	HILIC	positive	3.3	1019.4350	0.00	41.07	13.06	0.00	18.04	0.00	4.44E-02
168	HILIC	positive	3	1032.3850	75.06	246.68	150.41	199.24	198.78	0.38	8.60E-17
169	HILIC	positive	2.44	1037.2180	34.26	100.35	164.43	117.93	127.57	0.27	3.21E-19
170	HILIC	positive	3.15	1055.5400	50.25	58.94	138.68	123.92	107.18	0.47	3.62E-03
171	HILIC	positive	3.24	1083.5150	121.93	50.79	50.67	37.47	46.31	2.63	4.80E-05
172	HILIC	positive	3.24	1087.5650	50.70	71.65	167.95	106.10	115.23	0.44	1.83E-02
173	HILIC	positive	4.84	1096.4000	782.37	321.50	299.31	399.13	339.98	2.30	5.83E-08
174	HILIC	positive	2.85	1105.4970	26.45	139.23	65.64	99.46	101.44	0.26	1.28E-15
175	HILIC	positive	2.86	1107.5130	207.15	824.45	467.89	586.86	626.40	0.33	4.75E-18
176	HILIC	positive	3.28	1107.5530	0.00	28.28	140.54	151.33	106.72	0.00	1.56E-10
177	HILIC	positive	3.1	1109.4910	28.06	121.86	57.10	48.48	75.82	0.37	1.82E-05
178	HILIC	positive	2.87	1129.4950	55.92	237.94	126.88	172.70	179.17	0.31	2.12E-17
179	HILIC	positive	2.85	1143.4530	26.66	132.47	62.34	72.76	89.19	0.30	1.13E-12
180	HILIC	positive	2.87	1145.4680	169.07	625.78	389.08	407.25	474.04	0.36	4.04E-18
181	HILIC	positive	3.31	1145.5110	198.37	0.00	0.00	0.00	0.00	n/a	4.04E-13
182	HILIC	positive	3.28	1151.5020	0.70	144.14	127.46	143.60	138.40	0.01	3.99E-33
183	HILIC	positive	3.05	1161.5670	238.08	0.04	0.00	0.17	0.07	3364.22	1.59E-10
184	HILIC	positive	2.84	1195.5500	140.69	0.11	0.13	0.02	0.09	1634.79	1.85E-10
185	HILIC	positive	3.05	1199.5230	348.05	1.34	0.06	3.72	1.71	203.67	2.00E-10
186	HILIC	positive	2.94	1203.5770	1039.17	0.09	0.00	0.00	0.03	35084.55	5.75E-11
187	HILIC	positive	2.4	1209.2530	175.08	126.33	0.01	0.00	42.11	4.16	1.29E-15
188	HILIC	positive	2.84	1211.5230	165.80	0.32	0.34	0.23	0.30	560.91	1.60E-12
189	HILIC	positive	2.95	1225.5590	470.75	0.00	0.00	0.00	0.00	n/a	1.34E-12
190	HILIC	positive	3	1233.5880	0.16	149.98	143.46	181.71	158.38	0.00	3.98E-29

191	HILIC	positive	2.4	1234.3070	220.01	148.59	0.00	0.00	49.53	4.44	8.10E-17
192	HILIC	positive	2.57	1234.6270	2.29	22.74	26.21	7.47	18.81	0.12	2.43E-02
193	HILIC	positive	2.4	1239.2630	391.47	299.14	0.01	0.03	99.72	3.93	1.51E-14
194	HILIC	positive	2.95	1241.5330	812.00	0.00	0.00	0.00	0.00	n/a	1.52E-11
195	HILIC	positive	3	1255.5700	0.36	224.25	186.99	269.22	226.82	0.00	9.87E-18
196	HILIC	positive	2.39	1264.3180	324.89	235.63	0.00	0.05	78.56	4.14	1.87E-15
197	HILIC	positive	2.44	1265.6210	26.53	47.37	131.70	37.90	72.32	0.37	7.03E-05
198	HILIC	positive	2.82	1267.5700	0.48	371.14	358.19	336.99	355.44	0.00	1.71E-37
199	HILIC	positive	2.4	1269.2730	498.95	385.84	0.06	0.00	128.64	3.88	3.05E-14
200	HILIC	positive	3.01	1271.5430	1.08	576.27	470.73	561.93	536.31	0.00	1.37E-24
201	HILIC	positive	3.29	1273.0840	2.42	180.23	32.10	29.17	80.50	0.03	2.85E-07
202	HILIC	positive	3.28	1273.6450	260.80	188.86	9.17	4.04	67.36	3.87	4.02E-04
203	HILIC	positive	2.92	1275.5980	2.49	793.42	736.21	898.97	809.54	0.00	6.10E-36
204	HILIC	positive	2.82	1283.5440	0.44	394.59	426.78	317.75	379.71	0.00	8.23E-29
205	HILIC	positive	3.28	1291.5600	2.51	236.35	46.54	42.94	108.61	0.02	1.88E-07
206	HILIC	positive	3.28	1292.0620	4.72	324.73	83.40	69.15	159.09	0.03	2.02E-08
207	HILIC	positive	2.39	1294.3280	301.38	237.64	0.01	0.00	79.22	3.80	2.04E-13
208	HILIC	positive	2.93	1297.5800	0.85	588.21	367.51	489.42	481.71	0.00	4.46E-22
209	HILIC	positive	2.39	1299.2840	414.36	356.07	0.05	0.00	118.70	3.49	5.34E-12
210	HILIC	positive	3.28	1299.5560	0.92	77.20	118.67	137.81	111.22	0.01	1.60E-21
211	HILIC	positive	3.28	1300.0590	0.61	100.42	155.18	179.51	145.03	0.00	3.83E-21
212	HILIC	positive	2.93	1313.5540	2.45	1468.48	939.55	1062.42	1156.82	0.00	1.15E-22
213	HILIC	positive	3.41	1323.6200	196.03	0.23	0.05	0.20	0.16	1231.19	6.60E-09
214	HILIC	positive	4.84	1338.9640	181.69	43.75	27.61	59.72	43.69	4.16	4.04E-10
215	HILIC	positive	4.84	1339.4670	217.11	54.99	37.17	85.21	59.12	3.67	2.77E-09
216	HILIC	positive	4.84	1348.4840	458.70	182.50	165.36	237.25	195.04	2.35	8.40E-07
217	HILIC	positive	4.84	1348.9850	465.67	175.75	180.93	256.08	204.25	2.28	3.52E-06
218	HILIC	positive	3.25	1349.6350	130.13	0.04	0.06	0.01	0.03	3724.81	1.52E-08
219	HILIC	positive	3.41	1361.5750	712.38	1.48	2.48	0.72	1.56	455.85	6.24E-10
220	HILIC	positive	3.31	1365.6300	804.89	0.45	1.94	0.83	1.07	751.78	7.19E-13
221	HILIC	positive	3.25	1387.5910	261.44	0.00	0.00	0.07	0.02	11031.30	1.07E-11
222	HILIC	positive	3.36	1395.6410	0.32	119.90	90.31	120.91	110.37	0.00	1.26E-24
223	HILIC	positive	3.31	1403.5860	1644.19	1.68	1.32	1.68	1.56	1054.50	7.91E-12
224	HILIC	positive	3.23	1421.6560	0.21	160.52	130.28	162.36	151.05	0.00	9.32E-32
225	HILIC	positive	3.31	1423.0650	123.34	0.37	0.07	0.21	0.22	568.17	6.48E-08
226	HILIC	positive	3.1	1429.6010	0.10	203.69	154.44	144.95	167.69	0.00	3.14E-27
227	HILIC	positive	3.36	1433.5970	1.96	497.93	415.08	515.07	476.03	0.00	1.83E-27
228	HILIC	positive	3.29	1437.6510	3.46	969.18	835.65	947.60	917.47	0.00	1.67E-34
229	HILIC	positive	3.31	1441.5420	282.29	0.35	0.70	0.41	0.48	583.46	5.17E-11

230	HILIC	positive	3.28	1457.1320	0.01	106.00	125.81	165.22	132.34	0.00	1.32E-21
231	HILIC	positive	3.23	1459.6120	1.27	445.59	431.53	473.83	450.32	0.00	1.47E-34
232	HILIC	positive	3.29	1475.6070	16.28	2448.06	2259.12	2632.56	2446.58	0.01	1.14E-33
233	HILIC	positive	3.29	1494.5850	0.00	78.74	105.57	130.44	104.92	0.00	2.56E-19
234	HILIC	positive	3.29	1495.0860	0.06	119.69	154.48	182.72	152.30	0.00	2.29E-20
235	HILIC	positive	3.3	1513.5630	0.31	108.08	133.00	165.03	135.37	0.00	1.08E-21
236	HILIC	positive	3.07	1559.3180	46.80	72.91	167.99	126.44	122.45	0.38	2.65E-10
237	HILIC	positive	4.85	1591.5500	124.76	33.49	22.22	53.17	36.29	3.44	2.57E-09
238	HILIC	positive	4.84	1600.5690	190.42	73.10	62.99	105.90	80.66	2.36	9.82E-09
239	HILIC	positive	4.85	1601.0700	249.27	85.47	76.88	144.62	102.32	2.44	2.87E-06
240	HILIC	positive	5.26	1695.0190	48.70	176.92	114.85	165.98	152.58	0.32	7.49E-03
1	HILIC	negative	3.87	78.9580	854.53	0.00	0.00	0.00	0.00	n/a	1.11E-13
2	HILIC	negative	3.87	96.9688	256.92	0.00	0.00	0.00	0.00	n/a	3.86E-03
3	HILIC	negative	3.97	111.0076	0.00	671.67	749.01	688.41	704.65	0.00	1.68E-22
4	HILIC	negative	4.33	111.0076	343.20	0.00	47.99	0.00	16.00	21.46	1.54E-03
5	HILIC	negative	2.4	127.0390	896.18	707.42	4.62	1.48	237.68	3.77	6.64E-14
6	HILIC	negative	4.73	131.0811	3522.67	1417.07	1316.81	1537.72	1416.29	2.49	1.37E-11
7	HILIC	negative	4.8	154.0613	1967.19	561.01	444.62	658.28	545.91	3.60	3.31E-11
8	HILIC	negative	3.87	158.9246	1087.06	0.07	0.00	0.00	0.02	44298.43	4.69E-11
9	HILIC	negative	2.41	171.0288	851.56	743.64	0.66	0.86	248.43	3.43	1.38E-11
10	HILIC	negative	4.73	173.1034	5589.43	2327.67	2154.56	2242.17	2232.45	2.50	7.50E-12
11	HILIC	negative	3.88	176.9352	2484.79	0.00	0.00	0.00	0.00	n/a	2.44E-11
12	HILIC	negative	2.4	193.0496	919.39	692.69	7.78	5.05	234.96	3.91	9.32E-15
13	HILIC	negative	4.44	195.0500	232.61	0.00	0.00	0.00	0.00	n/a	8.54E-03
14	HILIC	negative	2.4	197.0446	1242.59	1097.97	0.00	9.97	371.12	3.35	7.39E-12
15	HILIC	negative	2.79	197.0446	1711.62	689.30	864.96	978.80	841.92	2.03	8.21E-13
16	HILIC	negative	3.87	198.9171	3441.10	0.00	0.00	0.01	0.00	820834.46	3.41E-13
17	HILIC	negative	2.4	215.0188	520.97	439.15	3.49	2.31	148.05	3.52	1.48E-12
18	HILIC	negative	3.88	216.9277	3347.75	0.00	0.00	0.00	0.00	n/a	1.02E-14
19	HILIC	negative	3.8	223.0449	1720.21	635.12	558.59	625.25	601.41	2.86	1.70E-07
20	HILIC	negative	2.4	223.0602	1064.82	872.55	2.21	1.90	292.22	3.64	3.18E-13
21	HILIC	negative	3.87	232.9017	439.46	0.02	0.00	0.01	0.01	35429.66	2.13E-05
22	HILIC	negative	2.4	265.0707	611.12	492.83	2.36	0.00	165.06	3.70	4.65E-13
23	HILIC	negative	4.9	265.0927	344.88	137.67	134.00	180.65	148.40	2.32	2.48E-07
24	HILIC	negative	2.61	275.0211	0.00	176.37	216.69	591.46	329.18	0.00	4.03E-06
25	HILIC	negative	2.31	279.0588	413.32	306.15	58.70	77.93	147.59	2.80	1.10E-07
26	HILIC	negative	3.86	280.9880	0.00	194.27	118.57	313.12	208.69	0.00	3.27E-07
27	HILIC	negative	4.9	283.1038	1160.46	450.89	469.21	595.79	499.45	2.32	1.99E-07
28	HILIC	negative	3.86	292.9227	327.69	0.01	0.00	0.02	0.01	29827.12	2.34E-05

29	HILIC	negative	4.72	293.0616	830.87	34.26	13.21	38.60	28.69	28.96	5.23E-19
30	HILIC	negative	4.73	299.0801	416.62	65.49	32.08	70.72	56.10	7.43	3.77E-04
31	HILIC	negative	4.78	300.0482	554.44	80.71	147.01	108.11	111.47	4.97	1.38E-11
32	HILIC	negative	3.86	300.8657	929.87	0.00	0.01	0.00	0.00	247464.33	1.20E-14
33	HILIC	negative	3.9	302.1344	1353.87	623.04	751.98	597.27	662.53	2.04	1.20E-10
34	HILIC	negative	4.91	305.0856	552.43	211.56	240.19	330.70	257.71	2.14	1.26E-14
35	HILIC	negative	3.87	314.9046	1365.94	0.00	0.01	0.00	0.00	366515.16	4.33E-13
36	HILIC	negative	3.87	318.8760	2546.28	0.00	0.00	0.00	0.00	n/a	1.29E-14
37	HILIC	negative	4.56	323.0277	5133.99	2315.58	2811.47	2277.44	2476.49	2.07	5.18E-06
38	HILIC	negative	4.05	328.9782	608.61	249.43	291.77	183.10	243.37	2.50	2.00E-02
39	HILIC	negative	4.79	331.1120	341.83	49.64	25.64	67.29	45.81	7.46	7.38E-12
40	HILIC	negative	3.85	334.1071	476.62	156.22	238.08	194.90	204.08	2.34	1.73E-10
41	HILIC	negative	3.87	334.8500	682.62	0.04	0.00	0.00	0.01	54655.58	3.50E-14
42	HILIC	negative	3.82	335.0583	0.00	425.33	451.81	434.33	436.70	0.00	2.29E-46
43	HILIC	negative	4.08	336.8580	0.43	466.54	846.29	703.48	673.35	0.00	1.14E-15
44	HILIC	negative	4.02	336.8701	1288.89	85.63	100.32	68.47	86.57	14.89	7.82E-07
45	HILIC	negative	3.87	336.8869	3563.58	0.00	0.00	0.00	0.00	n/a	1.47E-13
46	HILIC	negative	3.97	337.0620	16.06	490.84	535.33	540.21	522.16	0.03	2.32E-35
47	HILIC	negative	5	341.1080	6887.78	2713.87	4060.88	2660.23	3166.76	2.18	4.26E-11
48	HILIC	negative	4.8	346.0544	3269.34	1464.30	1610.97	1732.85	1599.96	2.04	2.71E-05
49	HILIC	negative	4.72	347.2148	622.34	97.25	52.58	105.98	84.07	7.40	4.65E-09
50	HILIC	negative	4.79	350.1535	368.30	71.69	49.07	83.60	66.93	5.50	6.38E-13
51	HILIC	negative	3.87	352.8606	1317.83	0.00	0.00	0.06	0.02	65617.33	1.04E-13
52	HILIC	negative	2.4	353.0505	351.28	335.21	0.00	0.00	111.74	3.14	2.45E-10
53	HILIC	negative	4.72	353.2336	774.08	68.36	33.36	141.01	69.79	11.09	5.45E-26
54	HILIC	negative	4.38	354.0665	2259.18	880.52	987.05	726.78	870.05	2.60	5.18E-09
55	HILIC	negative	3.97	359.0442	0.00	474.53	485.56	520.75	493.33	0.00	9.48E-34
56	HILIC	negative	2.81	359.0972	3545.04	1144.39	1527.64	1748.12	1466.96	2.42	2.09E-16
57	HILIC	negative	2.43	361.1399	0.00	95.21	336.79	475.84	297.72	0.00	4.81E-11
58	HILIC	negative	3.92	364.0541	10.31	365.77	357.20	435.91	384.22	0.03	1.40E-33
59	HILIC	negative	2.4	365.0864	409.73	307.79	1.19	2.82	103.99	3.94	7.78E-15
60	HILIC	negative	2.4	369.0811	619.43	509.24	0.00	0.00	169.75	3.65	4.37E-13
61	HILIC	negative	4.73	369.1965	2795.90	725.56	632.88	810.28	716.90	3.90	4.73E-11
62	HILIC	negative	2.62	373.1854	871.41	443.43	347.56	369.00	385.60	2.26	2.98E-12
63	HILIC	negative	3.85	374.1558	535.74	13.58	12.12	40.85	22.18	24.15	5.46E-22
64	HILIC	negative	2.42	383.0608	1837.97	1519.79	19.75	17.89	519.48	3.54	1.03E-12
65	HILIC	negative	5	387.1129	802.32	244.21	423.88	291.57	326.01	2.46	1.69E-10
66	HILIC	negative	4.43	391.0995	2907.33	1798.69	779.80	1782.79	1419.99	2.05	2.62E-02
67	HILIC	negative	2.4	395.0968	398.24	330.12	1.70	0.00	110.61	3.60	4.51E-13

68	HILIC	negative	2.86	395.1544	5864.13	1223.20	1970.51	1359.56	1532.78	3.83	7.18E-10
69	HILIC	negative	2.64	403.0870	1818.63	451.29	522.75	486.10	480.67	3.78	6.94E-23
70	HILIC	negative	3.84	407.0852	835.31	0.00	0.00	0.25	0.08	9959.46	1.35E-05
71	HILIC	negative	2.4	409.0765	3775.82	2812.58	41.87	34.71	963.08	3.92	6.90E-15
72	HILIC	negative	2.41	413.0738	3744.39	3345.72	505.59	413.93	1425.64	2.63	6.88E-12
73	HILIC	negative	3.95	418.0594	2049.50	6.88	0.31	0.00	2.73	751.50	5.60E-04
74	HILIC	negative	3.11	421.0017	4000.12	1965.82	1577.89	1740.98	1691.68	2.36	9.56E-04
75	HILIC	negative	5.16	421.0739	6159.69	1406.58	1669.94	1216.84	1441.96	4.27	1.41E-09
76	HILIC	negative	2.81	427.0852	343.95	113.96	139.13	154.10	134.53	2.56	2.50E-13
77	HILIC	negative	3.9	427.1080	345.49	9.71	9.15	7.84	8.90	38.82	3.17E-19
78	HILIC	negative	3.82	429.0671	1321.24	0.00	0.00	0.00	0.00	n/a	7.98E-06
79	HILIC	negative	2.4	431.0822	639.42	518.62	1.19	2.19	174.00	3.67	1.38E-12
80	HILIC	negative	2.63	433.2348	667.18	355.84	308.95	238.55	312.21	2.14	1.64E-20
81	HILIC	negative	3.87	434.8634	2075.87	0.62	0.11	0.20	0.31	6658.31	2.71E-14
82	HILIC	negative	4.26	439.0814	32398.34	9047.91	6965.58	10458.99	8749.79	3.70	1.17E-03
83	HILIC	negative	5	439.0851	541.21	71.67	112.67	141.85	104.89	5.16	4.24E-05
84	HILIC	negative	2.4	439.0871	2729.06	2260.10	8.95	12.69	761.05	3.59	4.44E-13
85	HILIC	negative	3.94	440.0465	2405.38	220.85	297.39	124.00	217.31	11.07	1.16E-06
86	HILIC	negative	3.57	442.9835	0.00	91.57	0.00	54.15	48.57	0.00	2.35E-02
87	HILIC	negative	3.13	442.9836	2380.44	1103.71	1167.76	1103.20	1110.04	2.14	6.39E-04
88	HILIC	negative	3.81	445.0413	192.34	0.22	0.78	0.00	0.33	580.22	2.27E-02
89	HILIC	negative	3.32	445.1267	727.56	399.43	191.46	297.46	283.16	2.57	5.41E-09
90	HILIC	negative	3.87	450.8378	1135.02	0.00	0.01	0.14	0.05	21833.13	8.97E-12
91	HILIC	negative	3.81	451.0488	322.73	0.00	0.00	0.00	0.00	n/a	7.51E-05
92	HILIC	negative	5.18	453.0119	807.06	302.45	444.39	212.20	332.28	2.43	1.23E-11
93	HILIC	negative	3.94	456.0196	499.43	101.70	129.20	107.37	112.82	4.43	2.58E-07
94	HILIC	negative	4.01	456.8304	830.73	13.63	6.95	9.53	9.70	85.60	1.27E-07
95	HILIC	negative	3.86	456.8461	2230.89	0.00	0.00	0.00	0.00	6577037.75	1.66E-15
96	HILIC	negative	4	472.8054	531.25	3.85	10.40	1.19	5.37	98.94	3.27E-05
97	HILIC	negative	3.86	472.8198	1222.13	0.03	0.00	0.00	0.01	132700.60	5.66E-14
98	HILIC	negative	2.91	476.2772	5574.54	1849.09	2807.49	2333.45	2281.52	2.44	2.64E-04
99	HILIC	negative	5.15	489.0615	510.42	0.00	0.00	0.00	0.00	n/a	1.58E-04
100	HILIC	negative	4.8	496.1768	1290.65	654.29	560.31	649.98	616.14	2.09	1.75E-07
101	HILIC	negative	4.02	503.0850	919.31	324.36	520.33	199.92	355.28	2.59	2.36E-11
102	HILIC	negative	3.82	505.1069	26.90	624.88	518.67	730.89	616.08	0.04	1.33E-20
103	HILIC	negative	4.3	507.0721	552.78	0.00	0.00	0.00	0.00	n/a	9.04E-06
104	HILIC	negative	4.68	513.1187	2729.30	1138.06	1235.13	1317.25	1224.71	2.23	1.23E-07
105	HILIC	negative	4.28	516.2034	1693.36	446.66	605.74	592.59	544.80	3.11	2.88E-31
106	HILIC	negative	2.4	517.0978	1635.26	1274.11	0.00	0.00	424.70	3.85	5.82E-14

107	HILIC	negative	4.44	521.0879	653.64	207.28	186.74	160.41	185.68	3.52	9.33E-40
108	HILIC	negative	4.72	521.3214	722.60	9.65	0.00	41.23	16.96	42.60	3.35E-06
109	HILIC	negative	3.9	525.0852	425.06	0.00	0.00	0.57	0.19	2247.46	1.50E-08
110	HILIC	negative	4.73	527.3466	1525.86	427.88	364.98	422.94	401.27	3.80	2.56E-11
111	HILIC	negative	3.87	532.8408	919.51	0.00	0.08	0.00	0.03	34844.29	9.83E-14
112	HILIC	negative	2.3	535.1083	517.33	750.06	5.78	3.31	253.37	2.04	1.52E-04
113	HILIC	negative	3.94	538.0263	630.50	21.30	32.26	12.35	21.77	28.96	3.54E-15
114	HILIC	negative	2.4	547.1084	2282.77	1700.73	27.94	22.70	583.69	3.91	7.66E-15
115	HILIC	negative	3.87	548.8152	490.81	0.10	0.00	0.00	0.03	14720.75	2.04E-05
116	HILIC	negative	2.44	551.1035	1053.27	704.35	0.00	0.00	234.78	4.49	3.09E-15
117	HILIC	negative	3.86	554.8234	1574.73	0.00	0.00	0.23	0.08	20885.77	6.49E-15
118	HILIC	negative	4.29	559.0416	465.58	0.00	0.00	0.00	0.00	n/a	4.55E-12
119	HILIC	negative	3.94	560.0054	755.86	99.74	145.98	41.25	100.13	7.55	1.33E-11
120	HILIC	negative	2.97	569.0758	0.00	356.61	42.67	254.11	203.57	0.00	1.01E-04
121	HILIC	negative	3.87	570.7968	1169.83	0.07	0.00	0.00	0.02	50175.63	9.39E-14
122	HILIC	negative	4.72	571.3120	470.61	135.59	69.98	144.89	114.58	4.11	8.96E-20
123	HILIC	negative	2.83	573.1565	1674.59	765.63	894.72	728.20	800.67	2.09	1.85E-09
124	HILIC	negative	3.85	576.8051	846.76	0.00	0.00	0.05	0.02	52707.02	7.69E-14
125	HILIC	negative	2.41	577.1194	31410.55	25617.31	250.96	185.75	8686.24	3.62	2.62E-13
126	HILIC	negative	2.97	589.0439	0.00	73.43	0.00	156.14	76.52	0.00	2.51E-02
127	HILIC	negative	4.7	589.2560	2636.88	379.63	314.96	676.47	448.73	5.88	1.05E-04
128	HILIC	negative	4.85	601.1346	9994.62	4409.29	4878.26	4357.45	4515.28	2.21	1.66E-11
129	HILIC	negative	3.82	603.0841	390.39	12.58	12.67	13.30	12.85	30.38	5.79E-08
130	HILIC	negative	2.81	603.1676	1471.33	489.82	614.41	525.98	545.06	2.70	2.89E-10
131	HILIC	negative	2.4	607.1299	43585.41	34614.07	489.74	438.27	11848.70	3.68	7.34E-14
132	HILIC	negative	2.42	612.1272	750.16	489.96	7.64	6.47	167.64	4.47	9.98E-16
133	HILIC	negative	2.99	619.0540	0.00	106.39	223.96	376.97	230.39	0.00	8.38E-06
134	HILIC	negative	4.71	619.3027	161.06	0.00	0.00	2.32	0.77	208.42	2.03E-02
135	HILIC	negative	2.38	621.1451	3.60	325.64	646.72	812.06	589.00	0.01	3.05E-18
136	HILIC	negative	4.72	625.3219	565.30	78.89	50.77	55.30	58.74	9.62	9.18E-11
137	HILIC	negative	3.1	635.0107	555.27	91.13	37.82	89.49	72.81	7.63	2.77E-03
138	HILIC	negative	2.4	637.1405	52777.20	48379.38	303.73	228.55	16305.15	3.24	2.96E-11
139	HILIC	negative	2.37	651.1554	0.00	150.27	584.37	800.84	503.38	0.00	1.11E-12
140	HILIC	negative	3.87	652.7991	928.35	0.00	0.00	0.00	0.00	n/a	4.01E-15
141	HILIC	negative	2.39	656.3292	6.66	20.97	303.48	325.07	214.88	0.03	2.37E-10
142	HILIC	negative	4.69	657.2446	195.55	11.20	10.32	25.96	13.72	14.26	5.43E-06
143	HILIC	negative	2.4	657.3577	0.00	0.00	207.50	318.34	175.68	0.00	1.78E-05
144	HILIC	negative	3.94	657.9842	631.55	11.39	33.81	6.02	18.27	34.58	1.38E-16
145	HILIC	negative	4.84	658.2307	10952.36	2923.24	2140.00	3086.06	2675.25	4.09	8.36E-15

146	HILIC	negative	2.83	659.1820	290.56	63.74	103.36	114.83	93.66	3.10	4.14E-09
147	HILIC	negative	5.18	667.1251	1466.67	269.20	700.91	242.57	419.44	3.50	1.05E-07
148	HILIC	negative	2.99	667.2811	334.75	52.21	39.90	12.45	36.37	9.20	4.04E-03
149	HILIC	negative	3.87	668.7742	781.72	0.05	0.00	0.14	0.06	12394.36	1.87E-12
150	HILIC	negative	4.89	669.1257	777.58	332.93	338.44	290.62	322.17	2.41	8.25E-26
151	HILIC	negative	3.01	669.2963	159.27	17.08	14.02	9.20	13.44	11.85	6.56E-03
152	HILIC	negative	4.98	672.2464	767.41	208.90	137.41	161.82	168.19	4.56	4.09E-09
153	HILIC	negative	3.93	673.9543	325.93	2.34	4.02	0.72	2.36	138.02	4.74E-12
154	HILIC	negative	3.86	674.7807	669.85	0.00	0.00	0.13	0.04	15188.51	1.56E-14
155	HILIC	negative	2.43	680.2178	338.55	385.64	0.00	18.67	134.77	2.51	8.92E-08
156	HILIC	negative	3.86	690.7563	669.24	0.00	0.00	0.00	0.00	n/a	5.70E-14
157	HILIC	negative	4.98	691.2881	638.57	343.98	342.07	242.40	310.48	2.06	4.86E-06
158	HILIC	negative	4.38	696.1875	1266.62	505.33	575.87	418.24	503.89	2.51	4.24E-05
159	HILIC	negative	3.82	697.1335	0.00	328.45	263.76	375.99	318.19	0.00	1.35E-20
160	HILIC	negative	2.62	698.2289	562.26	224.41	102.36	497.60	257.24	2.19	7.44E-03
161	HILIC	negative	4.86	699.2196	812.67	299.88	340.87	272.40	303.35	2.68	3.10E-08
162	HILIC	negative	4.72	701.4586	400.72	11.39	0.00	25.77	12.39	32.35	1.94E-06
163	HILIC	negative	4.72	707.4791	339.17	30.88	0.00	50.49	27.12	12.50	2.38E-05
164	HILIC	negative	4.37	710.2034	737.76	231.62	254.90	165.51	219.01	3.37	3.11E-08
165	HILIC	negative	2.41	710.2290	459.58	491.93	18.95	14.82	176.01	2.61	9.10E-09
166	HILIC	negative	3.37	719.3125	4.50	340.38	291.58	307.82	310.80	0.01	3.03E-25
167	HILIC	negative	2.4	740.2398	696.25	838.31	15.95	13.39	289.24	2.41	4.44E-07
168	HILIC	negative	5.78	745.1797	958.95	460.93	546.72	394.96	463.99	2.07	6.96E-06
169	HILIC	negative	3.86	750.7756	232.40	0.00	0.00	0.30	0.10	2289.89	6.99E-03
170	HILIC	negative	2.21	754.4749	171.17	68.94	12.74	14.96	32.21	5.31	1.67E-02
171	HILIC	negative	5.17	755.1382	547.39	9.94	11.25	11.17	10.78	50.76	1.00E-07
172	HILIC	negative	5.25	763.1858	2389.16	1217.96	1215.77	953.08	1144.91	2.09	2.62E-08
173	HILIC	negative	2.65	770.4958	256.95	26.85	102.89	113.41	77.22	3.33	1.82E-02
174	HILIC	negative	3.86	772.7600	543.11	0.00	0.00	0.15	0.05	11175.30	2.61E-14
175	HILIC	negative	5.17	777.1256	520.49	18.44	0.00	6.55	8.33	62.48	1.32E-08
176	HILIC	negative	4.28	781.1983	2260.80	876.76	895.70	779.27	853.85	2.65	1.10E-14
177	HILIC	negative	4.88	787.2737	797.22	264.53	274.40	370.05	298.45	2.67	2.48E-05
178	HILIC	negative	3.86	788.7327	481.56	0.01	0.00	0.03	0.01	34135.71	1.57E-05
179	HILIC	negative	2.78	797.3202	1197.88	590.18	559.15	451.10	537.98	2.23	2.68E-10
180	HILIC	negative	2.78	813.2941	590.43	294.82	317.85	224.18	283.05	2.09	9.09E-07
181	HILIC	negative	2.59	818.5527	271.51	102.33	134.46	122.35	120.44	2.25	1.05E-04
182	HILIC	negative	5.17	837.2276	658.06	299.05	334.47	219.26	294.50	2.23	4.19E-05
183	HILIC	negative	2.62	858.5479	541.88	221.93	124.81	123.88	148.15	3.66	1.66E-02
184	HILIC	negative	2.78	878.4383	348.28	150.57	138.77	140.21	142.86	2.44	7.14E-07

185	HILIC	negative	2.38	884.2452	399.34	281.18	13.15	13.15	102.49	3.90	2.58E-16
186	HILIC	negative	3.47	884.6095	1405.54	604.22	653.69	742.72	662.94	2.12	9.97E-05
187	HILIC	negative	5.16	911.2650	234.46	91.57	112.63	63.39	90.34	2.60	3.15E-07
188	HILIC	negative	2.38	914.2554	588.46	475.84	14.20	10.80	166.93	3.53	1.59E-13
189	HILIC	negative	4.31	923.1338	394.61	170.80	179.36	152.55	164.84	2.39	2.84E-06
190	HILIC	negative	5.58	925.2378	1017.48	424.19	498.85	326.78	419.69	2.42	9.39E-06
191	HILIC	negative	4.38	931.2980	0.00	329.72	488.57	232.03	344.81	0.00	2.11E-07
192	HILIC	negative	2.51	954.5695	388.11	131.16	160.27	150.46	146.31	2.65	6.41E-10
193	HILIC	negative	3.47	968.5710	914.02	416.68	487.85	259.41	387.85	2.36	2.17E-03
194	HILIC	negative	5.26	1047.3090	302.48	155.01	140.73	85.87	127.21	2.38	1.70E-03
195	HILIC	negative	3.48	1052.5320	546.69	12.78	97.56	102.34	70.90	7.71	4.97E-04
196	HILIC	negative	2.63	1071.2250	0.00	0.00	611.99	427.91	355.53	0.00	3.57E-09
197	HILIC	negative	3.3	1103.5180	1410.95	1450.68	363.11	259.06	692.43	2.04	3.91E-04
198	HILIC	negative	4.86	1105.3060	823.92	282.73	293.71	276.66	284.02	2.90	1.14E-11
199	HILIC	negative	2.95	1129.5410	402.86	0.72	0.00	0.36	0.38	1054.00	1.17E-14
200	HILIC	negative	3.3	1141.4730	350.92	305.65	9.83	0.00	105.16	3.34	8.78E-08
201	HILIC	negative	2.41	1155.2450	1128.54	1535.23	2.93	3.24	513.80	2.20	2.68E-05
202	HILIC	negative	3.05	1159.5520	1283.23	2.66	18.18	13.09	12.65	101.44	7.01E-12
203	HILIC	negative	2.84	1171.5520	1110.36	5.32	7.72	2.28	5.28	210.41	3.45E-12
204	HILIC	negative	4.85	1181.4400	318.27	130.45	126.41	135.81	129.83	2.45	1.61E-11
205	HILIC	negative	2.41	1185.2560	1254.25	837.53	0.18	0.28	279.37	4.49	1.74E-12
206	HILIC	negative	2.94	1201.5630	5725.46	434.03	340.79	201.64	327.11	17.50	8.10E-19
207	HILIC	negative	2.4	1215.2670	2436.65	1754.48	0.12	0.36	584.98	4.17	2.73E-12
208	HILIC	negative	3	1231.5750	0.90	463.47	505.14	655.62	556.00	0.00	2.05E-05
209	HILIC	negative	2.82	1243.5740	5.24	2668.31	2535.00	2366.05	2525.93	0.00	1.04E-42
210	HILIC	negative	3.22	1243.5910	2.11	251.68	277.78	288.19	271.27	0.01	1.23E-24
211	HILIC	negative	3.23	1244.0940	2.58	320.50	356.64	359.67	344.32	0.01	1.03E-24
212	HILIC	negative	2.4	1245.2780	2428.53	1815.19	0.07	0.23	605.16	4.01	3.57E-12
213	HILIC	negative	4.27	1245.8870	792.96	399.31	187.45	448.64	329.48	2.41	2.69E-12
214	HILIC	negative	3.28	1259.5850	10.00	487.98	429.37	403.55	439.92	0.02	1.71E-24
215	HILIC	negative	2.93	1273.5850	64.40	6426.42	5434.30	6063.75	5953.60	0.01	2.19E-33
216	HILIC	negative	2.4	1275.2880	1545.29	1327.63	0.00	0.00	442.54	3.49	9.22E-11
217	HILIC	negative	2.41	1288.3560	624.10	468.18	0.88	0.27	156.41	3.99	6.92E-15
218	HILIC	negative	2.4	1318.3670	1223.16	955.85	0.30	0.33	318.78	3.84	4.50E-14
219	HILIC	negative	3.42	1321.6060	1098.76	6.43	5.17	3.44	4.94	222.64	5.88E-11
220	HILIC	negative	3.25	1347.6200	438.99	0.01	1.01	0.75	0.59	743.15	4.02E-14
221	HILIC	negative	2.4	1348.3770	1221.67	961.08	0.02	0.26	320.45	3.81	5.53E-14
222	HILIC	negative	3.31	1363.6160	2521.63	0.00	0.00	0.00	0.00	n/a	9.42E-16
223	HILIC	negative	5.3	1365.3940	0.00	184.50	136.78	70.17	138.51	0.00	1.36E-06

224	HILIC	negative	2.4	1378.3880	793.81	712.79	0.30	0.02	237.70	3.34	1.80E-11
225	HILIC	negative	3.31	1399.5910	1000.70	5.02	7.05	3.66	5.26	190.39	1.74E-11
226	HILIC	negative	3.41	1405.5660	497.86	3.79	2.80	1.45	2.85	174.81	4.61E-11
227	HILIC	negative	3.2	1405.6260	0.00	320.19	233.06	190.33	248.54	0.00	4.93E-22
228	HILIC	negative	3.31	1409.6200	604.20	0.59	3.85	2.70	2.25	268.32	7.36E-17
229	HILIC	negative	3.24	1419.6410	2.96	702.82	558.74	556.62	603.11	0.00	3.76E-26
230	HILIC	negative	3.29	1435.6380	55.69	4719.68	4585.06	4557.37	4602.71	0.01	6.00E-27
231	HILIC	negative	3.31	1437.5470	610.78	6.18	2.77	2.18	4.04	151.12	4.89E-12
232	HILIC	negative	3.32	1447.5780	375.73	2.17	0.23	1.65	1.35	278.23	1.73E-12
233	HILIC	negative	3.23	1465.6480	3.09	674.35	508.90	534.35	570.87	0.01	1.43E-25
234	HILIC	negative	3.3	1471.6110	9.85	1070.27	1006.28	1009.19	1021.35	0.01	8.75E-25
235	HILIC	negative	3.37	1477.5880	1.06	380.75	315.56	348.47	345.10	0.00	1.14E-24
236	HILIC	negative	3.29	1481.6420	9.25	647.58	587.31	567.06	598.35	0.02	2.87E-27
237	HILIC	negative	2.82	1487.6410	0.10	255.20	240.73	181.71	227.44	0.00	7.39E-28
238	HILIC	negative	3.3	1509.5680	6.18	574.71	567.76	571.71	567.96	0.01	9.44E-26
239	HILIC	negative	3.29	1519.5990	6.51	658.72	658.53	669.49	660.98	0.01	4.31E-26
240	HILIC	negative	4.85	1560.4870	386.82	172.12	176.17	210.42	184.58	2.10	1.93E-08
241	HILIC	negative	4.85	1560.9880	468.90	197.70	218.87	255.93	221.85	2.11	5.07E-07
242	HILIC	negative	2.93	1629.6550	0.00	202.46	153.90	247.07	199.82	0.00	1.23E-13
243	HILIC	negative	3.29	1665.6200	1.81	248.65	261.07	275.29	259.84	0.01	4.65E-26

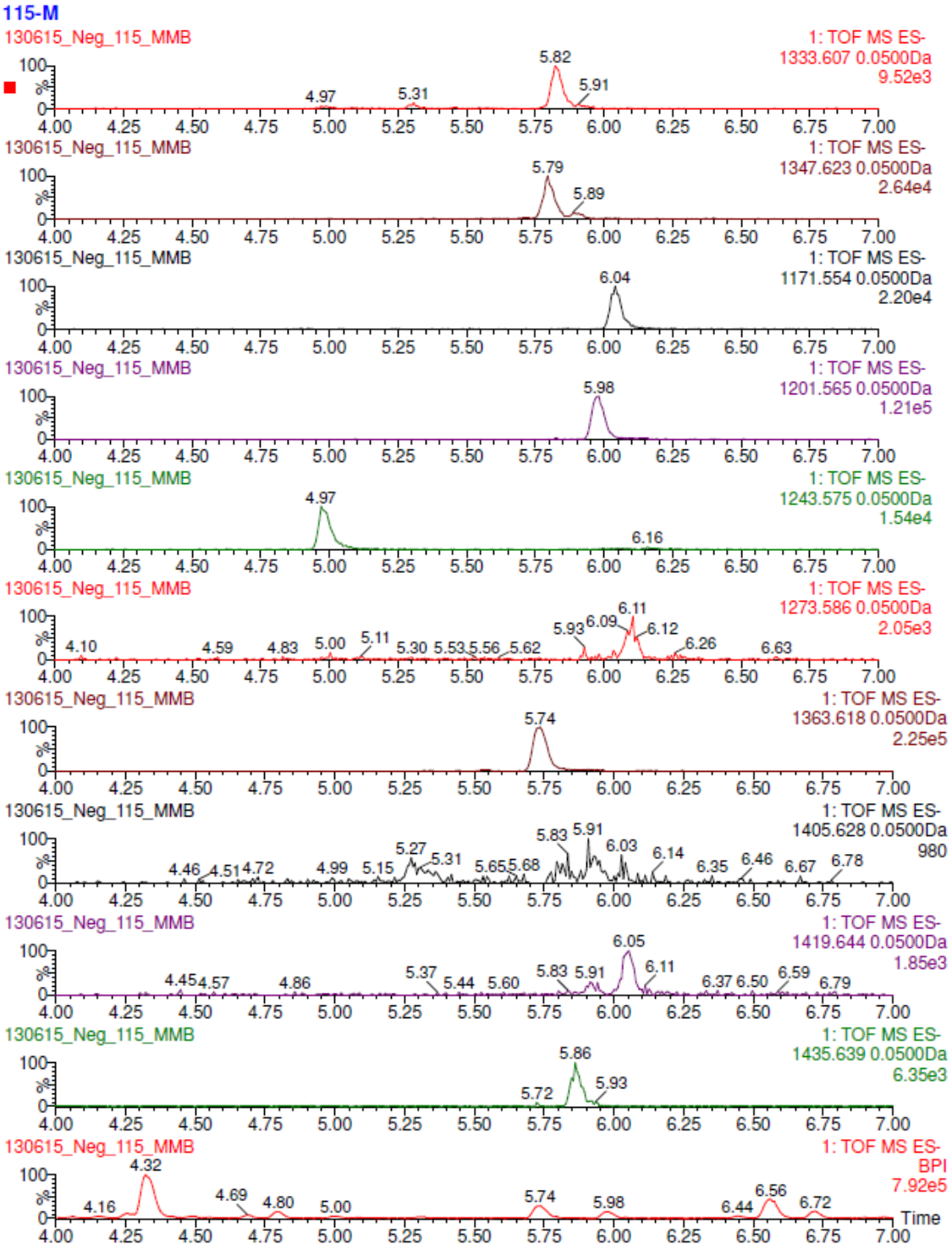
Table S3.3. EMRTs with a *p*-value < 0.05 and a factor of change less than 0.5 or greater than 2 separated into all the comparison between the low phytate line and all normal phytate lines. These EMRTs do not show up in any normal phytate vs. normal phytate comparisons for both columns and ionization modes but in all low phytate vs. normal phytate comparisons. The averages of the classes represent the peak areas.

Column	Ion mode	RT	m/z	average mrp(3)/ *mrp*(19)	average mrp(3)/ MRP(19)	average MRP(3)/ *mrp*(19)	average MRP(3)/ MRP(19)	all normal phytate (NP) lines	factor of change all NP/LP	p Value
RPC	positive	3.08	587.1583	53.58	130.44	128.54	132.37	130.45	0.41	2.18E-12
RPC	positive	3.81	255.0657	283.20	723.89	840.21	728.11	764.07	2.70	1.77E-15
RPC	positive	5.75	1107.5560	506.99	0.68	0.80	0.16	0.54	0.00	2.18E-08
RPC	positive	5.75	1365.6300	463.88	0.12	0.11	0.05	0.09	0.00	3.87E-06
RPC	positive	5.88	975.5144	1.22	1173.73	936.64	965.80	1025.39	842.66	7.09E-23
RPC	positive	5.88	1107.5570	0.00	305.40	260.99	231.27	265.88	864.63	8.55E-22
RPC	positive	5.88	1437.6510	0.09	1020.30	765.24	804.34	863.29	9434.25	1.72E-17
RPC	positive	5.99	1203.5770	78.47	0.00	0.04	0.01	0.02	4186.97	8.03E-05
RPC	positive	6.11	813.4625	0.17	251.39	245.32	244.07	246.93	0.00	1.88E-19
RPC	positive	6.74	617.4044	35.85	143.55	139.59	149.50	144.21	0.25	3.01E-09
RPC	positive	6.74	935.4959	74.20	209.69	217.49	218.50	215.23	0.34	2.97E-09
RPC	negative	2.44	447.1137	293.13	628.74	691.07	920.18	746.66	0.39	1.09E-13
RPC	negative	2.59	389.0715	268.25	404.13	664.60	582.08	550.27	0.49	3.05E-09
RPC	negative	2.67	355.0662	230.15	842.15	682.92	644.52	723.20	0.32	1.20E-07
RPC	negative	3.21	435.1295	107.69	241.69	234.54	217.33	231.18	0.47	1.46E-09
RPC	negative	3.44	587.2337	116.73	528.58	389.45	424.21	447.41	0.26	7.05E-17
RPC	negative	3.64	459.1289	136.09	323.45	239.62	256.44	273.17	0.50	5.28E-08
RPC	negative	3.76	565.1191	638.64	1485.51	2685.70	2067.59	2079.60	0.31	7.57E-14
RPC	negative	3.81	253.0500	711.60	2017.77	1875.65	1599.98	1831.14	0.39	3.56E-09
RPC	negative	3.95	1033.2250	495.34	1455.92	1325.13	1156.25	1312.44	0.38	3.08E-09
RPC	negative	4.08	656.2958	0.24	249.53	162.81	211.96	208.10	0.00	4.37E-16
RPC	negative	4.24	489.1024	112.00	361.23	376.57	278.48	338.76	0.33	1.15E-14
RPC	negative	4.35	1035.2040	3966.68	13500.03	13925.37	8974.51	12133.30	0.33	2.19E-11
RPC	negative	4.68	992.4154	67.93	279.51	150.60	193.10	207.74	0.33	7.56E-11
RPC	negative	4.93	698.3062	0.78	654.55	281.24	395.68	443.82	0.00	8.16E-07
RPC	negative	4.94	675.3033	2.87	488.71	314.36	405.94	403.00	0.01	4.36E-16
RPC	negative	5.03	668.3136	216.88	665.63	460.21	449.39	525.08	0.41	8.31E-06
RPC	negative	5.1	630.3059	188.81	715.05	705.02	634.63	684.90	0.28	2.37E-13
RPC	negative	5.1	653.3084	53.26	296.78	254.98	194.69	248.81	0.21	1.68E-07
RPC	negative	5.26	719.3116	1.71	1433.52	1059.74	1165.33	1219.53	0.00	2.54E-20
RPC	negative	5.31	711.3136	0.12	314.77	208.83	224.29	249.29	0.00	1.93E-13
RPC	negative	5.45	696.3092	2.43	1066.90	727.78	821.61	872.10	0.00	3.26E-18
RPC	negative	5.45	719.3119	6.40	1574.28	1145.68	1325.69	1348.55	0.00	8.58E-21
RPC	negative	5.49	748.3146	0.84	706.80	672.71	655.88	678.46	0.00	8.94E-22
RPC	negative	5.5	711.3136	0.62	403.04	276.31	248.81	309.39	0.00	3.82E-14
RPC	negative	5.53	740.3173	0.46	455.99	348.17	372.26	392.14	0.00	1.52E-18
RPC	negative	5.65	615.2827	0.74	420.47	274.84	304.37	333.23	0.00	1.44E-15
RPC	negative	5.65	638.2856	0.59	382.59	249.97	278.61	303.72	0.00	1.32E-12
RPC	negative	5.88	740.3174	46.87	3410.09	2866.62	2964.77	3080.49	0.02	7.73E-29
RPC	negative	5.95	732.3197	1.80	1056.59	844.19	849.89	916.89	0.00	7.07E-24
RPC	negative	6.05	717.3145	0.21	322.13	252.44	185.21	253.26	0.00	3.68E-17
RPC	negative	6.47	957.5046	1288.25	5219.21	4158.40	2810.99	4062.86	0.32	4.70E-09
RPC	negative	6.47	1025.4930	103.17	395.06	311.88	218.49	308.48	0.33	1.12E-08

RPC	negative	6.66	897.4838	72.48	444.45	351.26	225.10	340.27	0.21	4.26E-09
RPC	negative	6.74	979.4871	309.65	1090.82	1151.90	676.06	972.92	0.32	2.10E-12
RPC	negative	6.86	765.4420	324.11	1630.30	1665.95	856.75	1384.33	0.23	3.90E-10
HILIC	positive	2.8	383.0950	456.41	146.04	196.54	215.80	186.13	2.45	4.45E-15
HILIC	positive	2.81	439.3566	0.00	216.72	196.39	194.72	202.61	0.00	2.85E-17
HILIC	positive	2.82	1267.5700	0.48	371.14	358.19	336.99	355.44	0.00	1.71E-37
HILIC	positive	2.82	1283.5440	0.44	394.59	426.78	317.75	379.71	0.00	8.23E-29
HILIC	positive	2.85	419.1522	832.30	208.18	334.14	247.59	263.31	3.16	2.15E-10
HILIC	positive	2.85	627.2051	150.63	520.20	403.36	385.60	436.38	0.35	6.52E-13
HILIC	positive	2.87	1145.4680	169.07	625.78	389.08	407.25	474.04	0.36	4.04E-18
HILIC	positive	2.92	813.4627	324.38	1537.51	1759.80	2125.55	1807.62	0.18	2.21E-30
HILIC	positive	2.92	1275.5980	2.49	793.42	736.21	898.97	809.54	0.00	6.10E-36
HILIC	positive	2.92	209.1541	15.45	93.81	81.92	62.54	79.42	0.19	1.77E-06
HILIC	positive	2.93	1297.5800	0.85	588.21	367.51	489.42	481.71	0.00	4.46E-22
HILIC	positive	2.93	1313.5540	2.45	1468.48	939.55	1062.42	1156.82	0.00	1.15E-22
HILIC	positive	2.99	421.1107	84.53	185.24	170.91	199.67	185.27	0.46	2.28E-12
HILIC	positive	3.01	1271.5430	1.08	576.27	470.73	561.93	536.31	0.00	1.37E-24
HILIC	positive	3.09	393.0793	827.64	366.19	291.01	284.40	313.86	2.64	4.75E-11
HILIC	positive	3.1	1429.6010	0.10	203.69	154.44	144.95	167.69	0.00	3.14E-27
HILIC	positive	3.23	1459.6120	1.27	445.59	431.53	473.83	450.32	0.00	1.47E-34
HILIC	positive	3.23	1421.6560	0.21	160.52	130.28	162.36	151.05	0.00	9.32E-32
HILIC	positive	3.24	1083.5150	121.93	50.79	50.67	37.47	46.31	2.63	4.80E-05
HILIC	positive	3.28	738.3078	2.64	279.63	302.14	332.10	304.62	0.01	4.07E-38
HILIC	positive	3.28	1151.5020	0.70	144.14	127.46	143.60	138.40	0.01	3.99E-33
HILIC	positive	3.28	1300.0590	0.61	100.42	155.18	179.51	145.03	0.00	3.83E-21
HILIC	positive	3.28	1457.1320	0.01	106.00	125.81	165.22	132.34	0.00	1.32E-21
HILIC	positive	3.29	757.2854	22.52	1787.13	1902.64	2167.64	1952.47	0.01	4.03E-36
HILIC	positive	3.29	975.5145	161.23	1440.97	1338.24	1549.78	1443.00	0.11	2.91E-35
HILIC	positive	3.29	1437.6510	3.46	969.18	835.65	947.60	917.47	0.00	1.67E-34
HILIC	positive	3.29	1475.6070	16.28	2448.06	2259.12	2632.56	2446.58	0.01	1.14E-33
HILIC	positive	3.29	1494.5850	0.00	78.74	105.57	130.44	104.92	0.00	2.56E-19
HILIC	positive	3.29	1495.0860	0.06	119.69	154.48	182.72	152.30	0.00	2.29E-20
HILIC	positive	3.3	776.2632	4.59	556.46	585.60	664.10	602.06	0.01	1.12E-34
HILIC	positive	3.3	1013.4700	0.00	431.56	427.70	478.52	445.93	0.00	7.38E-31
HILIC	positive	3.3	995.4605	28.69	188.92	118.18	100.73	135.95	0.21	1.06E-06
HILIC	positive	3.3	1513.5630	0.31	108.08	133.00	165.03	135.37	0.00	1.08E-21
HILIC	positive	3.31	1403.5860	1644.19	1.68	1.32	1.68	1.56	1054.50	7.91E-12
HILIC	positive	3.31	1441.5420	282.29	0.35	0.70	0.41	0.48	583.46	5.17E-11
HILIC	positive	3.32	740.2527	407.58	0.26	2.17	0.96	1.13	359.81	7.12E-15
HILIC	positive	3.33	202.8982	125.47	269.33	342.29	257.82	289.81	0.43	5.31E-04
HILIC	positive	3.36	755.2590	1.43	228.31	203.90	228.02	220.08	0.01	2.51E-34
HILIC	positive	3.36	1433.5970	1.96	497.93	415.08	515.07	476.03	0.00	1.83E-27
HILIC	positive	3.36	1395.6410	1395.64	0.32	119.90	90.31	120.91	110.37	2.86E-03
HILIC	positive	3.37	736.2806	6.16	629.87	547.90	643.86	607.21	0.01	1.45E-34
HILIC	positive	3.41	700.2702	355.41	3.66	4.07	3.50	3.74	94.91	6.11E-12
HILIC	positive	3.41	1361.5750	712.38	1.48	2.48	0.72	1.56	455.85	6.24E-10
HILIC	positive	3.41	765.2829	0.44	177.32	176.12	196.37	183.27	0.00	3.68E-37
HILIC	positive	3.69	629.2418	270.71	835.34	758.95	714.61	769.63	0.35	1.27E-35
HILIC	positive	3.8	371.0616	243.69	38.01	0.00	61.82	33.28	7.32	7.07E-15
HILIC	positive	3.83	291.1300	223.12	13.66	75.43	75.37	54.82	4.07	1.98E-12
HILIC	positive	3.86	376.1715	264.62	62.37	76.85	96.18	78.47	3.37	9.97E-19
HILIC	positive	3.89	198.1857	280.99	3.37	13.53	9.39	8.76	32.06	4.84E-07
HILIC	positive	3.94	197.0933	19.05	136.46	147.26	144.91	142.88	0.13	2.47E-09
HILIC	positive	3.96	243.1707	152.22	3837.28	2372.44	3631.29	3280.34	0.05	6.88E-19
HILIC	positive	3.96	302.2440	63.38	3306.44	2039.35	3060.60	2802.13	0.02	4.71E-20

HILIC	positive	3.97	151.6261	253.03	1194.52	974.15	1201.10	1123.26	0.23	5.03E-19
HILIC	positive	4.01	400.2111	860.66	2342.86	1763.35	1961.27	2022.49	0.43	5.05E-07
HILIC	positive	4.09	288.2282	8.92	369.58	248.36	292.02	303.32	0.03	1.65E-10
HILIC	positive	4.11	151.6261	16.32	174.00	163.67	133.78	157.15	0.10	2.81E-17
HILIC	positive	4.31	260.1973	77.60	256.29	257.26	297.64	270.40	0.29	5.00E-08
HILIC	positive	4.47	243.1708	34.44	167.92	135.08	181.02	161.34	0.21	6.13E-09
HILIC	positive	4.62	272.1722	1429.94	434.69	432.32	416.03	427.68	3.34	2.61E-08
HILIC	positive	4.63	375.2242	259.06	84.15	103.14	105.04	97.44	2.66	6.07E-05
HILIC	positive	4.68	184.0740	504.05	81.73	91.75	88.48	87.32	5.77	1.60E-04
HILIC	positive	4.74	157.1088	204.04	15.49	21.02	54.95	30.49	6.69	1.76E-06
HILIC	positive	4.75	70.0656	372.64	99.38	73.03	115.10	95.84	3.89	5.36E-17
HILIC	positive	4.75	116.0717	927.23	361.87	369.82	432.05	387.91	2.39	8.60E-10
HILIC	positive	4.75	130.0981	608.01	201.39	208.91	243.33	217.88	2.79	4.27E-11
HILIC	positive	4.75	158.0930	1346.93	529.51	487.43	607.00	541.31	2.49	1.71E-09
HILIC	positive	4.75	175.1195	11762.38	4546.25	4368.65	5074.05	4662.99	2.52	1.16E-08
HILIC	positive	4.82	110.0719	213.71	21.66	8.76	22.58	17.67	12.09	3.50E-04
HILIC	positive	4.87	286.1406	160.82	58.86	56.29	62.78	59.31	2.71	7.81E-07
HILIC	positive	5.16	445.0716	271.68	15.25	16.01	16.94	16.06	16.91	5.80E-07
HILIC	positive	5.31	305.0718	46.38	132.44	171.53	169.79	157.92	0.29	9.10E-04
HILIC	negative	2.64	403.0870	1818.63	451.29	522.75	486.10	480.67	3.78	6.94E-23
HILIC	negative	2.78	797.3202	1197.88	590.18	559.15	451.10	537.98	2.23	2.68E-10
HILIC	negative	2.78	878.4383	348.28	150.57	138.77	140.21	142.86	2.44	7.14E-07
HILIC	negative	2.81	359.0972	3545.04	1144.39	1527.64	1748.12	1466.96	2.42	2.09E-16
HILIC	negative	2.81	603.1676	1471.33	489.82	614.41	525.98	545.06	2.70	2.89E-10
HILIC	negative	2.82	1243.5740	5.24	2668.31	2535.00	2366.05	2525.93	0.00	1.04E-42
HILIC	negative	2.82	1487.6410	0.10	255.20	240.73	181.71	227.44	0.00	7.39E-28
HILIC	negative	2.83	659.1820	290.56	63.74	103.36	114.83	93.66	3.10	4.14E-09
HILIC	negative	2.86	395.1544	5864.13	1223.20	1970.51	1359.56	1532.78	3.83	7.18E-10
HILIC	negative	2.93	1273.5850	64.40	6426.42	5434.30	6063.75	5953.60	0.01	2.19E-33
HILIC	negative	2.93	1629.6550	0.00	202.46	153.90	247.07	199.82	0.00	1.23E-13
HILIC	negative	3.1	635.0107	555.27	91.13	37.82	89.49	72.81	7.63	2.77E-03
HILIC	negative	3.11	421.0017	4000.12	1965.82	1577.89	1740.98	1691.68	2.36	9.56E-04
HILIC	negative	3.13	442.9836	2380.44	1103.71	1167.76	1103.20	1110.04	2.14	6.39E-04
HILIC	negative	3.22	1243.5910	2.11	251.68	277.78	288.19	271.27	0.01	1.23E-24
HILIC	negative	3.23	1465.6480	3.09	674.35	508.90	534.35	570.87	0.01	1.43E-25
HILIC	negative	3.24	1419.6410	2.96	702.82	558.74	556.62	603.11	0.00	3.76E-26
HILIC	negative	3.25	1347.6200	438.99	0.01	1.01	0.75	0.59	743.15	4.02E-14
HILIC	negative	3.28	1259.5850	10.00	487.98	429.37	403.55	439.92	0.02	1.71E-24
HILIC	negative	3.29	1435.6380	55.69	4719.68	4585.06	4557.37	4602.71	0.01	6.00E-27
HILIC	negative	3.29	1481.6420	9.25	647.58	587.31	567.06	598.35	0.02	2.87E-27
HILIC	negative	3.31	1399.5910	1000.70	5.02	7.05	3.66	5.26	190.39	1.74E-11
HILIC	negative	3.31	1409.6200	604.20	0.59	3.85	2.70	2.25	268.32	7.36E-17
HILIC	negative	3.31	1437.5470	610.78	6.18	2.77	2.18	4.04	151.12	4.89E-12
HILIC	negative	3.32	1447.5780	375.73	2.17	0.23	1.65	1.35	278.23	1.73E-12
HILIC	negative	3.42	1321.6060	1098.76	6.43	5.17	3.44	4.94	222.64	5.88E-11
HILIC	negative	3.8	223.0449	1720.21	635.12	558.59	625.25	601.41	2.86	1.70E-07
HILIC	negative	3.87	158.9246	1087.06	0.07	0.00	0.00	0.02	44298.43	4.69E-11
HILIC	negative	3.87	434.8634	2075.87	0.62	0.11	0.20	0.31	6658.31	2.71E-14
HILIC	negative	3.87	450.8378	1135.02	0.00	0.01	0.14	0.05	21833.13	8.97E-12
HILIC	negative	3.95	418.0594	2049.50	6.88	0.31	0.00	2.73	751.50	5.60E-04
HILIC	negative	4.02	336.8701	1288.89	85.63	100.32	68.47	86.57	14.89	7.82E-07
HILIC	negative	4.08	336.8580	0.43	466.54	846.29	703.48	673.35	0.00	1.14E-15
HILIC	negative	4.26	439.0814	32398.34	9047.91	6965.58	10458.99	8749.79	3.70	1.17E-03
HILIC	negative	4.28	516.2034	1693.36	446.66	605.74	592.59	544.80	3.11	2.88E-31
HILIC	negative	4.28	781.1983	2260.80	876.76	895.70	779.27	853.85	2.65	1.10E-14

HILIC	negative	4.37	710.2034	737.76	231.62	254.90	165.51	219.01	3.37	3.11E-08
HILIC	negative	4.38	696.1875	1266.62	505.33	575.87	418.24	503.89	2.51	4.24E-05
HILIC	negative	4.38	931.2980	0.00	329.72	488.57	232.03	344.81	0.00	2.11E-07
HILIC	negative	4.44	521.0879	653.64	207.28	186.74	160.41	185.68	3.52	9.33E-40
HILIC	negative	4.68	513.1187	2729.30	1138.06	1235.13	1317.25	1224.71	2.23	1.23E-07
HILIC	negative	4.72	293.0616	830.87	34.26	13.21	38.60	28.69	28.96	5.23E-19
HILIC	negative	4.72	521.3214	722.60	9.65	0.00	41.23	16.96	42.60	3.35E-06
HILIC	negative	4.72	571.3120	470.61	135.59	69.98	144.89	114.58	4.11	8.96E-20
HILIC	negative	4.72	625.3219	565.30	78.89	50.77	55.30	58.74	9.62	9.18E-11
HILIC	negative	4.72	701.4586	400.72	11.39	0.00	25.77	12.39	32.35	1.94E-06
HILIC	negative	4.72	707.4791	339.17	30.88	0.00	50.49	27.12	12.50	2.38E-05
HILIC	negative	4.73	131.0811	3522.67	1417.07	1316.81	1537.72	1416.29	2.49	1.37E-11
HILIC	negative	4.73	173.1034	5589.43	2327.67	2154.56	2242.17	2232.45	2.50	7.50E-12
HILIC	negative	4.73	299.0801	416.62	65.49	32.08	70.72	56.10	7.43	3.77E-04
HILIC	negative	4.73	369.1965	2795.90	725.56	632.88	810.28	716.90	3.90	4.73E-11
HILIC	negative	4.73	527.3466	1525.86	427.88	364.98	422.94	401.27	3.80	2.56E-11
HILIC	negative	4.78	300.0482	554.44	80.71	147.01	108.11	111.47	4.97	1.38E-11
HILIC	negative	4.79	350.1535	368.30	71.69	49.07	83.60	66.93	5.50	6.38E-13
HILIC	negative	4.8	154.0613	1967.19	561.01	444.62	658.28	545.91	3.60	3.31E-11
HILIC	negative	4.84	658.2307	10952.36	2923.24	2140.00	3086.06	2675.25	4.09	8.36E-15
HILIC	negative	4.85	601.1346	9994.62	4409.29	4878.26	4357.45	4515.28	2.21	1.66E-11
HILIC	negative	4.86	699.2196	812.67	299.88	340.87	272.40	303.35	2.68	3.10E-08
HILIC	negative	4.86	1105.3060	823.92	282.73	293.71	276.66	284.02	2.90	1.14E-11
HILIC	negative	4.88	787.2737	797.22	264.53	274.40	370.05	298.45	2.67	2.48E-05
HILIC	negative	4.89	669.1257	777.58	332.93	338.44	290.62	322.17	2.41	8.25E-26
HILIC	negative	4.98	672.2464	767.41	208.90	137.41	161.82	168.19	4.56	4.09E-09
HILIC	negative	5	439.0851	541.21	71.67	112.67	141.85	104.89	5.16	4.24E-05
HILIC	negative	5.16	421.0739	6159.69	1406.58	1669.94	1216.84	1441.96	4.27	1.41E-09
HILIC	negative	5.17	755.1382	547.39	9.94	11.25	11.17	10.78	50.76	1.00E-07
HILIC	negative	5.17	777.1256	520.49	18.44	0.00	6.55	8.33	62.48	1.32E-08
HILIC	negative	5.58	925.2378	1017.48	424.19	498.85	326.78	419.69	2.42	9.39E-06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4.97
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5.00
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5.30
5.53
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5.62
5.93
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6.11
6.12
6.26
6.63
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6.05
6.11
6.37
6.50
6.59
6.79
130615_Neg_115_MMB
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4.80
5.00
5.74
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6.44
6.56
6.72
Time
100
%
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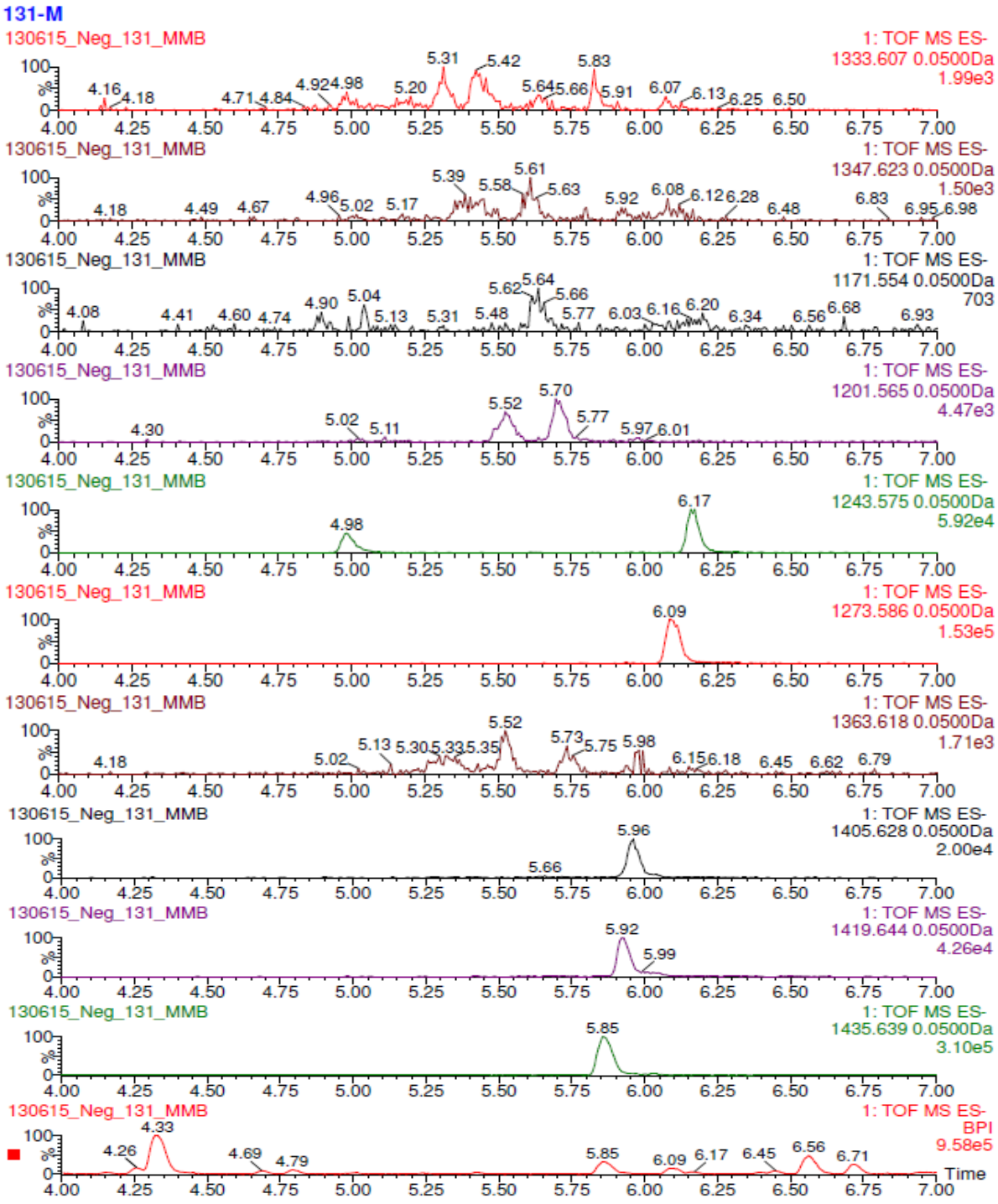

Figure S3.4. Extracted ion chromatograms of soyasaponins differing between the classes (115-M represents the master mix of *mrp*(3)/*mrp*(19), 131-M the master mix of MRP(3)/MRP(19)).The last line of each sample are normalized selected ion monitoring plots for the group A soyasaponins.

APPENDIX C. Supplementary Figures and Tables for Chapter Four

Table S4.1. EMRTs with a *p*-value < 0.05 and a factor of change less than 0.5 or greater than 2 in all the comparison between the low phytate line and all normal phytate lines for both ionization modes. The averages of the classes represent the peak areas.

Column	Ion mode	RT	m/z	average *mrp*(3)/ *mrp*(19)	average *mrp*(3)/ *MRP*(19)	average *MRP*(3)/ *mrp*(19)	average *MRP*(3)/ *MRP*(19)	all normal phytate (NP) lines	factor of change NP/LP	p Value
HSS	positive	3.12	599.5037	282.60	55.91	38.93	79.14	57.99	0.21	4.7E-03
HSS	positive	3.48	575.5024	1092.56	252.64	233.42	296.12	260.73	0.24	6.8E-03
HSS	positive	4.05	603.5346	418.13	92.55	102.91	134.25	109.91	0.26	1.8E-03
HSS	positive	4.06	516.4764	410.18	166.58	134.15	165.74	155.49	0.38	6.3E-04
HSS	positive	4.07	736.5327	361.65	123.99	100.44	126.21	116.88	0.32	2.2E-03
HSS	positive	4.07	262.2535	604.04	251.07	221.65	276.54	249.75	0.41	6.2E-04
HSS	positive	4.56	573.4871	340.12	127.57	121.59	135.26	128.14	0.38	1.6E-03
HSS	positive	4.57	939.6004	116.62	11.03	8.65	9.84	9.84	0.08	1.1E-02
HSS	positive	4.57	762.5008	205.73	63.87	64.08	86.91	71.62	0.35	3.3E-03
HSS	positive	4.57	599.5023	5641.28	2202.61	2111.95	2414.00	2242.85	0.40	2.2E-03
HSS	positive	4.58	740.5204	166.69	36.28	35.53	34.94	35.58	0.21	2.5E-02
HSS	positive	4.87	804.5518	427.48	30.69	45.09	112.79	62.86	0.15	1.2E-02
HSS	positive	4.87	86.096	213.73	13.03	40.55	61.44	38.34	0.18	1.4E-02
HSS	positive	4.87	502.3291	237.74	16.76	38.99	98.99	51.58	0.22	1.4E-02
HSS	positive	4.88	184.0741	6292.50	1122.98	1475.63	1964.69	1521.10	0.24	4.5E-03
HSS	positive	4.88	782.5686	3646.36	743.64	914.37	1228.04	962.01	0.26	7.1E-03
HSS	positive	4.99	716.5201	134.63	19.32	9.32	14.47	14.37	0.11	1.8E-02
HSS	positive	4.99	575.5019	5102.80	1773.92	1668.27	1868.88	1770.36	0.35	4.8E-03
HSS	positive	5	738.5015	226.53	69.53	47.48	65.48	60.83	0.27	3.3E-03
HSS	positive	5	754.4701	253.75	93.41	74.70	92.32	86.81	0.34	7.6E-04
HSS	positive	5.07	601.5186	825.94	255.48	172.84	307.73	245.35	0.30	1.2E-03
HSS	positive	5.07	195.0367	29.76	299.26	241.71	203.48	248.15	8.34	1.2E-05
HSS	positive	5.42	758.5681	1016.95	147.80	190.03	401.58	246.47	0.24	4.5E-02
HSS	positive	5.43	184.074	3838.72	480.38	671.82	994.99	715.73	0.19	6.0E-03
HSS	positive	5.45	784.5848	612.09	61.37	74.68	199.56	111.87	0.18	4.4E-03
HSS	positive	5.48	577.5183	398.27	122.87	94.88	145.62	121.12	0.30	2.5E-03
HSS	positive	5.61	604.5387	374.80	78.72	83.59	106.99	89.76	0.24	8.4E-04
HSS	positive	5.63	603.5348	898.14	273.32	287.95	319.57	293.61	0.33	2.3E-03
HSS	positive	6.14	866.6687	204.97	39.49	44.38	45.01	42.96	0.21	2.5E-04
HSS	positive	6.14	664.6238	292.62	75.16	70.17	64.29	69.87	0.24	3.7E-04
HSS	positive	6.14	682.635	324.19	81.94	87.35	86.83	85.37	0.26	5.1E-04
HSS	positive	6.15	282.2794	217.24	130.14	49.61	78.63	86.13	0.40	2.9E-03
HSS	positive	6.37	601.5192	1602.95	759.27	734.69	698.51	730.82	0.46	2.2E-06
HSS	positive	6.37	339.2905	361.84	205.15	150.83	143.44	166.47	0.46	1.3E-06
HSS	positive	6.38	620.598	493.94	172.94	128.46	206.68	169.36	0.34	6.5E-05
HSS	positive	6.38	660.5897	224.56	76.02	60.81	98.91	78.58	0.35	2.8E-05
HSS	positive	6.38	381.2998	3536.67	1597.85	1559.77	1465.04	1540.89	0.44	4.2E-06
HSS	positive	6.38	683.522	2031.60	990.92	1031.80	958.71	993.81	0.49	8.0E-07
HSS	positive	6.39	613.4831	220.76	93.00	27.47	54.41	58.29	0.26	4.7E-03
HSS	positive	6.41	933.7158	206.62	11.44	32.60	63.56	35.87	0.17	9.5E-03
HSS	positive	6.64	909.7139	350.90	72.83	97.38	125.81	98.67	0.28	1.0E-04
HSS	positive	6.69	577.5199	334.91	168.84	135.91	140.06	148.27	0.44	1.9E-06
HSS	positive	6.7	355.2843	426.94	184.78	186.09	162.53	177.80	0.42	4.9E-06
HSS	positive	6.73	381.2999	3455.08	902.50	895.20	820.65	872.78	0.25	3.0E-07
HSS	positive	6.73	599.503	187.38	51.59	104.85	57.36	71.27	0.38	4.8E-04
HSS	positive	6.77	603.5341	1097.52	441.47	462.17	442.42	448.69	0.41	3.1E-06
HSS	positive	6.78	685.5375	1144.38	468.99	518.27	516.44	501.23	0.44	4.0E-08
HSS	positive	6.85	648.6292	212.60	51.70	29.90	51.14	44.24	0.21	1.4E-04

HSS	positive	6.85	854.6859	175.02	49.33	44.06	40.91	44.77	0.26	1.4E-03
HSS	positive	6.85	842.6879	196.95	61.43	49.88	49.90	53.73	0.27	1.2E-03
HSS	positive	6.86	255.2113	280.01	130.75	153.94	123.85	136.18	0.49	2.2E-03
HSS	positive	6.86	395.3671	607.43	333.09	342.19	230.39	301.89	0.50	2.8E-03
HSS	positive	7	397.3824	7172.98	3652.88	3171.93	3623.15	3482.65	0.49	1.3E-04
HSS	positive	7.01	857.7055	300.01	60.10	50.01	48.70	52.94	0.18	1.7E-04
HSS	positive	7.13	381.2999	373.01	146.93	148.03	150.26	148.41	0.40	7.1E-07
HSS	positive	7.27	571.473	261.83	55.10	140.52	67.80	87.80	0.34	1.3E-02
HSS	positive	7.29	397.3819	2816.28	1346.45	1134.82	1498.93	1326.74	0.47	4.4E-04
HSS	positive	7.43	279.2327	251.17	203.05	81.64	62.47	115.72	0.46	4.4E-02
HSS	positive	7.45	912.7632	249.54	149.16	110.90	57.94	106.00	0.42	2.9E-03
HSS	positive	7.46	259.2063	211.29	105.24	69.27	0.00	58.17	0.28	1.0E-02
HSS	positive	7.46	243.2111	254.39	139.60	139.41	0.00	93.00	0.37	1.4E-03
HSS	positive	7.55	589.4825	1438.21	741.13	667.42	622.14	676.90	0.47	6.2E-04
HSS	positive	7.62	951.765	295.89	33.56	107.06	96.43	79.02	0.27	6.9E-04
HSS	positive	7.64	871.7372	524.97	353.54	198.67	219.09	257.10	0.49	6.1E-04
HSS	positive	7.66	599.5037	0.00	36.22	62.05	58.86	52.37	#DIV/0!	1.1E-02
HSS	positive	7.67	279.2328	256.98	139.04	81.30	78.26	99.53	0.39	6.7E-03
HSS	positive	7.67	879.7433	1060.44	568.27	412.98	337.45	439.57	0.41	1.7E-03
HSS	positive	7.67	575.5033	2418.18	1377.44	1105.70	799.64	1094.26	0.45	1.1E-03
HSS	positive	7.68	601.5189	8216.60	3656.00	3419.11	2966.61	3347.24	0.41	8.8E-07
HSS	positive	7.69	615.4982	4309.31	2516.41	1843.23	1640.40	2000.01	0.46	3.2E-03
HSS	positive	7.7	602.5234	2873.22	861.89	867.20	684.70	804.60	0.28	6.1E-05
HSS	positive	7.7	577.5189	2103.82	529.29	915.16	412.55	619.00	0.29	1.3E-04
HSS	positive	7.71	865.7642	553.45	243.93	220.47	202.26	222.22	0.40	4.6E-07
HSS	positive	7.72	339.2897	263.77	124.94	127.73	121.20	124.62	0.47	1.7E-02
HSS	positive	7.72	883.7744	7805.71	3790.41	3879.52	3401.27	3690.40	0.47	5.3E-06
HSS	positive	7.84	591.4983	859.54	229.43	122.34	51.24	134.33	0.16	1.2E-08
HSS	positive	7.86	603.5339	471.34	132.84	14.92	7.99	51.91	0.11	1.2E-03
HSS	positive	7.87	881.7559	363.18	62.88	36.75	36.95	45.53	0.13	5.2E-04
HSS	positive	7.87	953.7805	257.09	20.91	61.58	65.55	49.35	0.19	1.2E-04
HSS	positive	7.89	577.5188	334.64	195.24	93.16	20.78	103.06	0.31	2.8E-02
HSS	positive	8.05	631.5658	546.82	148.38	184.50	112.40	148.43	0.27	2.9E-05
HSS	positive	8.07	619.5293	1833.43	24.06	67.44	0.00	30.50	0.02	8.6E-09
HSS	positive	8.08	593.5139	188.41	48.20	27.22	26.65	34.02	0.18	1.1E-03
HSS	positive	8.08	577.5188	413.70	121.56	78.18	42.42	80.72	0.20	2.4E-03
HSS	positive	8.09	923.7671	1539.75	616.30	986.66	706.60	769.85	0.50	2.5E-05
HSS	positive	8.1	604.5382	392.45	27.66	33.73	7.15	22.85	0.06	3.1E-03
HSS	positive	8.11	493.3891	166.88	22.63	20.99	6.44	16.69	0.10	6.2E-04
HSS	positive	8.18	617.5136	108.40	247.68	287.56	259.95	265.06	2.45	1.2E-03
HSS	positive	8.66	319.2635	124.86	313.98	291.34	220.70	275.34	2.21	3.7E-03
HSS	positive	8.69	603.5341	7593.88	26166.66	15583.29	41453.87	27734.61	3.65	5.5E-03
HSS	positive	8.76	931.7713	48.97	548.10	475.63	721.08	581.60	11.88	3.7E-07
HSS	positive	8.79	953.8162	255.57	117.92	76.85	109.85	101.54	0.40	5.1E-10
HSS	positive	8.81	856.7441	213.66	491.80	492.29	485.83	489.97	2.29	1.0E-04
HSS	positive	8.82	313.274	100.64	351.56	328.36	375.29	351.74	3.49	1.3E-05
HSS	positive	8.87	575.502	2009.18	7701.78	8534.99	8322.04	8186.27	4.07	1.1E-09
HSS	positive	8.87	580.5372	190.34	912.74	851.91	1130.87	965.17	5.07	2.1E-04
HSS	positive	8.89	341.3049	187.37	390.16	260.07	588.27	412.83	2.20	1.5E-02
HSS	positive	8.96	591.5342	5362.01	2502.95	2606.28	2300.49	2469.90	0.46	4.1E-04
HSS	positive	8.97	617.5498	2373.66	989.42	1008.98	924.90	974.43	0.41	1.3E-06
HSS	positive	9.03	948.8061	102.17	216.93	197.68	243.73	219.45	2.15	1.8E-03
HSS	positive	9.18	619.5646	368.10	143.23	114.63	139.84	132.57	0.36	1.8E-04
HSS	positive	9.38	647.5966	1012.90	423.74	396.31	370.05	396.70	0.39	7.7E-03
HSS	positive	9.45	633.5814	53.32	161.84	130.33	144.92	145.70	2.73	2.5E-02
HSS	positive	9.47	661.6123	22399.12	10102.60	9430.63	11077.45	10203.56	0.46	2.8E-03
HSS	positive	9.48	603.5343	8650.42	4315.28	4385.52	3710.71	4137.17	0.48	1.6E-03

HSS	positive	9.49	195.0369	157.18	61.69	49.86	67.12	59.56	0.38	4.1E-02
HSS	positive	9.5	601.5188	2432.96	715.04	814.72	518.16	682.64	0.28	1.6E-03
HSS	positive	9.5	687.6281	6852.40	3342.89	3095.92	3683.09	3373.97	0.49	3.6E-02
HSS	positive	9.56	599.5037	78.01	0.00	0.00	0.00	0.00	0.00	9.2E-03
HSS	positive	9.57	675.628	2395.68	895.66	907.85	826.36	876.63	0.37	2.2E-03
HSS	positive	9.65	603.5344	3127.85	603.98	976.50	230.19	603.56	0.19	1.8E-02
HSS	positive	9.65	689.644	14529.91	5601.40	5333.63	5499.95	5478.33	0.38	4.7E-04
HSS	positive	9.67	988.9253	5608.77	2213.07	2256.49	1898.36	2122.64	0.38	9.9E-05
HSS	positive	9.68	1014.941	895.48	456.89	439.73	405.35	433.99	0.48	7.0E-04
HSS	positive	9.74	703.6594	1361.71	663.59	543.15	567.16	591.30	0.43	2.8E-03
HSS	positive	9.75	1002.941	492.16	227.78	213.92	211.44	217.71	0.44	5.9E-04
HSS	positive	9.81	605.5495	235.26	10.55	13.66	15.47	13.23	0.06	3.5E-02
HSS	positive	9.82	717.6749	2538.99	639.72	648.43	515.77	601.31	0.24	4.3E-04
HSS	positive	9.82	1016.956	1019.95	297.74	309.91	249.97	285.87	0.28	9.4E-05
HSS	positive	9.82	990.941	878.35	379.51	373.75	327.60	360.29	0.41	4.7E-04
HSS	positive	9.83	1022.916	655.73	264.82	282.58	258.42	268.60	0.41	8.0E-05
HSS	positive	9.83	1021.912	922.44	398.99	403.01	366.50	389.50	0.42	1.1E-04
HSS	negative	2.63	283.2636	12214.46	6031.03	3772.79	4422.04	4741.95	0.39	1.5E-02
HSS	negative	3.34	657.3476	340.94	199.60	142.92	110.47	151.00	0.44	4.3E-02
HSS	negative	3.34	675.36	172.96	110.60	61.07	41.68	71.12	0.41	3.6E-02
HSS	negative	3.89	695.4633	309.65	102.54	68.38	88.44	86.45	0.28	9.4E-03
HSS	negative	3.89	835.5322	810.20	208.43	173.58	180.77	187.59	0.23	3.3E-02
HSS	negative	4.05	861.5474	2408.48	764.23	905.59	794.64	821.49	0.34	4.5E-02
HSS	negative	4.14	913.5865	233.23	105.19	106.11	72.02	94.44	0.40	2.8E-02
HSS	negative	4.46	863.5635	188.12	30.22	31.98	34.04	32.08	0.17	2.6E-02
HSS	negative	4.9	217.0026	350.80	32.15	114.79	112.93	86.62	0.25	3.7E-02
HSS	negative	4.98	534.4877	278.99	88.99	75.22	115.53	93.25	0.33	4.1E-02
HSS	negative	5	409.404	388.39	43.03	39.50	62.22	48.25	0.12	2.1E-02
HSS	negative	5.45	816.5765	295.63	27.82	55.48	24.64	35.98	0.12	4.5E-02
HSS	negative	5.47	217.0028	304.95	42.85	87.81	71.24	67.30	0.22	4.3E-02
HSS	negative	5.63	814.6391	1438.47	633.83	613.27	547.58	598.23	0.42	3.2E-02
HSS	negative	5.63	850.6155	315.83	134.73	113.66	118.83	122.40	0.39	1.8E-02
HSS	negative	6.08	744.5527	311.65	58.38	57.42	118.63	78.14	0.25	4.3E-02
HSS	negative	6.12	688.5638	268.38	123.41	123.42	135.79	127.54	0.48	1.9E-02
HSS	negative	6.12	652.5869	1380.80	629.29	605.16	610.90	615.12	0.45	1.9E-02
HSS	negative	6.12	622.5764	378.89	190.11	122.04	188.51	166.89	0.44	2.2E-02
HSS	negative	6.14	952.7341	420.52	224.21	211.54	188.55	208.10	0.49	4.5E-02
HSS	negative	6.14	842.6703	2879.74	1069.76	1067.48	1030.69	1055.98	0.37	2.2E-02
HSS	negative	6.15	878.6459	649.33	256.98	240.97	267.76	255.23	0.39	1.7E-02
HSS	negative	6.21	978.7499	295.55	153.96	123.10	131.67	136.24	0.46	3.6E-02
HSS	negative	6.32	637.4161	56.47	330.98	430.43	130.86	297.42	5.27	2.6E-03
HSS	negative	6.34	824.6592	545.38	258.68	218.81	244.47	240.65	0.44	3.9E-02
HSS	negative	6.35	654.6019	170.70	70.39	81.04	80.42	77.28	0.45	3.7E-02
HSS	negative	6.36	339.3252	191.15	88.91	63.19	94.47	82.19	0.43	1.3E-02
HSS	negative	6.36	394.3672	315.89	143.80	100.22	154.10	132.71	0.42	1.3E-02
HSS	negative	6.36	382.3676	282.94	130.37	76.89	137.06	114.77	0.41	1.2E-02
HSS	negative	6.37	636.5924	6996.27	3019.36	2135.45	3361.17	2838.66	0.41	1.2E-02
HSS	negative	6.38	856.6849	229.00	93.16	87.32	110.36	96.95	0.42	3.8E-02
HSS	negative	6.6	716.5934	503.23	224.65	276.59	248.40	249.88	0.50	1.5E-02
HSS	negative	6.6	680.6181	2919.85	1183.13	1384.25	1267.48	1278.28	0.44	1.6E-02
HSS	negative	6.61	650.6073	1439.95	686.98	497.61	617.18	600.59	0.42	1.4E-02
HSS	negative	6.61	870.7015	488.11	175.56	170.02	196.56	180.71	0.37	2.0E-02
HSS	negative	6.77	798.5992	252.52	127.12	100.51	139.57	122.40	0.48	4.8E-02
HSS	negative	6.81	682.6339	517.96	217.06	235.12	263.58	238.59	0.46	1.3E-02
HSS	negative	6.83	664.6235	3345.39	1263.16	1116.18	1273.54	1217.63	0.36	9.4E-03
HSS	negative	6.94	717.5685	45.76	92.85	162.95	133.08	129.62	2.83	3.3E-02
HSS	negative	6.95	627.5191	43.92	207.04	261.16	169.21	212.47	4.84	5.1E-05

HSS	negative	6.96	819.5813	67.78	128.16	274.26	114.52	172.31	2.54	1.6E-02
HSS	negative	6.96	759.6152	457.30	842.41	1065.44	894.56	934.14	2.04	2.5E-03
HSS	negative	7.01	1676.325	399.06	152.48	107.91	155.68	138.69	0.35	2.1E-02
HSS	negative	7.04	666.6388	470.60	226.59	236.11	230.93	231.21	0.49	2.1E-02
HSS	negative	7.04	708.6492	492.38	175.03	232.66	229.39	212.36	0.43	1.2E-02
HSS	negative	7.05	678.6387	846.26	349.78	292.52	331.58	324.63	0.38	8.1E-03
HSS	negative	7.12	829.6543	430.68	160.84	220.55	170.03	183.81	0.43	6.8E-03
HSS	negative	7.13	281.2478	229.62	53.76	143.26	87.29	94.77	0.41	6.3E-03
HSS	negative	7.19	941.7807	79.95	279.99	216.17	241.58	245.91	3.08	1.6E-03
HSS	negative	7.2	933.6665	63.21	247.32	160.06	289.36	232.25	3.67	3.4E-04
HSS	negative	7.2	819.5816	470.89	1066.78	887.47	877.57	943.94	2.00	1.9E-03
HSS	negative	7.24	680.6535	215.20	118.10	101.06	101.31	106.82	0.50	1.2E-02
HSS	negative	7.26	692.6528	546.77	184.01	171.32	153.00	169.44	0.31	1.8E-03
HSS	negative	7.45	1055.866	33.44	121.61	63.42	174.62	119.88	3.58	2.3E-02
HSS	negative	7.51	887.6936	259.09	105.51	123.07	139.88	122.82	0.47	3.4E-02
HSS	negative	7.58	577.5911	320.83	107.42	48.96	63.34	73.24	0.23	6.6E-03
HSS	negative	7.75	279.2304	376.21	188.02	158.62	160.36	169.00	0.45	4.9E-03
HSS	negative	7.84	645.5294	72.93	133.91	187.99	184.38	168.76	2.31	2.0E-02
HSS	negative	8.08	645.5296	156.80	314.81	311.57	316.19	314.19	2.00	8.6E-03
HSS	negative	9.58	759.6137	20.11	112.90	292.74	94.39	166.68	8.29	2.5E-03