# NOVEL HYBRID RICE SEED PRODUCTION METHOD INCORPORATING HERBICIDE TOLERANCE

A Dissertation

by

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## ABSTRACT

The study relates to a hybrid rice seed production method to efficiently incorporate new herbicide tolerance into a systems using existing elite three-line hybrid rice parental lines, a WA-CMS female line, a maintainer line, and a restorer line. The method requires the following steps: (1) breeding for an isogenic maintainer line consisting of the trait of interest, herein herbicide tolerance using the elite maintainer parent and a donor of the trait of interest; (2) breeding for an isogenic restorer line consisting the trait of interest, herein herbicide tolerance using the elite restorer parent and a donor of the trait of interest; (3) introduction of the herbicide tolerance trait into the female line at the last step of basic seed production by crossing the isogenic maintainer line with the elite female line to generate a hemizygous female line; (4) introduction of the herbicide tolerance trait into the hybrid during the hybrid seed production by crossing the isogenic restorer line with the hemizygous female line. This study has validated the effectiveness of this invention with the modified hybrid seed production scheme. This system is versatile under field conditions and provides the possibility of utilizing heterosis both during the production of the hemizygous female seed and during yield trials of hybrid lines.

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# 1. INTRODUCTION

Rice (*Oryza sativa*) is one of the world's most important crops and is the staple food for more than half of the population (Khush, 2004). About 90% of rice production and consumption worldwide occurs in Asia (Gealy et al., 2003). China and India are currently the two countries with the largest population with 1372 million and 1314 millions of people respectively. Both of the countries are known to have rice as their staple foods. The world's population is projected to reach 9.7 billion by 2050. If rice remains a staple and land resources are limited, a large increase in rice production will be needed (Population Reference Bureau, 2015).

In order to meet the demand for rice consumption, it is necessary to increase grain yield potential to close the gap between demand and production to ensure food security. The discovery and commercialization of hybrid rice in China was an important development that increased yield in that country. Breeding and improvement of cultivation methods have been the main strategies to increase the yield potential of cultivated rice.

Many approaches have been taken to improve the yields in rice. Grain yield increases can be attributed to improved management practices and genetic advancements over the years. Various conventional breeding and selection techniques have been the oldest and most important methods of improving the genetic potential of yield through modification of yield components, various agronomic traits, and abiotic/biotic stress tolerances. During the last few decades, one of the predominant ways to increase the genetic potential of grain yield is utilization of heterosis within hybrid rice, which was made possible by the discovery in the 1970s of a commercially usable genetic tool for hybrid rice production - a wild abortive type of male sterility in rice. Hybrid rice has been reported to have 20-30% yield advantage over pure line varieties. Moreover, it requires roughly 1/4 amount of the seeds to plant an acre of field compared to the pure line

varieties, and requires less input for fertilizer, chemicals for pest and disease control (Salam et al., 2012).

Despite the steady increase of grain yields from 3,790 kg/ha to 8,373 kg/ha from 1959 to 2015 based on harvest records in the Mid-South of the United States (Rice-belt: Arkansas, Louisiana, Mississippi, Missouri, and Texas), the total planting acreage hasn't been increasing at the same pace as yield increases. It was reported that the total rice planting acreages in the Rice-belt area increased steadily from 1,607,000 in 1959 to 3,384,000 acres in 2005, but fallen back to below 3,000,000 acres since 2015 (USDA–ERS, 2016). Among the five states in the Rice-belt, Arkansas has the highest relative and absolute acreage of rice production with about 40.80% of US acreage or 439,000 acres being sown to hybrid rice in 2013 (Nalley and Tack, 2015). The primary determining factor of growers choosing to plant hybrid over conventional rice is the difference in the price for hybrid seeds versus pure line varieties. The price of hybrid seeds is usually 2-3 times higher than conventional pure line varieties because the cost of producing hybrid seed is much more expensive than with conventional varieties. A good hybrid may not be used by many growers unless it is feasible to produce hybrid seed at an economically competitive rate.

Researchers have been trying to incorporate various traits of interest into the existing rice germplasm to create more valuable rice through conventional breeding or genetic engineering. Breeding objectives have included increasing micronutrient content in grain, higher photosynthetic rates, herbicide tolerance, pests and disease resistance, and grain size (Boyle, 2011). However, only a small portion of hybrid lines is ever commercialized due to regulations or the feasibility of large-scale seed production. Clearfield rice varieties and Clearfield hybrids are the most widely grown types of rice in the US. They were bred by traditional breeding and selection techniques from mutation screening and not considered

genetically engineered. The incorporation of herbicide tolerance traits allows growers to lower the cost in production and more easily control weeds, which in turn improves grain yield, in comparison to normal rice. However, the lengthy process of converting the herbicide tolerance trait into the existing hybrids in order to develop a Clearfield version of that hybrid adds to the cost of the hybrid seeds.

Other than the issues of higher seed cost related to the seed production, for a new hybrid rice to be accepted by both growers and millers in the U.S., it must have high yield potential, high milling quality, lodging resistance, and meet established cooking quality criteria. However, it is widely believed that hybrids have higher yields but lower milling quality compared to conventional inbred rice varieties (Lyman and Nalley, 2013). Breeders often find the yield is frequently negatively correlated with quality and therefore breeders must compromise between these two important objectives when developing parental lines for the hybrid rice. Therefore the hurdles of the current hybrid rice system being largely adopted by the growers include the high cost of seed production and the hybrid seed quality.

Our proposed novel seed production method offers an alternative and effective way to incorporate traits of interest, such as herbicide tolerance (HT), into the current 3-line hybrid seed production system with a pair of existing elite parental lines. HT trait was introduced to the female seeds by the use of the converted isogenic HT B'-line to cross with the original A-line only at the last step of basic female seed production as opposed to the traditional way that requires the development of HT version of both A'-line and B'-line. The resulting hemizygous A-line/B'-line seeds will then be used as female seeds in the hybrid seed production field with the converted isogenic HT-R'-line as restorer male parents to produce HT 3way$F_1$ hybrids. The biggest advantages of this proposed novel seed production method is to reduce the time required for the breeders to develop promising HT hybrids, reduce the cost of hybrid seeds production,

and the hybrid seed costs to the farmers while allowing seed producers and farmers to more efficiently maintain the purity of the seeds by the use of corresponding herbicides.

The overall research was divided into five objectives to prove the actual feasibility of the proposed method in the hybrid production field conditions. Objectives 1 and 2 relates to the development of the isogenic HT version of A'-line/B'-line and R'-line respectively through the use of the parental lines of one of our promising hybrid and Puita-Inta-CL, one of the IMI-rice inbred cultivars, as the donor of the gene of interest (HT) through conventional repeated backcrosses. The development of A'-line is the most time consuming process, and it would not be needed if not to compare our proposed method with other traditional way of incorporating HT genes into hybrid rice production. Moreover, we tolerate minor differences between the original B-line and R-line with the converted B'-line and R'-line through only four generations of backcrosses to minimize the length of time required for the development while allowing us to make small improvements of the minor issues with the original parental lines through field selection during the backcrosses. Objectives 1 and 2 were done in collaboration with Brazilian coworkers previously with Bayer. Objective 3 was formulated to compare the seed production in the experimental seed production (ESP) field of the six hybrid combinations with 3 female lines (A-line, A'-line, and A-line/B'-line) and 2 male lines (R-line and R'-line). This serves to prove the incorporation of HT genes has no negative effects on the perceptivity of female and pollination of male lines. Most importantly, our hypothesis that the use of A-line/B'-line and R'-line as parents that produce no significant differences in the seed set yields on the female plants and other important agronomic traits with other combinations, would be tested. Objective 4 was formulated to compare the final grain yields, several important agronomic traits, and seed qualities of the hybrid seeds produced from various combinations in objective 3 in the actual yield trial. The hybrids will be referred to iso-hybrids throughout the article as they were produce

from combinations of isogenic parents, and the resulting hybrid produced from our proposed method would be referred to 3way$F_1$ hybrid as it has. Our hypothesis of objective 4 is that there are no significant differences of yield, important agronomic traits, and seed quality of the 3way$F_1$ hybrid compared to other iso-hybrids. The 5$^{th}$ objective was to seek for patent for this proposed new seed production method.

# 2. LITERATURE REVIEW

## *2.1 Hybrid rice*

Heterosis is the phenomenon utilized by every hybrid crop that describes the superior vigor of the hybrid offspring over the average performance of parents usually when the two inbred parents are adequately genetically distant. Heterosis can be positive or negative (Zhai et al., 2013; Nuruzzaman et al., 2002; Rahimi et al., 2010). It is often expressed as complex traits in plants, including plant height, duration to flowering, resistance to biotic or abiotic stress, and especially yield components that are probably controlled and influenced by many loci (Lippman and Zamir, 2007). In most cases, positive heterosis, especially in regards to yield related traits, is desirable, but negative heterosis can also be of value when it creates an early-maturing or short-statured $F_1$ plant.

Heterosis was first explained by Davenport (1908) as being the result of additive, dominance gene action. Others (East, 1908; East, 1936) described heterosis as being an over-dominance effect. The method of measuring the degree of over-dominance gene action was proposed by Comstock and Robinson (1952), who also tried to explain the mechanism of heterosis. However, all three hypotheses were later proven to be inadequate as more hybrid crop studies were conducted and information ascertained. Heterosis may also be a function of maternal effects, specifically when the cytoplasm of the female parent beneficially interacts with portions of the nuclear genome inherited from the paternal parent. There have been some studies reporting the different heterosis observed between same pollinator with female parents with different cytoplasmic male sterility sources, which can be an example of this type of heterosis (IRRI, 2000). This is in accordance with a later research in dissecting QTLs' responsible for hybrid cotton heterosis in five developmental stages. At single locus level, most QTLs related to

heterosis that were identified at later stages displayed over-dominance effects (Shang et al., 2016).

There have been multiple experiments examining the genetic basis of heterosis (reviewed by Schnable and Springer, 2013), including the complementation of allelic variation (e.g. Springer and Stupar, 2007) and variation in gene expression patterns (e.g. Guo *et al.*, 2006), as well as proteomic variation (e.g. Goff, 2011). There was even study suggesting the interaction between epigenetics and epistasis contributes to the overall performance of F1 hybrid as well (Chen, 2010; He *et al.*, 2010). In plants, most miRNA have non-additive expression pattern. In maize (*Zea mays*, L.) and Arabidopsis (*Arabidopsis thaliana*, L.), epigenetic modifications resulting from differentially expressed small RNAs have been linked to improved yield performance, and was thought to be the contribution to the heterosis (Ni *et al*., 2009; Groszmann *et al*., 2011). A new model explaining heterosis has been proposed dividing the growth of the plants into three phases, young vegetative, mature vegetative and reproductive stages (Baranwal et al., 2013). The additive expression pattern has been reported to be at higher level performance during the younger vegetative stages contributing biomass, and reduced significantly as the plant grow into later vegetative stage. Most non-additive gene actions contribute to phenotypic differences among plants in the reproductive stage (Baranwal et al., 2012). This is in accordance with a later research in diseecting QTL responsible for hybrid cotton heterosis in five developmental stages. At single locus level, most heyerosis related QTL identified at later stages displayed overdominance effects (Shang et al., 2016).

There has yet to be a straight forward and persuasive argument with a molecular basis that sufficiently explains the phenomenon of heterosis, especially for the more complex traits, such as grain yield. Most breeders tend to believe the phenomenon of heterosis is a joint result from the above hypotheses, while crops may have different degrees of heterosis due to unique

interactions of gene effects. Hybrid crops have become the predominant form of many cultivars since the launch of the first successful hybrid corn in the 1920s (Duvick, 1994; Stuber, 1994; Duvick, 2005; Wang et al., 2005). While additional studies are needed to fully explain the phenomenon of hybrid vigor, plant breeders continue to exploit combinations of inbred parents that result in hybrids possessing higher yield and better performance than current inbred cultivars.

Heterosis is often utilized within cross-pollinating crops such as maize, pearl millet, sorghums, onion etc.; but itis also applicable to self-pollinating crops, such as wheat and rice (Franklin-Tong, 2008). Hybrid seed technology has been used extensively in maize since 1930s and initially accounted for 30% increase in yield over conventional varieties (Duvick, 1992; Duvick, 1997). Differences in pollination mechanisms seem to relate to heterosis in terms of the relevance of epistasis in self-pollinating species which contributes more to heterosis than epistasis does in cross-pollinating crops as suggested by a series of studies investigating the genetic basis of heterosis in the model species *Arabidopsis* (Kusterer *et al.*, 2007; Melchinger *et al.*, 2007; Reif *et al.*, 2009). Heterosis is only expressed within the F1, and therefore growers must buy new seeds from the hybrid seed companies each growing season. Other crops utilizing three line hybrid seed production includes oilseed rape (WO92/05251, WO97/0737, or WO2005/002324), wheat (Wilson and Ross, 1962), sunflower (Chepurnaya et al., 2003), beet, carrot, maize, onion, petunia, rye, and sorghum (Kuck and Wrick, 1995).

Heterosis of hybrid rice is expressed mostly in terms of grain yield potential, in which hybrid rice consistently out-performs inbred varieties by 15-20% under similar growing conditions and requires nearly 50% less nitrogen fertilizer for maximum grain yield (Virmani, 2003; Yuan, 2003). Beyond grain yield performance, hybrid rice has better agronomic traits including tolerance to various biotic and abiotic stresses, which in turn, results in less usage of pesticides (Fujimaki and Matuuba, 1997; Sasaki, 1997; IRRI 2015). Consequently, acreage

worldwide over the past 10 years has increased substantially. There are two types of hybrid rice systems, three-lines and two lines. Hybrid rice employing three-line systems was initially developed in the 1970s by Chinese scientists (Li, 1977; Li et al., 2007). Research of a two-line system started in the late 1980s (Virmani et al., 1982; Yuan and Virmani, 1988; Mao and Virmani, 2003; Mou et al., 2003; Rongbay and Pandey, 2002; Rudger, 2001). Until now, most commercial hybrid rice was developed based upon the three-line system (Barclay, 2010).

Both three-line and two-line hybrid systems require stable male-sterile lines as female parents. Typically, the cause of male sterility can be classified into several categories, including cytoplasmic male sterility (CMS), cytoplasmic genetic male sterility (CGMS), genetic male sterility (GMS), environmental sensitive male sterility (EGMS), genetically engineered male sterility (GEMS), and chemically induced male sterility (CIMS). EGMS can be further sub-categorized into those that induce sterility or fertility of the plants due to temperature (TGMS) or induction based on photoperiod (PGMS). CMS and EGMS are most commonly used in the three-line and two-line systems respectively. Among all of the male sterility systems, the CMS is the most effective and popular method for commercial hybrid production. More than 20 different CMS sources have been identified in rice (Virmani et al., 2003). Some studies suggest the CMS female is associated with yield penalties or other undesirable traits (Kaul, 1988); however, numerous breeders have proven that this penalty can be abated with breeding efforts.

The three-line system involves the CMS (A-line), maintainer (B-line), and restorer lines (R-line). The male sterility relies upon the interaction between specific cytoplasm genomes in the mitochondria and nuclear genome (Virmani et al., 2003). The male sterility in the A-line requires both the nuclear restorer genes to be homozygous recessive (*rf*) and the cytoplasm genes in the mitochondria to be abnormal. Absence of either factor will possibly make the plant produce fertile pollens. Both A-line and the B-line have homozygous recessive nuclear genes

conferring male sterility. The B-line is the isogenic line that differs from A-line only in the cytoplasm that carries the normal fertility restoration genes in the mitochondria genome, which makes itself a fertile plant. B-line maintains the sterility of A-line by pollinating on the A-line plants. The A-line is multiplied by crossing with its maintainer B-lines, while B-lines or R-lines can easily be maintained and multiplied by selfing. The seed set harvested from the A-line will be further used as female in the hybrid production.

The Wild-Abortive type CMS (WA-CMS) has been the major source of the A-line in the three-line system since its discovery from the wild rice (*Oryza rufipogon* Griff.) and its *Rf* genes from the *indica* rice (*Oryza sativa* L. ssp. *indica*) cultivar (Lin and Yuan, 1980). It is accounted for almost 90% of hybrid rice production in China, and nearly all of hybrids developed outside of China (Sattari et al., 2007; Huang et al., 2014). It has the sporophytic manner of inheritance (Virmani et al., 1998). *WA352* gene in the mitochondria of WA-CMS was recently identified and found to be constitutively expressed in the WA-CMS lines. The encoded WA352 protein accumulates in the anther tapetum, where it interacts with the nuclear-encoded mitochondria protein, COX11, which intiates programmed cell death of the anther cells thereby causing male sterility (Luo et al., 2013).

The R-line, on the other hand, is a different line having the dominant restorer genes (*Rf*) in the nuclear genome and a normal mitochondrial genome. Several different restorer genes have been discovered. *Rf1a* and *Rf1b* were identified to be the restorer genes in the CMS-BT rice (Komori et al., 2004; Wang et al., 2006). *Rf17* was identified as a single nucleus gene that was able to restore gametophytically sterility of the CW-CMS type rice (Fujii and Toriyama, 2009). The genes *Rf3* and *Rf4*, on chromosome 1 and 10 respectively, are believed to be the two major genes responsible for counteracting the cytoplasm sterility in the WA-CMS system (Zhang et al., 1997; Zhang et al., 2002; Lu et al. 1997; Yao .et al. 1997; Tan et al. 2008; Jing et al. 2001;

Ahmadikhah and Karlov 2006; Ngangkham et al. 2010; Sure.sh et al. 2012). In some systems, one *Rf* gene can confer complete restoration the fertility of the CMS females, while sometime, it takes two or more *Rf* genes to restore adequate fertility (Schnable and Weise, 1998). The R-line is used as a pollinator in hybrid seed production. Strips of R-line are grown next to strips of A-line in hybrid seed production. The dominant restorer gene will restore the fertility in the derived $F_1$ hybrids.

There are several other requirements for a CMS line to become a usable A-line in a three-line system. First, it needs to be stable and completely male-sterile across different environments; second, it needs to be easily maintained by its isogenic line (B-line); third, fertility needs to be easily restored to 70-80% so that it can combine with different R-lines; fourth, it needs to have a favorable specific combinability with R-lines to promote heterosis in the F1 hybrid; fifth, it should have important agronomic traits that relate to yield and grain quality; last but not least, is the ability to produce seed via cross-pollination. Traits like floret opening, anther size, stigma size, anther extrusion, stigma exertion, stigma longevity, pollen longevity, pollen load, pollen size, and synchronized heading time between the A-line and the R-lines all significantly contribute to production of hybrid seed. A big and well-exerted stigma is an extremely critical character of an A-line to increase the outcrossing rates necessary for high production of hybrid seeds (Xu, 1988). It was reported that the vitality of a stigma can range from 3 to 7 days (Li et al., 2004). Therefore, increasing the rate of stigma exertion can not only increase the chance of pollination on the day of flowering, but also few days afterward, which can partially compensate for losses due to imperfect synchronization of the heading days between the A-line and the R-line. It was suggested that with an increase of 1% of stigma exertion rate of the A-line, the seed set rate of hybrid seed production can be increased by 0.74-

0.92% (Yang, 1997). Therefore the floret characteristics of A-lines are important for achieving high yield in the hybrid seed production.

Normal inbred rice varieties have an outcrossing rate of 0.5% or lower because rice is normally a self-pollinating plant. Male sterile rice plants, used as females in hybrid seed production, are grown together with male pollinator plants will have a 5.0-7.5% rate of cross-pollination with no artificial treatments (Kim, 2003). This rate is not high enough in order to make hybrid seed production economically feasible. Some important environmental factors known to influence the outcrossing rate include temperature (Beachell et al., 1938), relative humidity (Ramaiah, 1953), and wind speed (Kato and Namai, 1987). Methods to further improve cross-pollination include supplemental pollination by ropes, bamboo sticks, or in developed countries helicopters are used to move pollen from male to female strips. Gibberellic acid ($GA_3$) is used to extend the peduncle of the males panicles in strips extends over the female at the time of flowering to facilitate cross-pollination. $GA_3$ is also used to extend receptivity of the A-line stigmas. The outcrossing rate with these additional auxiliary methods has been reported to increase cross-pollination up to 30% (Mao and Vermani, 2003). However, these efforts require extensive labor, and therefore often only carried out in countries where labor cost is low. In areas with higher labor costs, helicopters are often used as a way to cross-pollinate in the hybrid rice seed production system.

Hybrid seed production practices were initially standardized in China by the father of hybrid rice, Long-Ping Yuan, during the 1970s. This technology has been introduced to growers in India, Vietnam, Philippines, Thailand, and United States, and many other rice producing countries. Because of the rice's natural tendency of self-pollination, the process of hybrid rice production is complicated. Increasing the outcrossing rate of A-lines has been one of the most challenging issues for the hybrid rice seed producers. The floret characteristics traits with A-lines

are critical to producing an abundance of F1 hybrid seeds. Breeding efforts in recent years has focused largely on modifications of floret traits and the duration of flowering time to have a better synchronization between A-lines and R-lines regardless of the two-line system or three-line system. Hybrids are typically grouped into three maturity durations, i.e. early (<120 days to harvest), medium (121 to 130 days to harvest), and late (>130 days to harvest).

Large variation in heading time has been found among cultivated varieties (Vergara and Chang 1985). Breeders often introduce naturally occurring early maturity varieties to make crosses with elite lines to modify the duration of the heading dates on both female and male sides (McKenzie et al., 1978; Carnahan et al., 1989; Rutger et al., 1987). However, the duration and flowering time are complex quantitative traits that can be regulated through the interaction of several QTL and the environment. Many studies have been reported from early researches (Chang et al., 1969; Yoko et al., 1980; Yamagata et al., 1986; Sato et al., 1988; Poonyarit et al., 1989; Sano 1992; Ohshima et al., 1993). However, the allelic relationships among these genes were not clearly explained. Several more studies were conducted later to indicate the pleiotropic effects of some major QTL on several different traits, including duration. In 2008, A QTL analysis revealed a gene, *GHD7*, has a large pleiotropic effect on the number of grains per panicle, heading date, and plant height in response to day-length (Xue et al., 2008). *GHD8* (also known as *days to heading8*, *DTH8*) was also identified later to be pleiotropically regulate grain yield, heading date, and plant height (Yan et al., 2011). The expression of both are enhanced under long day condition (LD), which acts upstream of *HD3A* and *EHD1* (*Heading Date 3a* and *Early Heading Date 1*) by repressing expression so that a phenotype develops with delayed flowering, increased plant height, increased panicle size, and an increased number of primary and secondary tillers, while no differences in the total number of tillers. Oppositely, short-day conditions diminish the effects. *GHD7* encodes for a CCT-domain proteins, which has been

identified in many plant species that regulate the process of photoperiodic flowering (Turner et al., 2005; Putterill et al., 1995; Yano et al., 2000), vernalization (Yan et al., 2004), circadian rhythms (Salome et al.,2006; Strayer et al.,2000), and light signaling (Kaczorowski and Quail, 2003). Therefore it is believed that the influence of *GHD7* on heterosis, which affects grain yield in rice hybrids is through the regulation of the duration of panicle differentiation that affects the panicle size (Huang et al., 2006). It was also suggested that *GHD7* plays an important role in both productivity and adaptability in rice globally (Xue et al., 2008). Another later study suggested that genes *HD1* (*Heading Date 1*) and *EHD1* jointly control the number of primary rachis branches and panicle size via regulation of flowering genes *HD3A* and *RFT1* (*Rice Flowering Locus T1*) in the rice leaves at floral transition stage (Endo-Higashi and Izawa, 2011). Because of the complex nature of this trait and *EHD1* alone was reported to be the major QTL responsible for at least 65% variation of the heading and most likely inherited additively (Yano., et al., 1996), breeders generally expect the progenies from two parents with different heading dates to segregate as a continuous pattern with most of the plants having heading dates in between the parents. Breeders will make selections from the progenies if they wish to adjust the headings.

2.1.1 Three-line hybrid rice seed production

Once the promising hybrids have been tested and parental lines determined, hybrid seed production using three-line system is a two-step process: female and male seed production (A-lines x B-lines; R-lines); and hybrid seed production (A-lines x R-lines).

To maintain genetic purity, all parental lines need to be maintained each year with a standard procedure where systematic pair-crosses of A-line and B-line single plants using only the A-line that possess 99.99% male sterility. Any plants that lack uniformity will be removed prior to the pair crosses. A-line and B-line seeds is harvested from each pair of crosses. Each A and B pair is planted as a row. Bulk pollinating of B-lines rows with the A-lines rows within each pair form Pre-Basic Seed. Pre-Basic seeds (A x B) are then used in a larger scale to produce Basic Seed. Basis Seed (A x B) is increased to becomes foundation seed. Breeder and Pre-Basic seeds have the highest genetic purity and require supervision of experienced breeders during production. The purity of an R-line is maintained by test-crossing it with the corresponding A-line and evaluation of $F_1$ progeny offspring. $F_1$ progeny are examined for pollen fertility to verify restoration. Phenotypes of $F_1$ progeny also need to be evaluated by breeders to ensure uniformity and nearly identical with the proposed hybrid lines. Any progeny row that looks different from the potential hybrid lines are discarded, as well as the corresponding R-line. Only seeds from R-lines with corresponding near-identical potential hybrid lines will be bulk harvested and kept as purified Nucleus Seeds. R-line Nucleus Seeds will then be grown and multiplied on a bigger scale to produce Pre-Basic, Basic, and Foundation Seed used in the hybrid seed production system as pollinator. Planting regulations, field plot designs, isolation distance, and pollen sterility examinations follow standard procedures from the hybrid rice breeding manual (IRRI, 1997).

2.1.2 Two-line hybrid seed production

EGMS is a male sterility line used in the two-line system in which the sterility is based upon two environment factors, temperature and photoperiod. The varieties with photoperiod conditioned fertility are called PGMS. Most of the PGMS lines do not produce fertile pollen during long days (> 13.75 hours) but will revert back to fertile plants under short days (<13.75 hours). The sterility of TGMS lines is sensitive to temperature, and the first TGMS was found in peppers (*Capsicum* spp.) (Martin and Crawford, 1995). Other examples of PGMS can be found in tomatoes (*Solanum lycopersicum)*, wheat (*Triticum* spp.), maize, and rice (Ku et al., 2003; Dwivedi et al., 2008; Shi et al., 2009). The rice TGMS system was found and utilized in a hybrid system in the 1980s (Cheng et al., 2007). Most of the TGMS rice lines are male sterile at higher temperature and the male fertility will be retrieved at lower temperature at a particular reproductive stage, which is around 15-25 days before heading or 5-15 days after panicle initiation. The actual temperature or day length for the critical points of fertility may vary from genotype to genotype. Hence the commercialization of a certain two-line hybrids will be extremely regional limited (Virmani et al., 2003). IRRI has suggested the maximum temperature determines the sterility/fertility pattern of the indica rice TGMS lines, which is around 29 °C (He and Yuan, 1989).

The nature of the fertility-alteration conditioned by the environment in EGMS is the biggest difference between two-line system and three-line production system. Unlike the three-line system, multiplication of the EGMS lines does not require another maintainer line. It can be done by self-pollination just like an inbred variety, which makes the process relatively simple and fast. Hence, only the EGMS and the pollinator are needed as female parent and male parent respectively for hybrid seed production. Some other advantages of the two-line system include a wider pool of possible male parents to search for good heterosis combinations in the F1 since

any fertile lines can be used as male parent. It was also reported that the EGMS system can be incorporated into any genetic background because it is governed by major genes, which again provides greater diversity when considering female parents in a hybrid (IRRI, 2003). The possible negative effect from the interaction between a sterile-inducing cytoplasm with another male is absent in the two-line system, which may allow a higher expression of heterosis.

The requirement for pollen sterility for the EGMS line under critical sterile condition should be more than 99.5% and the sterile phase should last for more than 30 consecutive days. During the fertile phase, the seed setting rate should be higher than 30%. Maintenance of EGMS lines and male parents through Nucleus Seeds are necessary to avoid genetic segregation that may affect sterility as generations advance. Nucleus Seeds of EGMS lines are produced by self-pollinating under conditions favorable for high seed set at a critical reproductive stage. The main panicle is scored for fertility after harvest and the top 50 plants with highest seed set are kept. A portion of the seeds from each of the selected 50 plants are grown as progeny rows under sterility favorable conditions, while remnant seed is stored. Sterility and uniformity of each progeny row is carefully examined, and only the remaining seed from the completely male sterile lines are bulked to form the Nucleus Seeds. Nucleus Seeds are used to produce Breeder Seeds under environmental conditions favorable for pollination. These plants are used to produce foundation seed of EGMS lines. The resulting EGMS lines are used in hybrid seed production. This is a standard purification procedure suggested by IRRI; however, in reality, the two-line system is notoriously for its potential instability of the female plant's sterility, EGMS. A sudden temperature changes in just few days during critical reproductive stages can lead to reversion of sterility of the EGMS lines used in the hybrid production field and causes many self-pollinated EGMS seeds to become mixed with hybrid seeds. The most ideal female line needs to be completely sterile for at leastfour consecutive weeks during the sterile phase with optimum

environmental conditions (Yuan 1998; Virmani et al., 2003). Many seed companies performing two-line hybrid rice system have experienced low purity of their commercialized products due to the instability of the EGMS lines used in hybrid production. This not only leads to apparent off-types in the field, but also causes the reduction of yield and grain quality.

Many efforts have been made to improve the floret traits of female lines that will enhance pollen reception. Breeders around the world have also worked to improve the pollen production from male parents which ultimately leads to cross-pollination rates for both 3-line and 2-line systems. Equally important as breeding efforts are developments of other techniques that improve cultivation and seed production technology. Together, these are a package allowing growers to improve profits from higher grain yield, especially in the areas where labor costs are high, such as in the United States. This was the motivation leading to the initial introduction of a herbicide tolerance trait into the hybrid rice systems.

*2.2 Weeds in the rice fields*

The biggest impact of weeds upon rice production is the negative effect on grain yield due to direct competition for sunlight, nutrition, and water. Poor weed management, especially early-season control, results in compromised yield and higher costs for late season use of herbicides or labor when attempting to control a mid-season flush of weeds. Weeds can reduce grain quality by mixing in weed seeds with the harvested rice grain, which can cause dockage. It has been reported that with intense weed density and high labor cost, herbicide applications are approximately 80% (about $US 200 per ha) more profitable than hand weeding (Beltran et al., 2012). Therefore, an efficient weed management system for rice production is critical.

It is estimated that more than 80 species belonging to 40 genera can be problem weeds in the US rice production. Weed species in rice can be regionally specific. In the United States, the most common weeds in rice production field include red rice (*Oryza sativa* L., AKA weedy rice), barnyardgrass [*Echinochloa crus-galli* (L.) Beauv.], California arrowhead (*Sagittaria montevidensis*Cham. & Schlecht.), eared redstem (*Ammania auriculata* Willd.), late watergrass [*Echinochloa oryzicola* (Vasinger) Vasinger], redstem (*Ammania coccinea* Rottb.), ricefield bulrush [*Scirpus mucronatus* (L.) Palla], rice flatsedge (*Cyperus iria* L.), and smallflower umbrella sedge (Heap, 2014). Among these, barnyard grass and red rice are two of the most serious yield-threatening weeds in rice grown in the United States, Brazil, Australia, Spain, and in most other rice-producing countries. In Brazil, the major representative of narrow leaf weeds are red rice, barnyard grass, the aquatic grasses (*Leersia hexandra* and *Luziola peruviana),* and the sedges (*Cyperus difformis*, *C. esculentus*, *C. ferax*, and *C. laetus*) with the increasing occurrence of Alexander grass (*Brachiaria plantaginea*), crab grass (*Digitaria horizontalis*), and goosegrass (*Eleusine indica*) as monocotyledonous weeds, while some perennial weeds are also seen in some areas with excess of moisture. The major broad leaf weeds in Brazil include morning glory (*Ipomoea* spp), Olive hymenachne (*Hymenachne amplexicaulis*), jointvetches (*Aeschynomene* spp.), alligator weeds (*Alternanthera philoxeroides*), water pepper (*Polygonum hydropiperoides*), and some aquatic weeds mainly in the water-seeded system fields (Andres and Theisen, 2013).

The barnyard grass is summer annual grass that germinates in the late winter or early spring through the summer. It will widely disperse its seed which makes it a troublesome weed. A mature barnyard grass plant can grow taller than most cultivated rice plants; therefore it poses a substantial threat due to its capacity to out-compete rice for sunlight and nutrients. Studies have shown that a single barnyard grass per square foot can reduce rice grain yield by about 25%, and

25 barnyard grasses per square meter can reduce the yield up to 50% . It can also serve as an alternate host for tungro and rice yellow dwarf viruses. Some researchers have shown the resistance in barnyard grasses to propanil and quinclorac or both, two of the most frequently used herbicides (Baltazar and Smith 1994; Lovelace, 2003; Norsworthy et al. 2009; Weed Science, 2005). Riceshot™ and Facet™ are the commonly seen Trade names for propanil and quinclorac respectively.

Red rice is a weedy relative of cultivated rice that is difficult to control because it is so closely related to domesticated rice (Chen et al., 2004). Red rice is highly competitive to rice not only due to the weed's early vigor, greater tillering, and greater height in comparison to rice; but also because of its long seed dormancy and propensity of shattering its panicle. Red rice can take up to 60% of applied nitrogen (N) fertilizer and has higher N use efficiency for biomass production than cultivated rice. The loss in yield of rice due to heavy red rice infestation can be up to 60% to 80% depending upon the degree of infestation (Burgos et al., 2006; Volgsaroj, 2000; Chauhan, 2013). It also reduces the quality of the milled rice (Burgos et al., 2008). For these reasons, red rice is important but also difficult to manage in direct seeded rice cultivation (Noldin et al., 2006). Growers in the United States reportedly lose and estimated $50 million per year because of dockages from millers for grain contaminated with red rice. Total economic losses due to red rice in southern United States rice production are estimated to be $500 to $750 million a year (Croughan, 2001). Due to the increasing discoveries of resistance to propanil and quinclorac in barnyard grass and red rice, the use of AHAS-inhibiting herbicides has increased worldwide in rice production system (Sudianto et al., 2013).

*2.3 Modes of action of herbicides*

One of the key advantages of this proposed new 3-way hybrid production method is to simplify the process to incorporate the gene of interest, most often the resistance to a certain herbicide, into the hybrids. Therefore it is crucial to understand different modes of actions of various herbicides that are most often used in hybrid crops for weed control. Knowing the basic modes of action of the herbicides will help the development of new herbicide resistance crops and the incorporation of it into the new hybrid production system.

There are various methods to classify herbicides, for instance: 1. broad spectrum vs. narrow spectrum herbicides; 2.systemic vs. contact; 3. residual duration in the soil; 4. means of uptake; 5.timing of application; 6. modes of action. Broad spectrum herbicides kill a large range of weeds, while the narrow spectrum herbicides target specific weeds, and also known as selective herbicides. The selectivity may be due to differences of translocation, absorption, or physiological effects between the crop and weed species in response to certain chemicals. Modes of action are the mechanisms by which herbicides with different chemical families affect plants at the tissue or cellular level. Herbicides with similar modes of action will generally produce similar injury symptoms.

2.3.1 Amino acid synthesis inhibitors

The most common class of herbicides is those that inhibit branched-chain amino acid synthesis enzymes in plants, acetolactase synthase (ALS), also called acetohydroxyacid synthase (AHAS). This enzyme catalyzes the first step in the synthesis of the branched-chain amino acids leucine, valine, and isoleucine essential for growing point or meristem (Avila et al., 2005). The herbicides that inhibit AHAS were discovered in the mid-1970s, and still widely used (Shaner et al., 1991; Stetter, 1994). The family includes the following five chemical classes: sulfonylureas

(SU), imidazolinones (IMI), triazolopyrimidines (TP, penoxsulam), pyrimidinyl-thiobenzoates (PTB), and sulfobylamino-carbonyltriazolinones (SCT). The sulfonylureas, imidazolinones, and triazolopyrimidines are known as ALS or AHAS inhibitors, and imidazolinones is the most commercially available herbicide to inhibit the activity of AHAS. Inhibition of this enzyme is normally fatal in plants. Most members in this category are phloem mobile, with exception of phosphorylated amino acids. Imidazolinones include imazapyr, imazapic, imazethapyr, imazamox, imazamethabenz and imazaquin, sharing an imidazole moiety in the molecular structure of each compound. They are further divided into three groups based on the second cyclic structure of their molecules. The first group, imazaquin, has a quinoline moiety; the second group, imazamethabenz, has a benzene ring; the last group, imidazolinones, has a pyridine ring composed of four analogs including imazapyr, imazapic, imazethapyr, and imazamox that differ only at position five of the pyridine ring. Imazapyr, imazapic, imazethapyr and imazamox have hydrogen (H), methyl and methoxymethyl functional groups at position five of the pyridine ring. All three groups have molecular sites of action on AHAS, but only the last group, imidazolinones, is used with imidazolinone-tolerant crops. The commonly seen trade names are Beyond™, NewPath™, Regiment™, and Permit™ for imazamox, imazethapyr, bispyribac-sodium, and halosulfuron respectively.

Another amino acid synthesis inhibitor family, glycine, includes glyphosate and glyphosate-trimesium, is an amino acid derivative. Glyphosate is a highly competitive inhibitor of the 5-enolpyruvyl-shikimimate-3-phosphate synthase (EPSPS), which is a key enzyme in the shikimate pathway synthesizing many aromatic compounds and formation of 5-enolpyruvyl-shikimimate-3-phosphate (EPSP) in plants from phosphoenolpyruvate (PEP) and shikimate 3-phosphate (S3P) (Steinrucken and Amrhein 1980).. The inhibition of EPSPS deregulates the shikimate pathway and reduces synthesis of aromatic amino acids, including phenylalanine,

tyrosine, and tryptophan, which are known to be precursors of many secondary products, such as lignin, anthocyanins, and certain growth regulators and is deleterious to plants (Franz et al., 1997; Duke et al., 2003). This strategy has been used to develop herbicide resistance in many crops since 1996 (Cajacob et al., 2004; Green et al., 2009; Duke et al., 2009).

Product names of this family include Classic, Pursuit, Roundup, and Liberty Link, which inhibit the synthesis of ALS, ALS, EPSPS, and Glutamine respectively. These herbicides are used to control a wide spectrum of grass and broadleaf weeds, including red rice found in rice fields. Several of these herbicides have residual activity and effective at low application rates, so that one treatment controls both existing weeds and weeds that emerge later, which is a significant advantage in rice production. This feature also allows growers to have flexibility in water management for weed control in rice fields by delaying the flooding time, which in turns, results in a greater control for water weevil (*Lissorhoptrus oryzophilus* Kuschel).

### 2.3.2 Growth regulator

Synthetic auxin is the oldest known action of mode for herbicides. It acts as the hormone agonist on the auxin receptor that mimics the action of auxin, and thus often called auxinic herbicide which was discovered in the 1910s (Went, 1926). Members of this family include phenoxy acids, benzoic acids, carboxylic acids (pyridine acids), and the picolinic acids. The most well-known common name is 2,4-D. Members in this family are all highly phloem mobile and affect both dicots and monocots.

### 2.3.3 Photosynthesis inhibitors

Chemicals that inhibit photosynthesis in plants work by blocking one of several binding sites in the process of photosynthesis were developed after the development of herbicides in the

growth regulator families. Members of this family can be further broken down into five subgroups:

a. Triazines, uracils, phenylureas, benzothiadiazoles, and pyridazines are known as inhibitors of photosynthesis at photosystem II site A, and block electron transportation and the transfer of light energy. They are usually only xylem upwardly mobile. Triazines herbicides are among the largest and most important family from this group first discovered in 1952, and commercialized in 1957. They control broad leaf and grassy weeds. The most important member in this family is atrazine, which blocks the electron transport on the reducing site of PSII (Trebst, 1980).

b. Substituted urea herbicides inhibit photosystem II at site β, and block the electron transport and transfer of light energy. This is xylem mobile.

c. Benzothiadiazole (bentazon), Nitrile (bromoxynil), Phenyl-pyridazine (pyridate) herbicides inhibit photosynthesis at photosystem II, Site α. This subgroup differs from the first two groups inthat plants are injured by contact only.

d. Bipyridylium herbicides act on the site of photosystem I as electron diverters, and is a contact only chemical.

e. Bentazon (Bentazone) is a selective herbicide manufactured by BASF belonging to the thiadiazine group of chemicals. Since herbicides with this mode of action inhibit photosynthesis, they only start working once plants have emerged and exposed to light, or light-activated. Injury symptoms occur after the cotyledons and first true leaves emerge. One of the most important differences between this type of herbicide and others is that they inhibit photosynthesis by binding with proteins encoded by chloroplast genes, which is only inherited from the female parent to the progeny.

Common herbicides include Atrazine®, Sencor®, Hyvar®, Karmex®, Basagran®, paraquat, and Buctril®. This is currently the second most common mode of action in herbicides.

2.3.4 Lipid synthesis inhibitors

Aryloxyphenoxypropionates (AOPP), phenylpyrazolines (PPZ), and cyclohexanediones (CHD) are the three families within this systemic mode of action herbicide. All three are used extensively at post-emergence by inhibiting the same enzyme, acetyl-coenzyme A carboxylase (ACCase), which acts on the substrate, Acetyl-CoA in annual or perennial weeds. They have little effect on dicots or non-graminaceous monocots (Harwood., 1988; Devine et al. 1993; Harwood, 1991; Duke and Kenyon, 1988). The ACCase is a pivot enzyme localized in the chloroplasts, which catalyzes the first step of ATP-dependent carboxylationof acetyl-CoA to form malonyl-CoA in the lipid biosynthesis pathway in plants. There are two isoforms of ACCase in plants, the plastid ACCase accounting for more than 80% of the total ACCase activity and the cytosolic ACCas involved in the long-chain fatty acid and flavonoids biosynthesis (Gronwald, 1991). Both isoforms carries two domains, the biotin-carboxylase (BC) domain and the carboxyl-transferase (CT) domain (Nikolau et al., 2003). Most of the herbicides work on the active domain of the plastid ACCase. Inhibiting the enzyme will block the production of phospholipids required for new membranes in new cells or cell growth. Other than the three major families, there is another active ingredient, cyhalofop-butyl that was intensively used in rice production. Cyhalofop-butyl acts as ACCase inhibitor, which breaks the structure of cell membranes (Oliveira Junior et al., 2011).

Most of these are known to systemically affect monocots, mainly used to control graminaceous weeds, with no effects on dicot weeds. Broadleaf species are naturally resistant to this herbicide family; therefore these herbicides are often used to control annual or perennial

grasses in broad leaf crops (Stoltenberg, 1989). Examples of trade names of products within this mode of action include Poast®, Assure II®, quizalofop, sethoxydim, fluazifop, and Select®.

2.3.5 Cell membrane disruptors

Chemicals including diphenylethers, aryl triazolinones, phenylpthalamides, and bipyridilium react with the cell membrane to form super oxides and hydroxyl radicals to destroy cell membranes. This family of herbicides is usually acting through contact and only affects the sprayed area of the plant. The herbicide families with this mode of action include: diphenylethers, aryl triazolinones, phenylpthalamides, and bipyridilium. Some common trade names of herbicides include Cobra®, Blazer®, Authority®, Aim®, and Gramoxone®.

2.3.6 Pigment inhibitors

The pigment inhibitor mode of action works by preventing the production of compounds that protect the plant from chlorophyll destruction. Instead of being green in color plant tissue turns white. Herbicides within this mode of action are typically preemergence treatments; however, a few have postemergence activity. Isoxazolidinones, isoxazoles, and pyridazinones make up the chemical families in this mode of action. Common trade names of pigment inhibitor herbicides include: Balance®, Callisto®, and Command®.

2.3.7 Seedling growth regulators

The seedling growth inhibition mode of action interrupts new plant growth and development. Herbicides within this mode of action must be soil applied and either inhibit root or shoot growth in emerging plants. Carbamothiates, phosphorodithioates, chloroacetamides, acetamides, and the dinitroanilines makeup of the herbicide families within this mode of action. Carbamothioates, Phosphorodithioates, chloroacetamides, and acetamides conjugate with acetyl

co-enzyme causing shoot emergence fail, while dinitroanalines and pyridines acts as the inhibitors of tubulin protein, which is responsible for microtubule assembly in cell division during mitosis and cause the root growth fail. Common trade names in this mode of action include Eptam®, Dual®, Harness®, Prowl®, and Treflan®.

*2.4 Types of herbicide tolerance and mechanisms in plants*

Herbicide resistance and tolerance are defined by Weed Science and Society of America in 1998 as following: "Herbicide resistance is the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. In a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis. Herbicide tolerance is the inherent ability of a species to survive and reproduce after herbicide treatment. This implies that there was no selection or genetic manipulation to make the plant tolerant, it is naturally tolerant" (Weed Science and Society of America, Weed Technology*,* 1998*)*

Crop resistance to herbicides has been long considered to be conferred by one of three mechanisms: resistance at the site of action, metabolic detoxification, and prevention of the herbicide from reaching the site of action. The degree of resistance is primarily due to the herbicide metabolic detoxification, secondarily due to differential resistance at the site of action, and lastly due to prevention of the herbicide from reaching the site of action (Devine et al., 1993). Recently it was found in several transgenic plants that overexpression of the target enzyme can also confer a fair amount of resistance to herbicides.

2.4.1 Prevention of herbicides from reaching the site of action

The first barriers designed to prevent herbicide uptake are external structure on the outside surface of plants, including cuticle, waxes, cell wall etc. The prevention of herbicide

uptake can be due to differences in interception and absorption. Herbicides absorption through roots occurs mostly at the apical ends through passive diffusion through the outer structure, epidermis and cortex, protecting the Casparin strips and the even inner structures in which the root endodermis contains vascular tissues. Different factors in plants, including the species, age of plants and environment, can contribute to varying degrees of chemical resistance by the leaf. Other mechanisms involved in the prevention of direct contact of herbicides with the more susceptible parts of the plants include translocation and compartmentalization (sequestration). It was found in some weed populations, plants that are naturally resistant to glyphosate accumulate glyphosate in the leaves rather than being translocated. This type of resistance is inherited as a single dominant or semi-dominant allele (Preston and Wakelin, 2008). Another mechanism utilizing compartmentalization was found in *Conyza Canadensis* and *Lolium rigidum* that shuttles and store 85% of the glyphosate to the vacuoles (Ge et al., 2010).

2.4.2 Metabolic detoxification

Detoxification of the chemical before it reaches the site of action is one of the most widely adapted mechanisms in plants providing resistance to certain herbicides. Metabolism-based resistance does not involve the binding sites of herbicides, but instead herbicides are broken down into non-toxic forms by chemical processes. The reactions of metabolic detoxification can be further grouped into four categories: reduction, oxidation, hydrolysis, and conjugation. Reduction plays a relatively less important role in herbicide detoxification in plants compared to other three reactions. Oxidation reactions are catalyzed by monooxygenases, known to possess functions of both oxidases and one of the following biochemistry actions: alkyl oxidation, aromatic hydroxylation, epoxidation, N-dealkylation, O-dealkylation, and sulfur oxidation.

Hydroxylation is common in plants resistance to several herbicides, including bromoxinyl (Buckland et al., 1973), propanil (Lamoureux and Frear, 1979), cyanazine (Benyon et al., 1972), and carboxylix acid ester such as 2,4-D (Loos, 1975). Conjugation is the most important type of reaction among the mechanism of detoxification. It is also the most commonly used approach among the four metabolic detoxification reactions when integrating herbicide resistance with improvement. Conjugation in plants typically converts the herbicide metabolite formed earlier by joining it with an endogeneous substrate to form a larger compound that is more water-soluble, and thus leads to metabolism. The cytochrome P450 mono-oxygenases (P450s) are a large family of herbicides that are involved in many plant metabolic processes. The enzymes are reported to detoxify the herbicide by catalyzing the hydroxylation reaction in plants. After the reaction, the active molecule from the herbicides will be inactivated by conjugating with the sugar and ready to be exported to the vacuole or to be incorporated into the cell wall. It is one of the predominant mechanisms utilized by plants to confer resistance to the ALS-inhibiting herbicides (Siminszky B, 2006).

There are three major types of conjugation detoxification mechanisms utilized by plants, including glucose conjugation, amino acid conjugation, and glutathione conjugation. Glutathione conjugation has a wider range of herbicide substrates compared to the other two and therefore is the most important type of conjugation conferring resistance to herbicides in plants. The glutathione conjugation reaction involves nucleophilic displacement between herbicides and glutathione by the action of glutathione-s-transferases (GSTs) with different specificities to different herbicides leading to direct detoxification of the herbicide active ingredients. GSTs belong to a family of enzymes that attach to the tripeptide glutathione through the cysteine residue to electrophilic, hydrophobic compounds. GSTs are involved in the metabolism of herbicides, such as triazine and chloracetanilide, providing herbicides resistance in corn and

sorghum. Once glutathione is bound to the herbicide, the herbicide is no longer toxic and may be moved to the vacuole. Studies indicate that the GST-endowed resistance is inherited as a single, partially dominant, nuclear-encoded trait. However, despite the relatively important role of glutathione conjugation in plants, there has been little interest in modifying gene encoding enzymes conferring herbicide detoxification in plant development (Bakkali et al., 2007).

Another enzyme that's also found to be responsible for metabolism based resistance is the aryl acylamidases. Aryl acylamidase is an enzyme responsible for the metabolism of propanil in rice, and it has been found that the resistance to the herbicides is significant in the population where the expression of aryl acylamidase is at higher level (Santos et al., 1998). The cytochrome P450 monooxygenases are a large family of enzymes involved in many plant metabolic processes (Fischer et al., 2000b; Osuna et al., 2002; Yun et al., 2005; Yasuor et al., 2009, 2010).

2.4.3 Resistance at the site of action

Most of the herbicides work on single sites of action controlled by single or few genes, and many times even just a single gene mutation can confer to resistance to the corresponding herbicides. Therefore this mechanism of modifying the sites of action by single gene mutation of single gene transformation has been the largest focus on developing herbicide resistance plants. Numerous studies have identified point mutations in different plants that changes only one amino acid in the target sites of the herbicides conferring resistance to different herbicides. However resistance to the same herbicide may not sue to the same mutation at the same locus of a gene. Several target-site mutations have been identified in plants to be responsible for the resistance to the herbicides, including Photosystem II (PSII), microtubule assembly, and the enzymes, acetolactate synthase (ALS), acetyl-CoA carboxylase (ACCase) and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), etc.

Seven point mutations on ACCase gene had been identified in *Avena sterilles* (a wild sterile oat species): Ile-1781-Leu in the chloroplast, Trp-1999-Cys, Trp-2027-Cys, Ile-2041-Asn, Asp-2078-Gly, Cys-2088-Arg, and Gly-2096-Ala that all confers some levels of resistance to various ACCase-inhibiting herbicides at different rates (Powles and Preston, 1995; Zagnitko et al., 2001; Liu et al., 2007; Delye 2005; Deley et al., 2008; Papapanagiotou et al., 2015). Herbicides in the family include the aryloxyphenoxypropionic acid (APP) and cyclohexanedione (CHD) herbicides, which target the plastid enzyme ACCase in plants, which are selectively lethal to many Graminaceae but not to dicot species, therefore they have become widely employed for grass weed control in many dicot crop production systems (Devine and Shimabukuro, 1994). A different type within this mode of action involves overproduction of the target enzyme, ACCase in *Sorghum halepense* as an example here.

Several PS2 inhibiting herbicides, triazine, phenylurea, and uracil, bind to the D1 protein at different sites in plants and block the transport system of electrons to the plastoquinone within the PS2 reaction center. Amino acid substitution at Ser-263-Gly on D1 protein in most plant species confers the resistance to symmetrical triazine herbicides only. The Ile-219-Val substitution in *Poa annua* was responsible for resistance to the phenylurea, diuron, and to metribuzin (an asymmetrical triazine). A Ser-264-Thr substitution in D1 protein in *Portulaca oleacea* provided resistance to the phenylurea, linuron, and to the symmetrical triazine herbicide families. The substituted urea and triazine herbicides bind to overlapping, but not identical, sites in PS2. As a result, mutating at one site doesn't affect the binding of another chemical to the enzyme (reviewed by Trebst, 1991). The inheritance of this resistance is maternal only since the D1 protein is encoded by genes in the chloroplasts (reviewed by Gronwald, 1994). Different levels of resistance were observed in different plant species reacting to the same chemical, this phenomenon was explained by the possibility of the chloroplast envelopes probably provide

differential barrier to these herbicides, as well as some species have differences in the reaction center proteins surrounding the active site (Trebst, 1991).

Site of action is so far the only mechanism found to be involved in the plants that are resistant to the AHAS–inhibiting herbicides. AHAS exhibits a wide range of mutations in the target sites (AHAS isoenzymes) found in many weed types that confers resistance to at least fifteen chemical classes of herbicides. Among them, sulfonylurea, imidazolinone, and triazolopyrimidine herbicides have been commercialized and widely use (Saari et al. 1994). In most cases, the sulfonylurea-resistant biotypes due to altered AHAS enzyme exhibit varying levels of target site cross resistance to the chemically dissimilar, but AHAS-inhibiting, imidazolinone, and triazolopyrimidine herbicides (Hall and Devine, 1990; Christopher et al., 1992; Saari et al., 1990; 1992; 1994). The resistance to the AHAS-inhibitors usually exhibit a semi-dominant trait conferred by a single dominant or partially dominant nuclear-encoded gene (Saari et al., 1990). The wide range of vitiation in target sites cross resistant weed biotypes implies there are a number of different functional mutations of the *AHAS* gene. There are two domains in the AHAS enzyme, domain A and domain B, which are involved in the resistance/sensitivity. It was initially found that amino acid substitution at Pro-197-His, a highly conserved region, in *Lactuca sativa* confers resistance to the AHAS-inhibiting herbicides (Eberlein et al., 1999). More studies conducted revealed that substitutions of Pro-197-Ser, Pro-197-Glu, or Pro-197-Ala in domain A can all result in the resistance to the herbicides (Saarri et al., 1994; Lee et al., 1988; Tranel and Wirght, 2002). Many other studies were also conducted in many weed species indicating point mutation at Pro-173 confers some levels of resistance to one or two families among all the AHAS-inhibiting herbicides, including *Kochia scoparia* and *Lactuca serriola* (Guttieri et al. 1992). Other than mutations at Pro-173 of domain A, several other point mutations were discovered to result in the resistance to sulfonylurea and/or

imidazolinone resistance in higher plants, such as Ala-122, Ala-205, Asp-376, Trp-574, Trp-753, Ser-653, and Gly-654 in the *AHAS* gene in *Arabidopsis thaliana* and *Nicotiana tabacum* to sulfonylurea and/or imidazolinone (Lee et al., 1988; Sathasivan et al., 1991; Yu et al., 2010; Heap, 2014). Obviously this is suggesting that there are several point mutations of the *AHAS* gene which will confer resistance to sulfonylurea and imidazolinone herbicides possibly due to subtly different binding by different herbicides on the AHAS enzyme and different mutation of AHAS. The wide variation in target sites conferring resistance to different herbicides implies that there are a number of different functional mutations of the *AHAS* gene (Durner et al., 1991; Landstein et al., 1993).

It is worth noting that other than the resistance, some of these mutations were associated with poor agronomic phenotypes. Examples include the Trp-574-Leu mutant of *Amaranthus powellii* were found to have thinner roots and stems, and also reduced number of leaves, which all influenced the yield of seed production (Tardif et al., 2006). Similar negative effects were also seen in the rice that Gly-654-Glu showed 5 to 100 percent of yield loss (Sha et al., 2007). It was therefore speculated that the active site and binding site in AHAS are different, and also explains why Pro-173 are most widely adapted naturally in the weeds for the change in this amino acids renders resistance to specific AHAS-inhibiting herbicides while causing no penalties on the normal catalytic functions (Warwick et al., 2010).

A common mechanism found in most plants that were found to be resistant to glyphosate is through the modification of the site of action in the EPSPS at Pro-106. Pro-106 is not at the binding site, but the amino acid substitution at position 106 from Pro to Ser, Thr, or Ala causes the disorientation of the enzyme that causes the reduced affinity of the actual binding site to the glyphosate, which thus confers to some degree of resistance to glyphosate (Bostamam et al., 2012).

The mechanisms of the resistant to the auxinic group are considered relatively complex because it was suggested that resistance in several weeds is due to multiple sites of action to auxinic compounds (Gressel and Segel, 1982; Morrison and Devine, 1994). Mechanisms can also be quite different across plant species, it was shown that the resistance to 2,4-D in *Sinapis arvensis* was controled by a single dominant gene (Jasieniuk et al., 1995; Jugulam et al., 2005). Later it was found that resistance in *Arabidopsis thaliana* to 2,4-D is due to a recessive mutation caused by a single gene, *axrl* (Estellel and Somerville, 1987), while actually there are at least 5 *axrl* alleles that can confer the similar phenotypes and reactions to the 2,4-D (Lincoln et al., 1990). Mutations in the five auxin-signaling F-box (*tir1-1*, *afb1*, *afb2*, *afb3*, and *afb5*) were also found to be conferred to resistance in different auxinic herbicides (Gleason et al., 2011). This recessive resistance is in contrast to most reports of herbicide resistance being controlled by a dominant or semi-dominant gene.

Another example of target-site based resistance controlled by recessive nuclear gene is the resistance to Dinitroanaline herbicides, such as prodiamide, oryzalin, and trifluralin. They bind to tubulin α and block the interaction between the tubulin, α and β, and thus inhibits the formation of microtubules, which are important in cell division. A point mutation in the gene that encodes for α-tubulin results in dinitroanaline resistance in plants, which has been reported to be controlled by a single, recessive nuclear gene.

2.4.4 Overexpression of the target enzyme

When a mutated enzyme identified to be responsible for the resistance to the herbicide, it is often to find the increase of resistance related to overexpressing the same protein. Therefore it is logical for the breeders to develop a resistant line through this approach.

It was suggested that the EPSPS expression level was positively correlated with the number of copies of *EPSPS* gene in plants (Powles, 2010; Gaines et al., 2010). The *EPSPS* genes are amplified through jumping gene, and at least 30 to 50 genome copies of *EPSPS* are necessary to allow plants to survive at the glyphosate rates between 0,5 to 1.0 kg/ha (Gaines et al., 2011). Several transgenic crops had been engineered to overexpress the *EPSPS* gene were reported to be successfully maintain normal metabolism after absorbing glyphosate at even four times the dose of herbicide needed to kill non-transgenic plants. This technique has been applied to many transgenic glyphosate-resistance crops to commercialize (Dill et al., 2008). The most well-known product is the Roundup Ready® Soybean, which was launched in US in 1995 (Delanney et al., 1995), and the CP4 EPSPS has still been the most widely used trait within all the genetically modified crops grown in the world (Dill et al., 2008).

Photoporphyrinogen oxidase (PPO) is the key enzyme in the chlorophyll/heme biosynthesis pathway, which is in charge of oxidizing photoporphyrinogen IX into protoporphyrin IX (Smith et al., 1993). It was found that plants overexpressing *PPO* gene confer resistance to herbicide containing diphenyl ether that causes light-dependent membrane damage (Lee et al., 1993; Li et al., 2003), which also serves as an effective selectable marker in the transformation in maize and rice, with transformation frequency similar to *pat o*r *pmi* systems (Lee et al., 2007). Other research was conducted to transfer the over-expressed *Arabidiopsis PPO* genes into tobacco, maize, and rice, which conferred tolerance to acifluorfen, butafenacil,

and oxyfluorfen respectively (Lermontova and Grimm, 2000; Ha et al., 2004). Transgenic over-expression of the *PPO* gene from *Myxococcus xanthus* confers high resistance to PPO inhibitors including oxadiazon, butafenacil, oxyfluorfen, and acifluorfen in rice (Jung and Back, 2005; Jung and Kuk, 2007; Lee et al., 2007; Jung et al., 2008).

As mentioned above, the enzyme P450s responsible for detoxifying many herbicides through hydroxylation followed by exporting the inactivated herbicide molecules to the vacuoles or the cell wall. It has also been reported that by overexpressing the P450s genes increased the P450-based metabolism and confers higher resistance or quicker detoxification in several weeds (Fischer et al., 2000b; Osuna et al., 2002; Yun et al., 2005; Yasuor et al., 2009, 2010).

Sometimes the resistance to certain herbicides can be a function of two mechanisms, such as the resistance to s-triazine in some weeds due to a combination of both alteration to the site of action, the D1 protein in chloroplast, and metabolic exclusion before reaching the target sites (Arntzen et al., 1982).

2.4.5 Different genetic actions of the resistance

The resistance/sensitivity responses in plants to herbicides with various mechanisms are controlled by different genetic actions. Most of the resistance or even semi-resistance responses are conferred by dominant or partial-dominant genes. Target-site resistance is often controlled by a single gene, such as resistance to AHAS inhibitor herbicides, which have been reported to be controlled by single dominant or semi-dominant gene. Heterozygous plants are often found to be injured, but do not die after application of the herbicides. This type of resistance is often found when strong selection pressure is applied, while other resistance that doesn't involve in the site of action (non-target-site resistance) usually are screened and identified under moderate selection pressure that's regulated by multiple alleles. Moreover, resistance controlled by a single or just a few genes possess major effects and less likely be affected by the environments, while the

polygenic resistance often involves higher proportion of additive genetic effects with mostly minor effect and higher GxE interactions. Hence the sites of action regulated by a single gene is more widely used by breeders to incorporate into cropping systems among all of the modes of actions (Preston, 2003; Delye et al., 2013; Powle and Yu; 2010). A point mutation causing a single amino acid substitution at Leu-1780-Ile in the chloroplast ACCase was identified in a grass species, *Setaria viridis*, which confers resistance to sethoxydim, a cyclohexanediones (one of the ACCase inhibitor herbicide chemicals). The inheritance of this resistant trait is controlled by a single, semi-dominant gene.

AHAS inhibiting herbicides includes five chemical families. The resistance and the level of resistance in plants to each of the families can be conferred by different point mutations at different sites on the AHAS as stated previously. Some mutations contribute to resistance to more than two chemical families; it is also possible to have multiple point mutations in the *AHAS* gene that can provide a greater level of resistance to multiple chemical families of AHAS inhibiting herbicides. The resistance to the AHAS inhibitors has been reported to be controlled by a single, dominant or semi-dominant gene (Tranel and Wright, 2002; Tranel et al., 2014; http://weedscience.com). Heterozygous plants are often reported to possess less resistance to the herbicide that they became injured, but not killed by the herbicides.

Some herbicide resistances were reported to be controlled by a single recessive gene. The development of frequency distribution of the resistance alleles in a population throughout the breeding process also depends upon the types of pollination. In cross-pollinating crops, dominant resistance alleles will increase in the population more rapidly than recessive alleles. The recessive resistance trait will evolve in the population at a slower rate than dominant resistant because the dominant homozygotes and heterozygotes will be removed by herbicide application. However in self-pollinating plants, dominant or recessive alleles are likely to

accumulate at equal frequency in population under selection pressure, which also explains why most of the recessive dominance were conducted and identified in self-pollinating crops (Charlesworth 1992; Jasieniuk et al., 1996). The types of chemicals that have been reported in this type of action are also limited. Resistance to Dinitroanaline in goosegrass (*Eleusine indica*) is an example of recessive resistance. Dinitroanaline is one type of growth regulator herbicide that binds to the tubulin and blocks the formation of microtubules and further affects the cell division. The replacement of Thr-239-Ile239 in the *α-tubulin* gene causes the resistance in the goosegrass (Yamamoto et al., 1998). Studies also were conducted showing resistance to herbicides clopyralid and picloram in a wild oat, yellow starthistle (*Centaurea solstialis*), is controlled by a single recessive gene (Van et al., 2004). There also were attempts, although rare, of utilizing the mutant of the α2-tubulin gene in maize to create a herbicide resistance line through traditional breeding and genetic engineering (Landi et al., 1999; Anthony and Hussey, 1999).

As briefly mentioned previously, the resistance in *Arabidopsis thaliana* to 2,4-D , which can be a result from point mutation of any of the 5 *axl* alleles demonstrating another recessive resistance example (Lincoln et al., 1990).

*2.5 Uses of herbicide tolerance in crop development*

The adoption of herbicide-tolerance has increased considerably in the last few years in different cropping systems. Currently, three herbicide tolerant systems are most commonly used. These include Glufosinate (LibertyLink®) resistance commercialized by Bayer CropScience, glyphosate (Roundup Ready®) resistance commercialized by Monsanto (Franz, 1970), and Imidazolinine tolerance (Clearfield®) commercialized by BASF (Duke, 2005). The occurrence of mutated sites of action within plants can be the sources of potential resistance development. One of the sources of tolerance or resistance can occur as a result of random mutation, which is

relatively infrequent. Human induced mutation can come from mutagen induction, including, radiation, ethyl methane sulphonate (EMS), or chemical herbicide-induces mutations. There is also an increasing acceptance of transgenic traits improving the crop performance and even more and more widely used cisgenic technique. Commercial development of respective varieties resistant to each herbicide of different crops is underway. Despite various ways of creating allelic variants, the resistance cannot be observed without the selection pressure of corresponding herbicide exposure. The selection of herbicide resistance plants followed by the selection pressure application can be accomplished by either traditional plant breeding or/and biotechnological techniques. DNA responsible for the herbicide resistance in these plants are not inserted from foreign species, and therefore not considered to be GMO.

Screening for individual plants possessing resistance to herbicides in a mutagen treated population has been an effective way to identify plants with mutant enzymes withenzymatic activity directly resistant to normally-inhibitory levels of a herbicidally-effective active ingredient. Herbicide tolerant crops developed through this conventional process have an advantage in commercialization with fewer regulation hurdles compared to transgenic herbicide tolerance crops, and therefore more readily acceptable by growers (David et al., 2003).

Selectable marker genes confer resistance to herbicides such as glyphosate, glufosinate, imidazolinine, or broxynil were often used in the selection process (Comai et al, 1985; Gordon-Kamm et al, 1990; and Stalker et al, 1988). Genetically modification (GM) was used as strategy to incorporate herbicide resistance genes into crops to manage weeds since 1996. There are many ways for plans to acquire the trait of tolerance transgenically. One way is to incorporate a gene producing the herbicide tolerant form of the target enzymes. Another way is to incorporate the gene producing herbicide degrading enzyme. Modern biotechnology has greatly widened the efficiency of the procedures to identify, purify, and transfer the genes from one organism to

another (Osuntoki, 2005). Today, GM crops incorporated with herbicide resistance accounts for more than 80% of the currently grown GM crops worldwide (James, 2012).

One substantial category of the current GM herbicide resistant crops is the glyphosate-resistant crops including corn, soybean, canola, alfafa, sugarbeet, and cotton, commonly known as Roundup Ready® cropping system (Bertges et al., 1994; Cajacob et al., 2004; Green et al., 2009; Duke et al., 2009). Glyphosate is a non-selective herbicide has been the mostly widely used herbicide even before the development of any herbicide resistant crops (Gianessi and Reigner, 2006). There are several approaches of generating glyphosate-resistant crops. The most popular way is to alter the structure of EPSPS enzyme by transferring a naturally occurring gene, *CP4 EPSPS*, from an *Agrobacterium tumefaciens* strain CP4 encoding a modified form of EPSPS that confers natural resistance to glyphosate. This has been done intensively since it was discovered and has been found to be effective in many transformed crops, such as cotton, corn, and soybean (Barry et al., 1992; Padgette et al., 1995; Padgette et al., 1996). It has been reported that the CP4 EPSPS alone makes soybean roughly 50-fold more tolerant to glyphosate (Nandula et al. 2007). Some other glyphosate-resistant crops rely on a mutant E coli gene fused to EPSPS enzyme chloroplast transit sequence to create transgenic plants, have been continuously grown (OECD, 1999; Michiels and Johnson, 2001). Researchers from China also developed a transgenic glyphosate resistance rice line, EP3, by transferring a native rice *EPSPS* gene into an existing popular rice variety, Minghui86 (Xu et al., 2002; Yan et al., 2011). A range of modified *EPSPS* genes were utilized to develop transgenic rice resistant to glyphosate herbicides (Charng et al., 2008; Zhao et al., 2011; Chandrasekhar et al., 2014). A recent research introduced a codon-optimized *mCP4-EPSPS* gene (modified *CP4-EPSPS*) with N-terminal chloroplast targeting peptide from *Petunia hybrida* into a popular inbred rice variety, IR64. The transformed rice plants exhibit high EPSPS activity even treated with high concentration of glyphosate

herbicide compared to the control plants, which showed tolerance to 1% of commercial Roundup (equivalent to 10 $g^{-1}$ or 10,000 ppm, or 10 mM (175 ml)), which is roughly 5 times higher of dosage used to kill regular weeds in the field conditions at seedling stage (Chhapekar et al., 2015).

Another strategy that has been reported is to metabolically degrade glyphosate into non-toxic products in plants (Coupland, 1985; Thompson et al., 1987; Pline-Srinc, 2006; Duke, 2011). Some crops, such as canola can use a gene transferred from a microbe *Ochrobatrum anthropic* that encodes glyphosate degradation enzyme, glyphosate oxidase (GOX), which also detoxifies the glyphosate into and yield the less-toxic products, aminomethyl phosphonic acid (AMPA) and glyoxylate, by cleaving the carbon-nitrogen bond of glyphosate that are treated on the plants (Padgette et al., 1996). Canola and some other crops, such as tobacco, were reported to be able to use the glyphosate oxidoreductase enzyme isolated from *Pseudomonas* sp. Strain LBr to metabolize the treated glyphosate (Franz et al., 1997). The *GOX* gene isolated are often put in combination with *CP4 EPSPS* and transformed into plastids for the efficiency of transformation and an elevated tolerance was found (Zhou et al., 1995; Mannerlof et al., 1997). *GOX* and *CP4* genes together provide approximately 50-fold of resistance to glyphosate in canola (Nandula et al., 2007). A fungal gene encoding glyphosate decarboxylase was patented and used to develop different transgenic glyphosate resistant crops (Hammer et al., 2005). Other researches transferred another gene encoding for glyphosate acetyl-transferase (GAT) from *Bacillus licheniformis* into crops to detoxify the glyphosate (Castle et al., 2004; Siehl et al., 2007). GAT can convert the glyphosate into N-acetylglyphosate that doesn't inhibit the function of EPSPS in plants. Glycine oxidase (GO) from *Bacillus subtilis* also were found to be able to convert glyphosate into AMPA and glyoxylate through a different reaction mechanism. The genes encoding for GO were successfully transformed in rice plants as a source of resistance to

oxyfluorfen herbicide (Jung et al., 2004). The GO genes were also utilized to develop other transgenic glyphosate-tolerant crops (Nicolia et al., 2014). A number of assays worked on recognizing GO variants with improved affinity with glyphosate through site saturation mutagenesis on the active sites. Different genes were therefore used to combat glyphosate in various crops (Pedotti et al., 2009; Pollegioni et al., 2011; Zhan et al., 2013; Nicolia et al., 2014). Some of the latest research associated with *Arabidopsis* involved transferring a gene, the *DAAO* gene, from *Bradyrhizobium japonicum* responsible for encoding the D-amino acid oxidase (DAAO), which can oxidatively cleave the carbon-nitrogen bond on the carboxyl side of glyphosate to AMPA and glyoxylate. The transformed *Arabidopsis* plants were found to be significantly more tolerant to glyphosate than untransformed plants (Han et al., 2015). DAAO and GO belong to the same structural family with different substrate specificities. They show modest sequence similarity to GO (Pedotti et al., 2009).

Glufosinate is another fast-acting, non-selective post-emergence herbicide chemical ingredient that has been widely used to control weeds by inhibiting the enzyme glutamine synthetase in plants (WSSA, 1994; Wild and Manderscheid, 1984). Glufosinate is a synthetic version of the natural product, phosphinothricin. Glutamine synthetase is the key enzyme catalyzing the reaction of forming the amino acid glutamine from glutamate and ammnonia (Bayer et al., 1972; Lea et al., 1984). This enzyme is known to have two types of isoforms (GS1 and GS2) in the roots and the leaves respectively. The GS1 in cytoplasm of roots shows greater sensitivity to the glufoniate than the isoform GS2 in chloroplast the leaves due to different kinetic effects (Wild and Manderscheid, 1984; Manderscheid and Wild, 1986). The inhibition of the enzyme caused the accumulation of ammonia, reduced synthesis of glutamine, and further reduced the other amino acids including glutamate, aspartate, alanine, glycine, and serine. The depletion of these amino acids in plants will lead to inhibition of the glycolate pathways,

resulting in accumulation of phosphoglycolate, glycolate, and glyoxylate (Wild and Wendler, 1991). Glyoxylate accumulation is toxic to the ribulose bisphosphate (RuBP) carboxylase activity (Ziegler and Wild, 1989), and therefore leads to the rapid inhibition of the photosynthesis, chlorosis of contact tissues, and the ultimate death of the treated part of the plant (Sauer et al., 1987; Wild et al., 1987; Lacuesta et al., 1992). Glufosinate has been reported to be better at controlling broadleaf weeds, such as s morningglories (*Ipomoea* spp.), hemp sesbania (*Sesbania herbacea* (P. Mill.) McVaugh), Pennsylvania smartweed (*Polygonum pensylvanicum* L.), and yellow nutsedge *(Cyperus esculentus* L.) compared to the glyphosate. However the limitation of glufosinate is that it is a contact herbicide, and translocation is significantly slowed down 24 hours after absorption. Therefore it must be applied to the smaller plants and not as effective as glyphosate on perennial weeds that require translocation for complete control (Ullricj et al., 1990; Steckel et al., 1997).

The *pat* and *bar* (bialaphos resistance) genes, were later discovered from *Streptomyces viridochromogenes* and *Streptomyces hygroscopicus*by respectively researchers are known to be homologous and encode for structurally similar proteins that's able to detoxify the glufosinate via acetylation (Wehrmann et al., 1996). The *bar* gene from *Streptomyces* encoding acetyl transferase is responsible for detoxification of glufosinate by actylating the free $NH_2$ group (Thompson et al., 1987; Wohlleben et al., 1988). The *pat* gene is found to be encoding for enzyme, phosphinothricin-acetyltransferase, which inactivates glufosinate by acetylation the phosphinothricin, which serves as an active ingredient in the broad spectrum herbicides. These two genes are mostly infused with a strong promoter when transferred into various crops to allow high expression of the acetyl transferase that confers the resistance, including rice, alfafa, corn, wheat, canola, poplar, soybean, rapeseed, potato, sugar beet, tobacco, and tomato crops known as LibertyLink®cropping system (De Block et al., 1987; Botterman and Leemans, 1989;

Mullner et al., 1993; Vasil et al., 1992; Cobb, 1992, Christou et al., 1991; Gordon-Kamm et el., 1990; D'Halluin et al., 1992; De Greef et al., 1989; De Block et al., 1987; Bertges et al., 1994). Both BAR and PAT enzymes selectively acetylate only glufosinate, but not other amino acids (Wehrmann et al., 1996). LLRICE06 and LLRICE62 were the two transgenic lines produced by Bayer by transforming *pat* gene and *bar* gene in the regulatory region into the existing variety, M202 and Bengal respectively, which were approved to plant in USA in 2006. LLRICE601 is called the LibertyLink rice possessing the herbicide resistance pattern is similar to LLRICE06 (WO2000/026356) and LLRICE62 (US20082289060), but with PAT expression lower than detectable (Bayer, 2006). Different levels of resistance to glufosinate were observed in different rice varieties transformed with *bar* gene, which is also seen in the transgenic barley with *bar* gene. This phenomenon was explained by the possibility of pleiotropic effects due to different genetic back grounds of the recipient plants (Oard et al., 1996; Bregitzer et al., 2007).

It is noteworthy that reportedly when treated with glufosinate, transgenic rice transformed with *pat* gene was less susceptible to the fungi, *Magnaporthe grisea* and *Rhizoctonia solani*, that causes blast and sheath blight disease in rice (Tada et al., 1996; Uchimiya et al., 1993).

A new gene isolated from a marine bacterium *Rhodococcus* sp. Strain YM12, named *RePAT*, was found to encode for protein RePAT, a novel phophinothricin N-acetyltransferase (Wu et al., 2014). This protein shows 37% similarity to the phophinothricin N-acetyltransferase encoded by *bar* or *pat* genes. The *Agrobacterium RePAT* transgenic plant (PAT7, PAT 11), transformed japonica rice Zhonghua11, showed stable inheritance, no negative effect on the agronomic traits, and most survived at high glufosinate concentration at 5000 g/ha, which is about 10 time higher than recommended dosage (Cui et al., 2016). The relatedness of the expression level of RePAT and the tolerance level to glufosinate was also validated, and this

newly cloned *RePAT* can be used as selective marker in the development of new crops tolerant to glufosinate.

Development of crops with double tolerance to multiple herbicides or even pathogens provides growers a choice between two broad spectrum herbicides and other optional selective herbicides. Crops stacked with genes resistant to both glufosinate and glyphosate are commercially available in cotton, soybeans, corn, and rice (Deng et al., 2014). "WideStrike" or "WideStrike3" are the cotton hybrid produced by Dow AgroSciences by crossing two transgenic cotton lines, DAS-21023-5 and DAS-24236-5, that are resistant to glufosinate herbicide and lepidopteran insects respectively. The *pat* gene, was used as the selectable marker during the Agrobacterium-mediated transformation process of *cry1F* and *cry1Ac* genes for its known to be closely linked to both genes. However the tolerance to glufosinate of WideStrike is not as robust as in LibertyLink Cotton, developed by Bayer. The triple transformation cotton hybrid was later developed by crossing "WideStrike" with a MON88913 event, which is a line resistant to glyphosate. The line derived thus expresses the four transformed, CRY1F, CRY1Ac, PAT and CP4 EPSPS proteins (WideStrike Roundup Ready). "TwinLink" is the cotton product developed by Bayer that is also built to incorporate *cry2Ae* and cry1Ab genes conferring resistance to lepidopteran larvae, and the *bar* gene that's resistant to glyphosate by crossing two lines T304-40 X GHB119 (Reviewed by Rao, 2014). In soybean, there are several products are commercialized or close to commercialization engineered or stacked to express multiple traits, so crops are tolerant to multiple herbicides, such as Roundup Ready 2 Xtend™ soybeans, which are tolerant to both glyphosate and dicamba; Enlist™ soybeans which are tolerant to 2,4-D choline, glyphosate, and glufosinate; Balance™ Bean which is tolerant to glyphosate and isoxaflutole; and Bolt™ soybeans with enhanced tolerance to ALS-inhibiting herbicides and glyphosate. There are recently developed corn plants tolerant to both glyphosate and glufosinate.

Other platforms are looking to combine the tolerance to glyphosate and sulfonylurea class of herbicide (ALS inhibitor), or the triple combination of 4-hydroxyphenylphenylpyruvate dioxygenase (HPPD) inhibitor with glyphosate and glufosinate varieties.

Bromoxynil is a nitrile herbicide that blocks photosynthesis through the action of 3,5-dibromo-4-hyroxybenzonitrile on inhibiting photosynthesis by binding to the Photosystem II complex of chloroplast membranes and blocking electron transport (Ahrens, 1994). The *bxn* genes (Bromoxynil specific nitrilase) was first identified by McBride et al., in 1986 encoding bromoxynil-specific nitrilase, which is a critical enzyme that converts the 3,5-dibromo-4-hydroxybenzonitrile to its metabolite 3,5-dibromo-4-hydroxybenzoic acid that is at least 100-fold less toxic (McBride et al., 1986). The codon-optimized synthetic gene of *bxn* has been developed and been transferred into cotton, tomato, potato, and rapeseed (Dyer et al., 1993). The advantages of codon optimization is that it allows secondary structure modification of the transcribed mRNA, increase of the protein level expressed in the transferred organism from 2 to 10 fold by adding signal peptide sequence at the 5' end and the retention peptide signal sequence to the 3' end of the foreign gene. It can be designed for specific crops or even specific subcellular sites by adding signal sequences to localize the foreign proteins (Wong et al., 1992; Welch et al., 2009; Kim et al., 2009). Therefore, transferring synthetic genes instead of natural genes are expected to cause higher tolerance to the herbicide and act more rapidly on degradation of the active ingredients in herbicides in plants. This approach of transformation has become more and more popular creating transgenic crops, including *TSP* gene in tobacco and *EPSPS* and *BAR* genes in rice (Wang et al., 2003; Deng et al., 2014).

Several reports are available for other synthesizing herbicide resistance genes and transferred into various crops conferring resistance to different herbicides. The enzyme 2,4-dichlorophenoxyacetate monooxygenase encoded by *tfd* gene was discovered to be resistant to

herbicide 2, 4-D , and later was synthesized and transferred to into crops, including pepper, apple, tomato, hirse, sunflower, tobacco, potato, corn, cucumber, wheat, soybean and sorghum (Perkins et al., 1990; Bayley et al., 1992; Fukumori and Hausinger, 1993; Lyon et al., 1993; Oliver et al., 2003). Using synthetic *cryIC* gene encoding a Bacillus thuringiensis δ-endotoxin protein confers spodoptera resistance has been reported in alfalfa and tobacco (Strizhov et al., 1996). The transgenic expression of synthetic *Bt* genes are reportedly effective for controlling insect pests in several major crop plants, including corn, rice, cotton, potato, tomato, tobacco, soybean and canola (Miklos et al. 2007).

The cyanamide hydratase gene, *Cah*, from the soil fungus *Myrothecium verrucaria* was found to confer resistance to cyanamide by converting this chemical to its metabolite urea that has a narrow substrate specificity (Maier-Greiner et al., 1991). Selection of resistance to cyanamide due to *Cah* has been conducted on different crops, including *Arabidopsis*, potato, rice, and tomato (Damm, 1998). The method of transformation in wheat with this gene and selection using cyanamide was patented in the United States in 2001 (Weeks, 2000; US6268547 B1). Direct introduction of *Cah* gene into seeds was also carried out in soybean and tobacco using hygromycin phosphotransferase as the selectable marker gene and hygromycin as the selection agent (Zhang et al., 2005).

Several forms of cytochrome P450 monooxygenases (P450s) are known detoxify herbicides by metabolizing the phytotoxic ingredients. Transgenic plants with mammalian cytochrome P450 genes were first developed to introduce herbicide resistance (Ohkawa et al., 2009). Rice endogenously expresses multiple P450 enzymes genes, and it was later found that the transgenic rice plants with other P450 isoforms conferred higher resistance to the herbicides, such as the human P450, CYP1A1. Transgenic rice (Nipponbare) with *CYP1A1* under the control of modified CaMV 35S had broad cross-resistance towards various herbicides,

xenobiotics chemicals, by metabolizing them at a higher pace (Kawahigashi et al., 2003; Kawahigashi et al., 2006). The *CYP1A1* transgenic rice plants also showed enhanced resistance to the actyl-CoA carboxylase-inhibiting herbicide, zalofop-ethyl, the very long-chain fatty acid (VLCFA) synthesis-inhibiting herbicide, mefenacet, and 10 other herbicides belonging to different chemical families while the germination of WT was inhibited by these chemicals. Similar results were observed in transgenic potato (*Solanum tuberosum*) plants when it was transferred with rat or human *CYP1A1* genes that showed broad resistance to herbicides chlorotoluron, atrazine, and diuron, suggesting that *CYP1A1* works efficiently in both monocots and dicots (Inui et al., 1998; Inui et al., 1999). The herbicide tolerance during germination might thus allow the *CYP1A1* transgenic rice plants to be directly seeded in the field with various herbicides for weed control. The transgenic *CYP1A1* rice plants were also proposed to be used as a phytoremediation tool to reduce various agrochemical residual contaminates in the environment (Kawahigashi et al., 2006).

Plant species differing in resistance level to AHAS can develop resistance to different classes of AHAS inhibitors. Most resistance cases are due to point mutation in AHAS gene that causes the reduction of affinity of the enzyme to the herbicide (Kolkman et al., 2004). AHAS-inhibiting herbicides resistant crop development can also be accomplished by the transfer of resistance genes between different higher plants species (Falco et al., 1989; Haughn et al., 1988; Miki et al., 1990). Various mutant forms of AHAS genes for herbicide resistance have been used as selectable molecular markers in the transformation and molecular assisted selection process (MAS). Among all the AHAS genes, *csr1* genes isolated from *Arabidopsis* is the most common used as selection marker (Haughn and Somervilee, 1986). It was later taken as selection marker in several transgenic crops, such as rice (Li et al., 1992), tobacco (Charest et al., 1990), maize

(Fomm et al., 1990), canola (Miki et al.,1990), common bean (Bonfim et al., 1992), and jujube (Gu et al., 2008).

AHAS inhibitors are generally highly active and selective, and therefore particular compounds have been designed for specific crops are favored for certain crops. Both sulfonylurea-resistance and imidazolinine-resistance have been used to develop the herbicide resistance crops. Sulfonylurea-resistance crops generated via mutant selection includes tobacco (Chaleff and Ray, 1984), flax (Jordan and McHughen, 1987), soybean (Sebastian et al., 1989), canola (Tonnemaker et al., 1992), sugar beet (Saunders et al., 1992; Hart et al., 1993), barley (Baillie et al., 1993), cotton (Rajasekaran et al., 1996), and rice (Terakawa and Wakasa, 1992; Wakasa et al., 2007). This herbicide resistance trait is a single gene mutation and is usually heritable as semi-dominant or dominant trait. Genetic engineered sulfonylurea-resistance crops were also developed with different sources of AHAS genes. A mutated tobacco AHAS gene was used to develop transgenic sulfonylurea-resistance cotton (Saari and Mauvais, 1994) and sugar beet (D'Halluin et al., 1992). A mutated Arabidopsis AHAS gene was used to transform tobacco (Gabard et al., 1989), flax (McSheffrey et al., 1992), canola (Miki et al., 1990), and rice (Li et al., 1992).

The Clearfield production system combines various imidazoline-tolerant crops, firstly developed in rice through seed mutagenesis, and later utilized in other crops, such as wheat, maize, oil seed rape and sunflower (Croughan 1994; Lincombe, 2004; Sha et al., 2007; Tan et al., 2005). Mutations of *AHAS* gene in various crops provide ways to develop more imidazolinone-tolerant crops. The selection for imidazolinone-resistance maize started in 1982 (Shaner et al., 1994). Since the first commercially launched imidazolinone-tolerant maize in 1992 in the US, many other major imidazolinone-tolerant crops have been developed and commercialized using mutagenesis mutating several variants of *AHAS* genes and through selection, including wheat,

canola, oilseed rape, rice, sunflower, cotton, and a few other vegetative crops. Generally, one single target site mutation in the *AHAS* gene is sufficient enough to confer tolerance to AHAS-inhibiting herbicide. The eukaryotic AHAS protein is made of two sub-units, one catalytic sub-unit and one regulator sub-unit. The regulatory sub-unit is also known as the smaller sub-unit of AHAS (SSU). The catalytic sub-unit is a homodimer formed by folding two large sub-units (LSU) monomers, and each of them has three domains of similar size, α, β, and γ. Structural analysis of wild type and mutated AHAS enzymes have suggested that the binding sites of imidazoline herbicide is within the herbicide-binding pocket near the actives site of located at the interface of the two LSU monomers of the catalytic sub-unit. The herbicide-binding pocket was later identified through molecular modeling to be at the entry channel for the substrate of the AHAS enzyme. The AHAS LSU is composed of about 670 amino acids varying from species to species.

There are at least 10 mutation sites in the AHAS-encoding gene that has been found to confer herbicide resistance without compromising enzyme activity significantly because of the herbicide binding sites are different from the enzyme active sites (Mazur and Falco, 1989). Most mutations causing amino acid substitutions in any of the three domains are found to be responsible for the reduced sensitivity of AHAS to the imidazolinones. It was later identified that the herbicides containing different classes of active ingredients bind to different, but overlapping sites within the herbicide-binding pocket. Both sulfonylureas and imidazolinones were found to be impeding the binding of the substrate to AHAS by binding sites in the channel where the substrates normally go through. Hence the enzymatic activity of AHAS is directly resistant to normally-inhibitory levels of imidazolinones herbicides. The most common mutations of amino acids substitutions that contribute to the tolerance to the AHAS-inhibiting herbicides are at the positions of Ala122, Pro 197, Ala205, Trp574, and Ser653 of the AHAS

LSU. It was found that plants with mutation at position Ser653 confer tolerance to imidazolinones, but not other classes of AHAS-inhibiting chemical families. A recent attempt to develop the imidazolinones-tolerant barley was done through induced mutation at the Ser653 of the AHAS enzyme, which led to high levels of resistance to the IMIs herbicides, but not to other AHAS inhibitors, such as sulfonylureas and trizolopyrimidines (Moody et al., 2015). Plants with mutation at Trp574 are cross-tolerant to different families of AHAS-inhibiting herbicides and therefore have been more widely used to develop imidazolinones-tolerant crops. Mutations at Ala122 and Ala205 also confer some level of tolerance to the imidazolinones and also were used in some crops. Plants with mutation at Pro197 have good tolerance to sulfonylureas, but low tolerance to imidazolinones, therefore less frequently used for imidazolinones-tolerant crop development. The most common mutations of all the commercialized imidazolinones-tolerant crops are found to be either one or a combination of Ala205, Trp574, and Ser653. The cross tolerance patterns to various AHAS-inhibiting herbicide families from mutations of combinations at different sites of AHAS are often similar across different crops.

Mutation at site Ala122 of AHAS in sugar beet was found to confer resistance to AHAS-inhibiting herbicide imidazolinones, and mutation at Pro197 was reported to confer resistance to sulfonylureas and triazolopyrimidines. Homozygous of the mutation at site Ala122 showed higher tolerance to the herbicides compared to the heterozygotes, which is suggesting the semi-dominant action of the resistance gene (Wright et al., 1998). A naturally occurring mutation in maize was used to develop a variety, ICI 8532IT, that exhibits a high level of resistance to imazethapyr and pyrimidinylthiobenzoates but a relatively low tolerance to sulfonylurea and triazolopyrimidine (Bernasconi et al., 1995). Similar mutation conferring similar tolerance pattern to the chemicals were also found in cotton and was commercialized (Rajasekaran et al., 1996).

The combination of imidazolinone-tolerance traits and imidazolinone herbicides is the basis of the Clearfield production system. Imazamox, imazethapyr, imazapyr and imazapic have been registered for imidazolinone-tolerant crops. Imidazolinone-tolerant sunflower was developed from a naturally occurring mutation at Ala205-to-Val205 in AHAS, which is different from most of other Imidazolinone-tolerant crops developed through mutagenesis. The imidazolinone-tolerant maize was reported to be derived from selections of cell culture after mutagenesis utilizing mutation at Ser653-to-sn653 and Trp574-to-Leu574 in AHAS. Similarly, Imidazolinone-tolerant oilseed rape was derived from mutagenesis of microspores at the same sites of mutation. Trp574-to-Leu574 is currently the only site of mutation that confers high tolerance to all other families of AHAS inhibitors. Imidazolinone-tolerant rice and wheat, on the other hand, were developed via mutagenesis of seeds. Several IMI-tolerant wheat cultivars have been developed and commercialized since 1992 by agriculture research institute in Chile in partnership with the BASF under the name "Clearfield Crops" (Newhouse et al., 1992; BASF, 2010). Several vegetable crops were also reported to possess AHAS-inhibiting herbicides via mutations at different sites in AHAS including lettuce, tomatoes, and tobacco. All of these commercialized imidazolinone-tolerance traits are reported to be semi-dominant and the tolerance level depends on the level of zygosity along with the rates of herbicide. These crops were all able to be launched as Clearfield crops because they are non-GMO and do not contain foreign DNA.

Imazethapyr, one of the AHAS inhibitor in the family of imidazolinone chemical, is the herbicide labeled for use in imidazolinone-resistance rice production (Masson and Webster 2001). Imazethapyr is widely used to control many key weeds, such as barnyard grass, broadleaf signalgrass, and rice flatsedge, especially red rice. Therefore it was used as a screening chemical for mutants resistant to it. Imidazolinone tolerant soybeans are genetically engineered to be

resistant to the ammonium salt of imazethapyr (active ingredient of BASF's herbicide Pursuit) and ammonium salt of imazamox (active ingredient of BASF's herbicide Raptor), and has yet to be deregulated for commercial sale (BASF, 2012). The first imidazolinone-resistance rice was developed in 1993, line 93-AS-3510 (Croughan, 1994), and has been used as donor of imidazolinone resistance for many rice cultivars through conventional breeding since then. The first two commercialized imidazolinone-tolerance rice varieties are 'CL121' and 'CL141' utilizing Clearfield system developed by mutation induced by ethyl sulfonate (Carlson et al., 2002; Lincombe, 2004). New imidazolinone-tolerance rice varieties, 'CL161' and 'Clearfield XL8', were developed from second mutation from a line 'PWC-16' in 2003 (Wenefrida et al. 2004). The segregation of progenies from the crosses using the initial herbicide-resistant mutants suggested the gene controlling the resistance trait is a dominant gene. However, it was found that CL 121 and CL 161 differ greatly in their level of tolerance to imazethapyr. This is due to the level of tolerance in the parents used in developing these two cultivars (Line PWC-16 was known to be eight times more tolerant to line 93-AS-3510) (Wenefrida et al. 2004; Bond and Walker, 2011). 'Puita-Inta-CL' was another rice variety initially developed in Argentina through the artificial mutation, the nontransgenic way that are also resistant to the imidazolinone (IMI-rice) (Livore, 2003). The Puita-Inta-CL has a point mutation of a single amino acid substitution at the Ala-122-Thr, whereas the other IMI-rice lines 93-AS-3510 and PWC-13/PWC-16 have mutations at the Gly-654-Glu, and Ser-653-Asn respectively (Roso et al. 2010). Mutations at different target sites on the same enzyme can confer different levels of imidazolinone herbicides due to conformation changes of the enzyme in each mutation (Avila et al. 2005). These IMI-rice cultivars were reported to increase the rice grain yield by 50% in southern Brazil (Merotto et al., 2016). Puitá INTA CL was reported to be the only cultivar of irrigated rice among the IMI-rice

in Brazil that was not negatively affected by the use of imazamox as the imazamox dose increased (Merotto et al., 2016).

Transgenic approaches were less common in developing imidazolinone-resistant rice, but it was also used to develop AHAS-inhibitor resistance rice via gene targeting technique through Argobacterium-mediated introduction of two point mutations *AHAS* genes into rice (Endo et al., 2007). Recently, a much more precise technique, CRISPR/CasR9-mediated genome targeting was adopted to generate point mutations or even gene replacement in several crops to develop AHAS-inhibiting herbicides resistant lines, including soybean, maize, and rice (Li et al., 2015; Svitashev et al., 2015; Sun et al., 2016).

Since the imidazolinone-resistant rice was developed through chemically induced seed mutagenesis and conventional breeding incorporating resistance to the imidazolinone group of herbicides, and therefore it is not considered to be transgenic thus receiving less regulatory scrutiny than transgenic crops (Gealy et al., 2003). However, scientists have raised the concerns about the escape of the resistance traits from Clearfield rice to the weedy rice. It was therefore predicted that the rice Clearfield technology would last only 8-10 more years due to accelerated evolution of herbicide resistance in weedy rice from gene flow (Sudianto et al., 2013). Moreover, some residual activity occured in the soil where there were frequent applications of imazethapyr and imazapic in Brazil (Kraemer et al., 2009). The residual activity was injurious to non-tolerant rotation crops, such as soybeans, corn, and sorghum. Therefore the discovery of new genes conferring resistance to the herbicide and the technology allowing quick introduction into elite varieties becomes increasingly important.

Glufosinate and glyphosate resistant rice were mostly developed through transgenic technologies and convey resistance to these broad-spectrum, non-selective herbicides. Both glufosinate-resistant and glyphosate-resistant transgenic rice have not been approved for

commercial use (Kumar et al., 2008  Demont et al., 2009).  Other than the three existing rice systems that have been used for decades since development, BASF is likely to launch a new rice system in 2017, Provisia™ Rice, which is proposed to provide a new tool for post-emergence control of broad range of grass weeds, including AHAS-resistant grasses, weedy rice, and red rice. The Provisia™ Rice was developed through natural selection and traditional breeding using ACCase inhibitor herbicides as selection tools. Provisia™ Rice is not a GMO technique; therefore, it can be used by the rice growers to rotate with the Clearfield system in the US, which reduces the risk of developing weeds resistant to a specific type of herbicide.

2.5.1 Uses of herbicide tolerance in hybrid crops

Hybrid crops of any kind involve male sterile plants as female parents and its pollinators, and the seeds that are harvested off of female plants are the final products to sell.

Herbicide was not only applied in the production field to control the weeds, but was also used in combination of the herbicide resistance/susceptibility in crops as a method to simplify the production procedure, lower the cost and labor, increase the yield, and improve the seed purity. The strategy of combining herbicide and utilizing herbicide resistance gene in crops to control the purity of hybrid seed production was first proposed by Yan in 2000 (US Patent 6066779). The differences in the responses of different lines in a hybrid system can be viewed as phenotypic marker.  The most predominant genetic mechanisms controlling the herbicide resistance/susceptibility are either the utilization of recessive sensitivity genes or the dominant resistance genes of the crops to the herbicides. Recessive sensitivity genes are usually screened for or introduced into the line that ought to be removed with corresponding herbicide, while the plants with dominant resistance genes are designed to be kept for harvesting after the application of corresponding herbicides. In most of the hybrid crop systems, usually the male pollinator, the

fertile /partially fertile female parents, or any other genetic off-type are the targets to be precluded in the field. The male-sterile female lines are the plants to be kept and harvested.

The use of the different levels of herbicide tolerance/susceptibility in the hybrid production systems generates several benefits that supposedly lead to cost reduction and increase the yield and purity. Mixed sowing the female and male seeds is one of the biggest differences that is convenient for growers compared to conventional strip planting. Advantages from this seed mixture includes: 1. Increase in the efficiency of pollination since there is less space in between parents, which further allows the increase the ratio of female : male plants which facilitates outcrossing resulting in higher seed sets and yield. It's even more substantial for self-pollinating crops 2. Allowing mechanical harvesting after application of herbicides after unwanted plants are killed. 3. Increases the purity of the seeds by chemically excluding contamination from various possible sources depending on the design of the system of which parents are resistant. All of mentioned can greatly increase the profits and decrease the invested cost and time.

It is thus crucial to determine whether to have one, two, or even three parents carrying genes conferring herbicide resistance traits in a hybrid system depending on the need from the growers and the level of weed infestation in the area.

It was reported the use of triazine resistance in the hybrid seed production in canola CMS hybrid system to eliminate the off-types in the hybrid seed production field (Conner and Christey, 1997). The transfer of the herbicide resistance was through somatic hybridization of protoplasts, which allows the regenerated cells carrying new combination of mitochondria and chloroplasts that is normally not allowed through conventional sexual hybridization due to the usual maternal inheritance of organelles (Conner and Meredith, 1989). This technique allows the chloroplast encoding for herbicide resistance and mitochondria encoding for CMS in one

hybrid, so the regenerated canola plants are thus male-sterile with resistance to herbicide and to be utilized as female plants in the canola hybrid production system. Randomly mixed planting of the female and male seeds were performed in the production field to allow more efficient transfer of the pollens. The male pollinators are removed by application of herbicide before the harvesting of seeds on the female plants since only male plants or other possible off-types are susceptible to the herbicide, and thus all the non-hybrid seeds are said eliminated. The same application of herbicide can also be applied to the next generation of F1 hybrid to remove any non-hybrid off-types, which also shows the inheritability of the triazine resistance.

2.5.2 Uses of herbicide tolerance in hybrid rice

The conventional protocol of hybrid seed production for either two-line or three-line system was established almost 40 years ago, that is female plants and male plants are planted in separate rows with ratios about (2 to 3 male): (8 to 10 female) repeatedly grown. There's usually about 20 cm space between each female rows and the adjacent male rows. Roguing to remove fertile male plants in the female rows is required to maintain the sterility system. This process requires experienced technician or breeders to correctly identify the offtypes. Supplementary pollination is required for hybrid seed production unless natural wind is constantly blowing 16 to 32 km/h. The major pollination tool in both systems to increase the outcrossing rate on the female plants is the application of $GA_3$ at about 30% heading of female plants and 10% of male plants. $GA_3$ as mentioned earlier helps with panicle exertion because plants with WA cytoplasm system are known to have poor or incomplete panicle exertions. $GA_3$ not only helps with panicle exertion on both male and female plants, but also increase the duration of flowering time, widens the flag leaf angle, and improve the stigma exertion and its receptivity on female plants. Other supplementary pollination methods include shaking the male plants using bamboos, ropes, sticks or even helicopter. This operation has to be done at least 3-4 times a day when florets open

during the peak of anthesis for 6- 10 days. The harvesting and threshing of the hybrid seed using this system is critical. Male plants have to be carefully cut and removed completely from the production field to prevent harvest of pollinator seeds with the hybrid seeds.

The purity and volume of hybrid seed is often the limiting factor of profit in hybrid rice production. Potentially good hybrids with high heterosis which can be difficult to produce and limited due to either purity or hybrid seed production volume. Therefore the incorporation of the herbicide resistance technique into hybrid rice production systems allows the simultaneous improvement of profits and reduction of the cost of production which is why it has a growing demand among customers. Utilization of herbicide resistance engineering as a strategy to provide efficient means of controlling purity in the production of hybrid seeds was first proposed by Yan (Yan, 2000). Since then, the hybrid rice production has been extensively studied to integrate the herbicide resistance to increase the purity and reduce the labor and cost. However, there are currently only a limited number of herbicide-tolerant hybrids available commercially. The need for developing herbicide-tolerant hybrid rice is continuously growing because it would allow growers not only to easily manage the weeds and seed purity, but retain other agronomically desirable characteristics and yield potential within hybrid rice. Backcross breeding has been a method of transferring target trait controlled by favorable allele from a donor line into the recipient elite line. However, there are two major disadvantages of this method: 1. The amount of time may be too long for a highly converted isogenic line. See the table below for the assumed percentages of homozygosity and the recurrent parent genome for each backcross generation; 2. The linkage drag may cause the simultaneous transfer of other genes closely linked with the target gene. Therefore breeders usually don't have direct control over the recombination rate or the actual size of the transferred gene through this backcross breeding method. The number of backcrosses depends on the desired level of homozygosity or

the percentage of the recurrent parent genome for different purpose (Semgan et al., 2006). As homozygosity increases through backcrossing, so does the contribution of the recurrent parent to the offspring's genome (Table 1).

| BC Generation | Homozygosity | Recurrent Parent Genome |
|---|---|---|
| | % | % |
| $BC_1F_1$ | 50.00 | 75.00 |
| $BC_2F_1$ | 75.00 | 87.50 |
| $BC_3F_1$ | 87.50 | 93.75 |
| $BC_4F_1$ | 93.75 | 96.87 |
| $BC_5F_1$ | 96.87 | 98.44 |
| $BC_6F_1$ | 98.44 | 99.22 |
| $BC_7F_1$ | 99.22 | 99.61 |

**Table 1. The theoretical percentages of homozygosity and recurrent parent genome with every generation of backcrossing.**

The use of herbicide resisatnce in hybrid rice can be discussed in different systems and the use of parental lines carrying herbicide resisatnce traits. In the three-line system, many researcher were introduced herbicide resistance gene from only restorer.

*Bar gene* was used to transform into some restorer lines for the CMS three-line systems, such as Minghui63 (Xue et al., 1998), R752 (Zhang et al., 2000, Rao et al., 2003), T461 and R402 (Rao et al., 2003), Jingyin119 (Zhu et al., 1996) Jingdao162 and Jingdao18 (Shi et al., 2004), and H84 (Wang et al., 2007). Most of the existing restorer lines are from indica varieties, while Jingyin119, Jingdao162, Jingdao18, and H84 are transformation from Japonica varieties. *Japonica* varieties are known to have much higher transformation efficiency than indica varieties.

Therefore most of the transgenic indica varieties are developed from continuous backcrosses of an elite *indica* line that was used in a cross with another transformed japonica line. Minghui63, Ce64 and Teqing (Li et al., 2000), and Milyang46 (Xue et al., 2001), R402 (Zhong et al., 2000) are all famous transformed restorer lines used in the three-line systems.

Development of herbicide resistance hybrid rice via transgenic was evaluated in regional trials under control in China since 2000 (Li et al., 2000, Xue et al., 2001, Xiong et al., 2004, Xiong et al., 2007), such as hybrid Xiang125S/Bar68-1 using the herbicide resistance restorer line, Bar68 -1 (Xiao and Yuan 2007). Bar68-1 was further used in other restorer line breeding and another line, Bar9311 was later developed from the pedigree selection. The resulting hybrid using this restorer line, Pei'ai64S/ Bar9311, 244S/ Bar9311, and 81S/ Bar9311 were evaluated in 2004 and 2006 in China, and all of them were reported to possess strong vigor, equal yield to the checks and resistance to the herbicides.

Recent studies used an elite R-line, indica Rice 93-11, treated with irradiation to screen and develop a library of mutant lines resistant to bentazon while still possessing desirable agronomic traits. Gene *CYP81A6*, which has been patented in 2005, was responsible for the resistance in these mutant lines. Genetic study of the segregation of wildtype and mutant plants is 3:1 suggesting this trait is controlled by a single recessive gene. This library of mutation can then serve as germplasm of elite R-line breeding in the three-line system (Tuan et al., 2015).

There are other researchers who developed herbicide resisatnce lines to serve as female parent of a hybrid.

*Bar gene* was used to transform into some CMS lines, such as II32A and LongtepuA. The sterility of these two lines was, however, reported to be unstable under abnormally high temperature, which could also lead to a decrease in hybrid seed purity.

05Z221A and 05Z227A are two CMS lines developed by the transformation of *Bar* gene and continuous backcrossing into the corresponding maintainer lines derived from FengyuanB/Bar68-1. The sterility of the CMS lines needs to be evaluated and confirmed to be completely sterile in every backcross generation before proceeding (Xiao, 2007). In recent years, a few herbicide-tolerant rice varieties and hybrids have been successfully introduced to the market. See U.S. Patent Nos. 5545822; 5736629; 5773703; 5773704; 5952553; 6274796, US8598080 (CL131), US8841525 (CL111); and 6,943,280. The published International Patent Applications include WO 00/27182, WO 01/85970, WO2007032807 A2 (CL131), WO2011044315A1 (CL111), etc. These herbicide-tolerant rice plants are resistant to or tolerant of herbicides that normally inhibit the growth of rice plants.

Another application of herbicide susceptibility/resistance in the three-line hybrid production system is again utilizing genes causing lethality to bentazone. Researchers transferred it into an existing B-line (B) to create an isogenic line (B') that's only different from the old B-line in the gene that's responsible for the lethality to the bentazone. Crosses between regular A-line and B-line (AxB) is still done to produce more A-line seeds, however AxB' still used to produce A-line seeds for AxR hybrid seed production. Since B and B' are isogenic lines differing by only one gene, the hybrids produced using A-lines from AxB or AxB' should have similar heterosis. The advantage of this method is to maintain the seed purity in the parental seed production step by spraying bentazone in the AxB' field to remove all the bentazone susceptible B-line plants that could self and mixed in the A-lines seeds (Zhu et al., 2002).

Herbicide resistance gene in both female and restorer has more limitations but it allows the growers to apply herbicide in the seed production field.

Atrazine is a slightly water-soluble triazine herbicide (6-chlro-N2-ethyl-N4-isopropyl-1,3,5-triazine-2,4,-di-amine) that is most commonly used in the sorghum, corn, and sugarcane

production (Udikovic-Kolic et al.,2012). A gene, *atzA* (*atrazine chlorohydrolase gene*), discovered and isolated from a soil bacterium *Pseudomonas* ADP was identified to encode for atrazine chlorohydrolase (Cai et al., 2003). A recent study indicated that transgenic *atzA japonica* rice lines showed resistance to the atrazine herbicide, specifically during germination and young seedlings stages. The transgenic lines include maintainers (Jindao7 and Jindao8) and a restorer (Jinhui3), therefore they were proposed to serve as parental lines in the three-line system. However the transgenic plants were only tested for resistance at germination and seedling stages, but not at reproductive stages, which is critical for utilizing mechanical harvesting. Almost all of the transgenic lines produced larger seedlings with similar or higher germination rate, taller, and higher total chlorophyll content than conventional control in the presence of atrazine (Zhang et al., 2014). The differences in the level of resistance in the F1 hybrid between using only one parent carrying the *atzA* gene or both parents carrying two *atzA* genes were also not determined. Therefore the actual application of this new gene in the 3-line hybrid rice production system will need more investigation in the future.

Other than the three-line system, two-line system is widely used to incorporate the herbicide tolerance into the hybrid through the development of parental lines. As mentioned, the two-line system has its own risk of instability of P/TGMS lines that can cause sterility fluctuation during the hybrid seed production. Using the herbicide-resistance trait was therefore investigated extensively since the discovery of the use of herbicide resistance in the two-line system to mainly minimize this potential yield loss due to seed impurity.

The introduction of herbicide tolerance into the hybrid solely via restoere lines in a two-line system includes many types of herbicide systems. Various herbicide resistance genes, such as *BAR*, *EPSPS*, *CPS*, and *AHAS*, were used in the development of restorer lines in the two-line system. The restorer lines and the hybrids then acquire the trait of herbicide resistance while the

female plants are susceptible to the same herbicides. This technique is used to allow the producers to kill all the plants selfed from P/TGMS plants in the $F_1$ hybrid field to increase the seed purity.

The herbicide resistant restorers, R187 and Xiushui04, in the two-line hybrid rice were successfully developed through transgenic approach by *Agrobacterium*-mediated transformation of *Bar* gene (Hu et al., 2000). D68 and E32 were developed through particle bombardment of *Bar* gene and pollen-tube pathway method, respectively (Xiao et al., 2007; Wang et al., 2004). 9311 was another indica type restorer line in the two-line hybrid system that's widely used (Wang et al., 2002).

There are more examples of incorporating herbicide tolerance traits into the femal lines than the restoere lines in the two-line system. The advantage of doing so is to allow the producers to reduce the cost of planting by mixing the male and female plants when sowing. Male plants can be removed simply by applying herbicides to kill them before harvesting. Mechanical harvesting of hybrid seeds on the female was carried out after all the male plants were killed. This technique is made possible by transferring the herbicide resistance gene in to the female line only (Xiao 1997; Kim et al., 2007).

*Bar* gene has been widely used in developing herbicide resistant lines in the two-line system resistant to herbicide glufosinate ammonium. The marker for the bar gene has ever since been used as an efficient tool to select for genetically modified crops (Miki and McHugh, 2004) and some crops pertaining the transformed bar gene were even commercialized in the US (Reddy et al., 2011). In rice, the *Bar* gene was transformed into a popular indica P/TGMS line, Pai'ai64, which was used in many two-line hybrid combinations with various elite restorer lines, such as Pai'ai64/Teqing, Pai'ai64/9311 (Fu et al., 2001). Kim et al. also transferred the *Bar* gene via *Agrobacterium* transformation into an elite PGMS line, 920S and develop a herbicide resistant

PGMS line, YA3530ms, in 2007. E coli strain DH10B was used as recipient of pCAMBIA3300 vector containing the *Bar* gene. Vector construction, rice transformation, and plantlets regeneration procedures were from publication by Lee (1999). A conventional pedigree breeding technique was used after successful transformation of the initial transgenic lines to include good agronomic characteristics. The seeds from the resulting herbicide resistance PGMS line, YA3530ms, are then mixed with seeds from the pollen plants with a ratio of 4:1 in weight to produce hybrid seeds. Herbicide was sprayed after the pollination to remove the pollinator plants, and harvest seeds only from the female plants YA3530ms.

Another gene, *EPSPS*, was also utilized for another herbicide resistance system into a japonica PGMS rice line to develop 7001S via the transformation through *Agrobacterium*. This line was later used in various superior hybrid combinations (Peng et al., 2008; Deng 2008, Deng et al., 2008).

The possibility of developing resistance to specific herbicide in weeds due to continuous use of a single herbicide has been a concern among agronomist for many years (Neve, 2007; Kuk et al., 2008; Heap, 2012). For instance, barnyard grass in rice fields was found to possess remarkable resistance to several herbicides, especially selective ones, such as propanil, molinate, thiobencarb, and etc (Fischer et al., 2000). On the other hand, non-selective herbicides seem to be difficult for plants to induce resistance since almost all plants are susceptible to them. Both glyphosate and glufosinate have been used in weed control for more than 40 years, and it is reported that only a few of weeds have resistance to glyphosate, and no reports finding resistance in weeds to glufosinate (Nandula et al., 2005). Moreover, it is logical to believe the alternative use of two or more herbicides should lower the chance of developing herbicide resistance in weeds compared to using a single herbicide. Therefore, this same group of researchers have developed a PGMS line tolerating two types of herbicides, glyphosate and glufosinate, by

transferring two genes with optimized codon, *EPSPS* and *Bar* genes, into the 7001S (a japonica PGMS line) to develop a new transgenic line, EB7001S (Della et al., 1986; Deng et al., 2014). The *EPSPS* gene from bacteria only expressed and accumulated in the cytoplasm leading to EPSPS protein transcribed in the chloroplast in the plant cell, resulting in the transformed plants resisting 3.332g glyphosate 2.7 times higher than the suggested rate by Monsanto (Barry et al., 1997). The hybrid generated from this PGMS line can therefore tolerate both glufosinate and glyphosate allowing the growers to control various weeds in the rice fields with lesser chance of developing herbicide resistance in the weeds (Deng et al., 2014).

EB7001S was proposed to serve as female in the two-line hybrid production system, mixed-grown with the herbicide-sensitive restorer line. Restorers can therefore be removed by application of herbicides after pollination to reduce the labor cost and possibilities of contaminating from the restorer seeds when harvesting. The F1 hybrids can also be applied with the corresponding herbicides due to the dominant inheritance pattern of the resistance genes from the female, EB7001S. A similar idea has been applied to develop a PGMS rice line that is resistant to both striped stem borer and glufosinate herbicide by introducing two genes, *Cry2A* and *Bar*, after codon optimization based on preferred codon in rice and transferred into a PGMS line, 4008S, via *Agrobacterium* transfer method. Again the developed line was also proposed to serve as a potential female germplasm in future two-line hybrid production combining resistance to both striped stem borer and glufosinate herbicide (Weng et al., 2014).

Unlike dominant resistance to the herbicides, which is more widely adapted in different crops systems, recessive sensitivity to herbicides was relatively rare, but was also used as a tool to be incorporated into crop protection. A herbicide sensitive mutant developed through radiation was first found to be lethal to bentazon, while it is bentazon was usually safe with normal rate on most graminaceous crops (Mori et al., 1984). Bentazone, an active ingredient of

herbicides such as Basagran and Bentazone, is used in rice production fields to kill weeds by inhibiting photosynthesis. A similar mutation found in maize also was sensitive to bentazon (Green et al., 1999).

The initial utilization of herbicide lethally sensitivity technology in the three-line hybrid rice production system started in 1984. Herbicide lethality was incorporated into R-lines instead of introduction of herbicide resistance in the A-lines. 'Norin 8 m', a mutant version of a japonica rice variety 'Norin 8', was found to be lethal to bentazone after screening of the induced mutants due to a recessive gene, *bls*. Mutated R-line consisting recessive homozygous of this gene in the three-line systems allows the growers to blend the A-line seeds with the R-line seeds and to be planted and transplanted together. The field was sprayed with bentazone herbicide after pollination to kill all the male plants in order to bulk harvest the A-line plants bearing all the hybrid seeds. The advantage of this technology is of the reduction of required labor to produce hybrid seed, as well as the increase of hybrid seed yield and purity. The limitation of this method is that the R-line and A-line have to have similar growth durations for synchronization of flowering time. Norin 8 m was therefore used as a donor plant for the lethality to bentazone to develop different R-lines with various durations to synchronize with various A-lines to create different hybrids. The genes responsible for the susceptibility to bentazone were mapped in rice and had been used as molecular marker to select for. In a study in 2004, this system with lethality to bentazone in R-lines was proven to outyield 42% more hybrid seeds than the conventional way of production, and 25% of which was from a higher seed set rate (Zhu et al., 2002).

In the two-line hybrid system, this lethality was later identified to be controlled by a single recessive gene, *bel*, found in a mutated PGMS line M8077S (Maruyama et al., 1991). M8077S carrying homozygous recessive *bel* was found to be lethally sensitive to bentazone and

sulfonylurea. This characteristic was used in a two-line hybrid production system to eliminate the selfed seeds of TGMS by spraying bentazone and sulfonylurea in the hybrid seed field since the real F1 generated from this TGMS with a normal male parent will be insensitive to the herbicide. A microsatellite marker was also found to be closely linked to this gene that can served as marker in the MAS breeding in the future. This method was proposed to solve the impurity problem caused by sterility instability of TGMS in the two-line hybrid system (Zhu et al, 2002). However the concentration of herbicide applied at different timing of rice stages is critical to determine the responses of the treated rice. If the concentration of herbicide is below the critical point, it will not cause damage to the self-pollinated PGMS plants mixed in the hybrid field, and if the herbicide concentration is higher than suggested, it may also cause damage on the actual hybrids. The previous scenario might be hard to detect unless the PGMS plants have obvious different phenotype than the hybrids for regular growers to identify. Otherwise not being able to kill any plants in the two-line hybrid seed field will only suggest that a seed lot has total purity to growers. The sensitivity of M8077S was also suggested to be decreased with other herbicide, such as Londax with sulfonylurea as the active ingredient. Moreover, the application of bentazon will only workwith the correct concentration, on the selfed PGMS lines, but may not work on other potential offtypes or other graminaceous weeds. Therefore the proposed use of the *bel* gene as insurance for purity of hybrid seeds still has its own challenges.

It was later found that the locus of *bel* in 8077S and *bsl* for Norin8m are allelic to each other and both mutants were due to single-base deletion. The two loci are renamed bel(a) and bel(b) relatively. The wild-type *Bel* gene encodes a novel cytochrome P450 monooxgenase, named CYP81A6, and the gene responsible for the tolerance was patented (Pan et al., 2006; EP1900817 B1).

# 3. MATERIALS AND METHODS

## *3.1. Research objectives*

### 3.1.1 Objective 1 – Development of the isogenic HT A'-line / B'-line

A1/B1 and R7 are the parents of an elite hybrid in our breeding program. A1 has good agronomic traits and most importantly it has the floral characteristics to be a good female in the hybrid seed production. It has good general combining ability (GCA), large stigma, high exertion rate (48-65%), and as a result a high out crossing rate. The complete sterility of A1 is also maintained in a stable state by B1, and it can be restored to 80-90% of fertility by the restorer parent R7.

We used this elite A/B lines (A1/B1) that has been tested before and known to produce a hybrid with the R-line (R7) with good heterosis in the $F_1$. The IMI herbicide resistance plant, Puita-Inta-CL, was used as a donor the glutamine resistance trait to cross with the B1 to obtain the HT1-line. Puita-Inta-CL contains the *INTA* gene with point mutation at the Ala122-to-Thr122 on the larger subunit of AHAS enzyme that contributes to the tolerance to the AHAS-inhibition herbicides. The inheritance of the herbicide resistance was validated in the progeny by spraying the NewPath herbicides at normally lethal rates and only the resistance plants were selected by simultaneously killing the susceptible ones. Without the markers availablem, four generations of conversion backcrosses with B1 were conducted to increase the B1 background genetic contribution. Validation of herbicide tolerance was done again by spraying NewPath at each backcrossing generation. Confirmation testcrosses were done using A1 at each generation of backcrosses. The sterility of the tested $F_1$ plants was tested by standard procedure of pollen staining with 1% iodine $I_2$-KI solution. Only plants that showed irregular shapes of pollen grains, and less than 0.01% of the pollens stained were considered as perfect maintainers. Panicles on

the same plants at each backcross generations were bagged to insure that there was zero seed set due to self-pollination.

Finally, selection was performed in field conditions based upon phenotypic ratings for the best line from all $HT_1$-$BC_4$ that met all the requirements as the derived converted B'-line (7019B). The development of the corresponding CMS line using the developed maintainer line is normally done through the standard procedure of repeated backcrossing the newly developed maintainer line to a known male sterile line with CMS cytoplasm for generations until the new CMS line has the same nuclear genome as the maintainer line and stable male sterility (Chen et al., 2011; Wei et al., 2012). In our proposed method, there would be no need to transfer the CMS cytoplasm to the 7019B to create its corresponding isogenic A'-line (7019A) if there were no attempt to compare the traditional methods of incorporating the GOI into the hybrid utilizing the CMS-converted A'-line, which usually takes 2-3 years to complete. This is one of the biggest advantages of this proposed method of incorporating GOI into the hybrid in a more efficient way. However, the development of the CMS-converted A'-line was needed in order to make proper comparisons. To develop the CMS line of the 7019B, the CMS were transferred from A1 with four generations of cytoplasm backcrosses (CBC). The sterility was validated by the same procedures of pollen staining with 1% iodine $I_2$-KI solution and bagging of panicle to ensure zero seed set from self-pollination to develop the corresponding CMS-BC4 line, A'-line (7019A). Herbicides were applied at each backcross population to rogue for non-HT plants before selecting for desired plants to advance with further backcrosses. Restorer- and heterosis-testcrosses (HTC) were done using 7019A with R7 and CFR7, which were generated from efforts detailed in the objective 2. This was to test the fertility of the F1 hybrid and verify that it could be fully restored and to compare the heterosis with the hybrid generated from the original parents, A1 and R7.

The most novel aspect of this approach is using the 7019B as a donor of herbicide resistance when producing female seeds for hybrid seed production by crossing 7019B with A1 only at the last step of basic seed production. This novel hybrid production system is referred to as a three-way $F_1$ hybrid production system because it comprises three parents in the hybrid, in contrast to the conventional two-way system using only A1/B1 and the R-line as the two parents of the hybrid.

### 3.1.2 Objective 2- Development of the isogenic HT R'-line

The elite R-line (R7) was crossed with the donor parent, Puita-Inta-CL, which is a glutamine herbicide resistant plant, to obtain the HT1-line. Inheritance of herbicide tolerance in progenies was validated by spraying the herbicides and selecting for resistance plants. Four generations of repeated backcrosses with R7 were made with a validation of herbicide tolerance at each generation of backcrossing to simultaneously remove susceptible plants. Heterosis testcrosses with A1 were made and the fertility of the resulting $F_1$ plants were tested to confirm the restorability using the pollen staining protocol with 1% iodine $I_2$-KI solution. Only parents with round-shaped pollen grains higher than 85% stained were considered a suitable restorer candidate. Bagging panicles on the rest of the panicles on the same plant was done to make sure plants had higher than 85% seed set at each backcross generation to further validate perfect restorability of the plants selected for advancement. Finally, selections were made in the field for a best-line based upon phenotyping from HT1-$BC_4$ that met all the requirements as the derived converted R'-line (CFR7). Heterosis testcrosses were made using the candidate plants, CFR7, with the A1 and 7019A, and the HTC $F_1$s were planted in the field to ensure the presence of the heterosis within the F1 and the restorability of the CFR7.

### 3.1.3 Objective 3- Seed production ability of various treatments in ESP

The third objective was to compare hybrid seed produce ability of various treatments in the ESP to look at the actual feasibility of our proposed method. The treatments are referred to six different methods composed of six different combinations of (Females x Males) that incorporate the herbicide tolerance (HT) into the hybrid seed production systems, including the use of HT A'-line (7019A), non-HT A-line (A1), or hemizygous A-line (A1/7019B) with HT R'-line (CFR7) or non-HT R-line (R7). Our proposed new method is treatment 4. The hypothesis of the objective 3 is that the treatment 4 does not have significantly lower seed set yield and negative effects on the important agronomic traits compared to the control and other treatments (Fig 1).

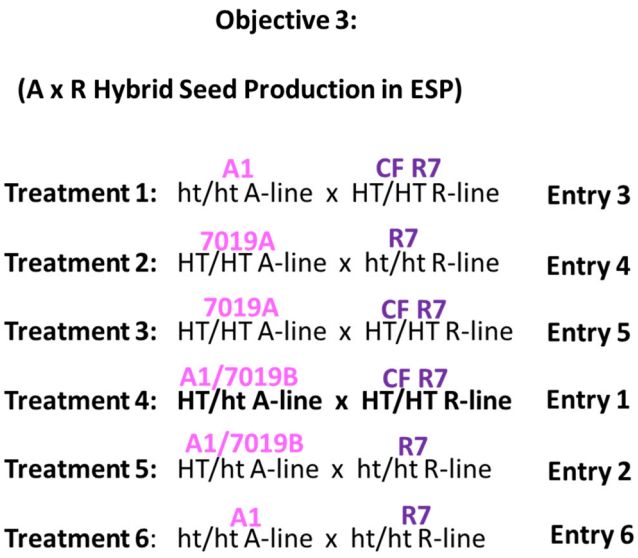


**Figure 1. Combinations of A-lines and R-lines used in each treatment in Objective 3 ESP, and the corresponding status of the HT gene.**

Treatment 1 is the most common method of seed production incorporating HT into the F1 hybrid seeds. It is effective because all F1 seed produced will be herbicide tolerant with the presence of one copy of HT gene in heterozygous state in the F1 hybrid supplied by the Restorer.

Treatment 2 is another method of seed production. It is less frequently utilized because it involves backcrossing the trait onto the B-line (maintainer) and the converting the maintainer into an A-line (female). The F1 hybrid is also expected to be tolerant to herbicide carrying one copy of HT gene inherited from the female parent.

Treatment 3 is coveted by seed producers because both the A'-line (7019A) and the R'-line (CFR7) have the HT gene in homozygous status and therefore the production field can be sprayed with the herbicide the system utilized.

Treatment 4 is a new method of seed production summarized by this thesis. The incorporation of the herbicide tolerance into the female seeds to be used in the ESP will only occur at the last step of foundation seed production by crossing the original A-line (A1) with the HT B'-line (7019B) to produce female seeds in a hemizygous state at the locus of GOI (A1/7019B). The incorporation of the herbicide tolerance will occur by crossing the HT R'-line (CFR7) with A1/7019B in the hybrid seed production.

Treatment 5 serves as a control using non-HT restore line that is known to have good combinability and heterosis with the female used, A1/7019B, comparing all the measurements with our proposed method in treatment 4 using isogenic HT R'-line (CFR7) as the restorer. It also can show outcrossing of the F1 female using two different males.

Treatment 6 serves as base control using both non-HT female and non HT-restorer identified by molecular markers as near iso-lines to the HT females and HT restorers. This hybrid combination was tested earlier and demonstrated good heterosis for yield.

Entry numbers (1 to 6) assigned to each of the hybrid combinations in the ESP were used in the statistical analysis instead of the treatment numbers.

Measurements on both female A-line plants and male R-line plants included important agronomic traits, such as plant height (cm), average tiller numbers per plant, days to 50% heading, days to $GA_3$ application (10% heading of the later heading parent), lodging rating (1-5; 1: no lodging, 5: flat on ground), phenotype (1-7; 1: poor, 7: excellent), moisture content of both R-lines, seed set on A-lines when harvested (%), and major pest or disease responses (1-5; 1: not affected, 5: severely infested). Measurements on only male plants included the following three floret characteristics that are most often used to determine a good pollinator: anther size (1-5; 1: small, 5: large), floret opening angle (1-5; 1: narrow, 5: wide), and the average of anther exertion

rate (%). Measurements on female A-line plants were related to floret characteristics that are often used to determine females with good outcrossing, including average stigma size (1-5; 1: small, 5: large), seed set rate (%), and eventually seed set yield (kg/ha). The seed set rate measures the number of filled grains per panicle, also known as receptivity, outcrossing rate (%), or spikelet fertility (%). The average of the seed set rate (%) from randomly selected 30 A-line panicles within the same ESP block has been used as an indicator of stigma viability (Li et al., 2008).

Physical comparisons of the agronomic traits and floret characteristics as pollinator were made between R7 and CFR7. Comparisons of final seed set between treatment 1 and treatment 6 (both use A1 as female), between treatment 2 and treatment 3 (both use 7019A as female), and between treatment 4 and treatment 5 (both use A1/7019B as female) allowed us to assess if there is any negative effect of incorporating the HT trait into the pollinators.

Heterosis has been widely utilized to increase the yield in hybrid rice by improving the performance of various yield-related traits in the $F_1$ hybrids. However, the floret characteristics, such as stigma size, stigma exertion rate, stigma receptivity, or stigma viability, have received very little attention because it is not directly related to the yield. Moreover, numerous studies have been suggesting the floret and stigma characteristics are complex quantitative traits controlled by many major and minor QTL (Li et al., 2001; Li et al., 2003; Yamamoto et al., 2003; Yu et al., 2006; Deng et al., 2009; Li et al., 2014). In the ESP of objective 3, comparison of all the aforementioned measurements of female characteristics and the number of tillers on female plants between treatment 2, 5, and 6 (all have the same pollinator R7, but different females) and between treatment 1, 3, and 4 (all have the same pollinator CFR7, but different females) would serve as a way to address if there is any positive heterosis effect on the receptivity of the stigma of A1/7019B and/or number of tillers. These measurements would suggest the existence of

heterosis in the A1/7019B could be validated by comparing final seed set yields. If the heterosis is proven to be expressed in the traits related to hybrid seed production, it could be stated as one of the advantages for this new seed production method that could largely reduce the cost of seed production.

3.1.4 Objective 4- Comparison of yields and quality of various treatments

The fourth objective is to compare the final yields of all the hybrids and control varieties in the yield trials at two locations in the two consecutive years for various treatments. The six hybrid types as specific outcomes from objective 3, one conventional Clearfield hybrid (XL745), two conventional Clearfield inbred varieties (CL131 and CL151) and the converted HT R'-line (CFR7) were the ten treatments entries in the model of Objective 4. Entry numbers (1 to 6) assigned to the iso-hybrids were used in the statistical analysis instead of the treatment numbers. The hypothesis in the Objectvie4 is that the treatment 4 (A1/7019B//CFR7) does not have significantly lower yield and negative effects on the important agronomic traits compared to the control and the iso-hybrids from other treatments (Fig 2).

Corresponding herbicides (NewPath$^{TM}$) were applied twice at suggested rate (4 oz/acre = 280.2 g/ha) to each of the treatments of $F_1$ hybrid trial possessing HT genes at homozygous or heterozygous states and did not segregate for non-tolerant plants for validation of the inheritance of the trait (treatments 1 to 4). Herbicide was also applied to the adjacent plot without any HT genes to serve as control to prove the effectiveness of the herbicide. Estimates of the levels of tolerance of hybrids with different states of HT genes at its locus were based on measurements 10 days following the 2$^{nd}$ herbicide application, including germination (1-5), plant stand establishment (1-5), seedling vigor (1-5), and phytotoxic effects due to the corresponding herbicide (1-5). More measurements were conducted to compare the $F_1$ performances generated from various treatments, including number of tillers, 50% flowering (days), major disease

response (1-5), lodging (1-5), plant heights (cm), phenotype rating (1-7), and final grain yields (kg/ha). NewPath herbicide was applied to the ratoon in every trial at 3X as suggested rate after harvest.

Grain quality was compared by measurements in seed sizes (length, width, and the length-to-width ratio), seed appearances ratings (color, translucency, and chalk %), head rice recovery (%), milling rice yield (%), and 1000 grain weights (g) for each treatment.

Comparisons of the yield and important agronomic trait performances between treatments 1 and treatment 6, and between treatment 2 and treatment 6 served as evidence of the similar or better heterosis in the $F_1$ hybrid to the original hybrid (treatment 6) that neither the incorporation of HT gene into $F_1$ from isogenic A'-line (7019A) nor isogenic R'-line (CFR7) will affect the heterosis and yields. In this case, the resistance to the herbicide will be a benefit and the only major difference between the three treatments.

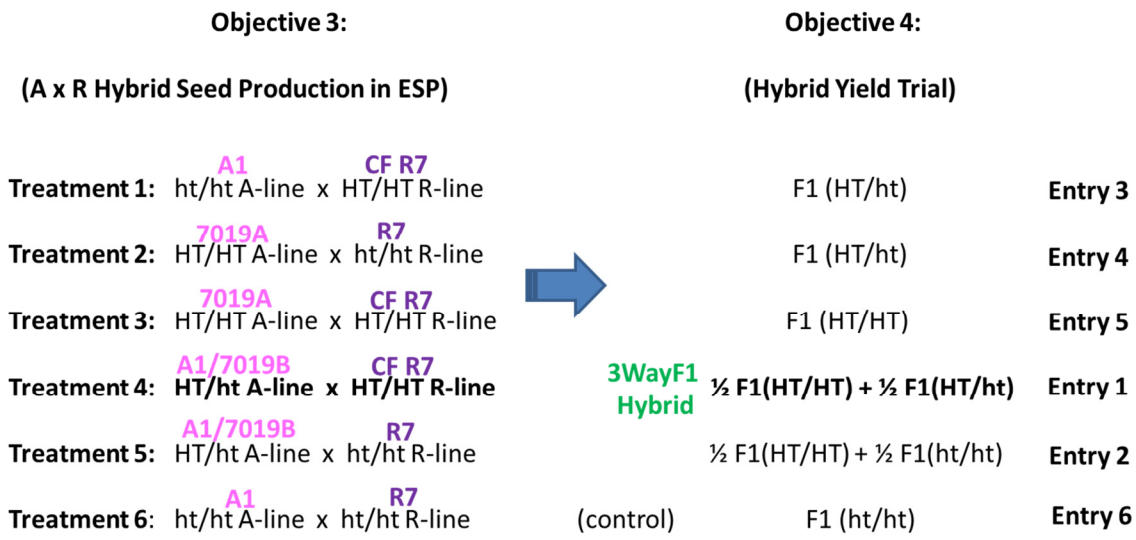


**Figure 2. Combinations of A-lines and R-lines used in each treatment in Objective 3 ESP, and the resulting hybrids tested in the yield trial for objective 4 with corresponding status of the HT gene.**

Comparisons made between treatments 1 and 2 (both have one copy of HT gene), treatment 3 (has two copies of HT genes), helped to answer if there are any positive or negative effects on grain yields or tolerance to the herbicide due to the copy number of the HT genes. Treatment 4 compared to treatment 3 showed if any yield penalty or gain is observed when ½ the population is homozygous and ½ is heterozygous for the HT gene. Treatment 5 versus treatment 3 also provided insight to the effect of HT percentages using a heterozygous HT female versus a homozygous HT female. Comparisons between treatment 4 with treatments 1 & 3 and comparisons between treatment 5 with treatments 2 & 6 showed if there are any yield advantages from including the 3$^{rd}$ parent in the 3Way$F_1$ hybrids. If the yields are higher, and the grain quality or other important agronomic traits are better than the iso-hybrids from other

treatments, there is a possible positive heterosis by incorporating the 3rd parent into the hybrid, which we called it 3way$F_1$ hybrid.

Finally, the grain quality analysis along with the total yield comparisons determined the actual feasibility of our proposed method of hybrid seed production. If grain yield, grain quality, and other important agronomic traits from treatment 4 are similar to other treatments, the feasibility of this new proposed method will have been proven.

3.1.5 Objective 5- Patent application

The objective 5 was to seek patent for the proposed new hybrid rice seed production method.

*3.2 Experimental designs*

3.2.1 Objective 1 - Development of the isogenic HT A'-line / B'-line

The first objective is to develop the isogenic HT B'-line (which was later named as 7019B) through four backcrosses using the donor plant, Puita-Inta-CL, to transfer the GOI, *INTA* gene at homozygous state (*HH*) (Roso et al., 2010), and the recurrent parent, the elite maintainer line (B1) of a known promising hybrid that is not in the recessive state of *INTA* gene (*hh*), thus has no tolerance to the herbicide.

The theoretical percentage of homozygosity of $BC_4F_1$ is 93.75% and the percentage of the recurrent parent genome is expected to be 96.87%. The objective was to have majority of the original parent genome transferred at the generation of $BC_4F_1$ to keep the heterosis, but allow for some differences for a few traits, which are not critical to grain yield, and to save time which may be needed for the backcrossing procedure.

The $F_1$ plants generated from the first cross between the Puita-Inta-CL and B1 were screened for the inheritance of the herbicide tolerance traits by application of the corresponding

herbicides, NewPath, at seedling stage at labeled rates. Since the HT trait is controlled by the *INTA* gene in a complete dominant pattern, all of the $F_1$ plants will survive the herbicide treatments. Confirmation test crosses (CTC) is a process using the corresponding A-line to cross with the new plants to validate the maintainer role of the plants. This process was done using A1 to cross with every plant from the $F_1$ population and the resulting CTC plants were tested for the sterility using two methods, pollen staining and self-bagging. Standard procedures are as following: Pollen staining using 1% $I_2$-KI iodine solution will be performed make sure the viable pollens are less than 0.05% to ensure the maintainer role of the 7019B. The pollen sterility and fertility identification was according to Chaudhary et al. (1981). The standard procedure of the pollen staining was to collect two spikelets from the upper, middle, and lower parts of a panicle one day after heading. Two to three anthers from each spikelet were crushed using tweezers in the 1% iodine $I_2$-KI solution and inspected under a microscope. Pollen grains that were round shape and were intensely stained were classified as fully developed and fertile; clear, irregular shapes of pollen grains were classified as sterile; anything in-between were classified as partially fertile or sterile. Pollen sterility of a single plant was calculated as the mean of sterile pollen rate from six spikelets from each of the three panicles from the same plant. Bagging the panicles on the same plant was done to ensure the results of the sterility of the plants. The rest of the panicles were bagged before flowering from the same plants from which pollen was stained with the sterility pollen rate of 99.99% or higher to validate the staining results. The self-seeding rate was calculated by the average of the seed set rate on the bagged panicles. Only the ones that had no self-seed set were considered as male sterile and to moved forward and crossed with the recurrent parent, B1, to generate $BC_1F_1$. The $BC_1F_1$ had the theoretical homozygosity percentage of 50% and the recurrent parent genome of 75%. Similar procedures of application of NewPath herbicide was conducted at the seedling stage to eliminate the plants

with *hh*. The remaining of the $BC_1F_1$ plants at the heterozygous state of *INTA* gene (*Hh*) were tested for the role of maintainer with same procedures and standards of pollen staining and self-bagging within CTC $F_1$ plants created by crossing A1 with each of the $BC_1F_1$ plants. Only the ones passed the 99.5% sterility standard were used to further cross with B1 to generate $BC_2F_1$. Theoretically, $BC_2F_1$ plants have 75% of homozygosity and 87.5% of recurrent parent genome. 50% of the $BC_2F_1$ population would inherit the herbicide tolerance at heterozygous state (*Hh*) while the rest of the 50% of the population would be at recessive homozygous state (*hh*) and would be killed and removed by the application of the herbicide. All of the remaining $BC_2F_1$ plants carrying *INTA* gene at heterozygous states (*Hh*) were tested for the role of maintainer with same procedures and standards of pollen staining and self-bagging within CTC $F_1$ plants created by crossing A1 with each of the $BC_2F_1$ plants. The ones that passed the sterility standards that reach 99.5% of sterility were used to further cross with B1 to generate $BC_3F_1$. The $BC_3F_1$ had the theoretical homozygosity percentage of 87.5% and the recurrent parent genome of 93.75%. 50% of the plants would carry *INTA* gene at heterozygous state (*Hh*) and the other 50% would carry recessive homozygous state of the gene (*hh*). Similar procedures of spraying NewPath herbicide were conducted at the seedling stage to eliminate the plants with *hh*. The remaining $BC_3F_1$ plants with *Hh* went through the same procedures to check the maintainer role within the $BC_3F_1$ plants through of pollen staining and self-bagging within CTC $F_1$ plants created by crossing A1 with each of the $BC_3F_1$ plants. Only the ones passed the sterility standards of 99.5% of sterility were used to further cross with B1 to generate a population of $BF_4F_1$. $BC_4F_1$ had the theoretical homozygosity percentage of 93.75% and the recurrent parent genome of 96.87%. Again, 50% of the plants would carry *INTA* gene at heterozygous state (*Hh*) and the other 50% would carry recessive homozygous state of the gene (*hh*), which would be removed from the tolerance screening by herbicide application. The remaining $BC_4F_1$ plants were tested for the

maintainer role by the same procedures and standards of pollen staining and self-bagging within CTC $F_1$ plants created by crossing A1 with each of the $BC_4F_1$ plants. Only the plants passed the sterility percentage of 99.9% were selected as possible candidates. The selected $BC_4F_1$ plants were grown under the field conditions in a typical growing season that allowed self-pollination to generate $BC_4F_2$ population. $BC_4F_2$ were planted as a population segregating for herbicide tolerance trait since $BC_4F_1$ is at heterozygous state for the herbicide tolerance (*Hh*). 25% of the population were at homozygous state possessing *hh*, which would be killed and eliminated from the population followed by NewPath herbicide application. The remaining 75% of the $BC_4F_2$ population consists 50% of homozygous *HH* and 50% of heterozygous *Hh*, which was identified by allowing selected plants to self-pollinate again without the identification of molecular markers associated with the trait of interest. Seed harvested from the selected $BC_4F_2$ were grown into $BC_4F_3$ panicle rows in the field following the application of herbicide to screen for segregation of the tolerance. Panicle rows that had about 50% of the plants killed were not further selected from since the plants survived in the same row are consisted of 50% of *HH* and 50% of *Hh*. The panicle rows that had no plants responded to the herbicide application and survive are consist of homozygous *HH* status for the GOI. Finally phenotypic selections were made based upon good agronomic characteristics and various floret characteristics as good female. The one with the best floret characteristics, such as stigma exertion rate (%), stigma size, and floret opening angle, and other suitable agronomic traits was selected as the converted B'-line (7019B). Seed was harvested from individual plants.

A1 was used as the donor of the CMS cytoplasm to cross with the resultant 7019B. The process is called the Confirmation backcrosses (CBC) and the resulting $F_1$ was checked for sterility using the same sterility testing procedures and standards. The validated $F_1$ containing the CMS cytoplasm with at least 99.99% of sterility were used to crossed with the 7019B again for

CBC to generate CMS-$BC_1F_1$. The same plant was crossed with the male parent in the promising hybrid, R7, for heterosis testcross (HTC). The resulting HTC $F_1$ seeds were grown under field conditions with the R7 side-by-side to evaluate the existence of heterosis and to make sure the sterility is restored by the R7 to at least 85% in the HTC $F_1$ plants. Only plants that met both of the requirements from the CTC and HTC were selected to make more backcrosses with 7019B. Similar procedures were repeated to obtain CMS-$BC_4F_1$ through three more times of repeated CBC using the resulting CMS-BC lines from each cycle and HTC using R7. Any CMS-plants with stained pollen of more than 0.01% were discarded. Application of the corresponding herbicide, NewPath, on the selected plants was conducted at seedling stage to validate the transfer of the HT gene and the trait of herbicide tolerance at each generation of CTC. Selections for good agronomic traits and floret characteristics as female were done throughout the process of CTC following the herbicide application at each generation of backcrosses. The resulting CMS-$BC_4F_1$ plants with CMS cytoplasm and herbicide tolerance transferred, good agronomic traits, and floret characteristics will be determined as 7019A, the CMS-converted line of 7019B. Again, the whole process of development of a new A-line by transferring CMS and conversion of the newly developed B-line is a time consuming and labor intensive work, whereas in this proposed new method, only 7019B is required to be developed through repeated backcrosses. This is one of the biggest advantages in this method in regards to time saved from not having to develop the corresponding 7019A. The purity maintenance and seed amplification of 7019A was made by crossing with 7019B. Each HT female, if developed independently, would require its own maintenance and seed amplification adding additional work load to a breeding program.

3.2.2 Objective 2- Development of the isogenic HT R'-line

The second objective is to develop the isogenic HT R'-line (which we later name it as CFR7) through repeated backcrosses using the same donor plant of *INTA* gene, Puita-Inta-CL, and the recurrent parent, the elite male parent (R7) of our promising hybrid. The conversion process is similar to the conversion of HT B'-line 7019B, except for the need to check for pollen sterility at each generation of backcrosses and the CMS transfer process at the end.

The $F_1$ plants generated from the first cross between the Puita-Inta-CL and R7 were screened for the inheritance of the herbicide tolerance traits by application of the corresponding herbicides, NewPath, at seedling stage at recommended rate. Since the HT trait is controlled by the *INTA* gene in a complete dominant pattern, all of the $F_1$ plants will survive the herbicide treatments. All of the $F_1$ plants were then further crossed with R7 to generate $BC_1F_1$ seeds. The $BC_1F_1$ had the theoretical homozygosity percentage of 50% and the recurrent parent genome of 75%. Similar procedures of application of NewPath herbicide were conducted at the seedling stage to eliminate the plants with *hh*. The remaining of the $BC_1F_1$ plants at the heterozygous state of *INTA* gene (*Hh*) were further crossed with R7 to generate $BC_2F_1$. Theoretically, $BC_2F_1$ plants had 75% of homozygosity and 87.5% of recurrent parent genome. 50% of the $BC_2F_1$ population would inherit the herbicide tolerance at heterozygous state (*Hh*) while the rest of the 50% of the population would be at recessive homozygous state (*hh*) and would all be killed and removed by the application of the herbicide. All of the remaining $BC_2F_1$ plants carrying *INTA* gene at heterozygous states (*Hh*) were further crossed with R7 to generate $BC_3F_1$. The $BC_3F_1$ had the theoretical homozygosity percentage of 87.5% and the recurrent parent genome of 93.75%. 50% of the plants would carry *INTA* gene at heterozygous state (*Hh*) and the other 50% would carry recessive homozygous state of the gene (*hh*). Similar procedures of spaying NewPath

herbicide were conducted at the seedling stage to eliminate the plants with *hh*. The remaining $BC_3F_1$ plants with *Hh* were backcrossed again with R7 to generate $BC_4F_1$. $BC_4F_1$ had the theoretical homozygosity percentage of 93.75% and the recurrent parent genome of 96.87%. Again, 50% of the plants would carry *INTA* gene at heterozygous state (*Hh*) and the other 50% would carry recessive homozygous state of the gene (*hh*), which would be removed from the tolerance screening by herbicide application. The remaining $BC_4F_1$ plants were grown under the field conditions in a typical growing season to allow self-pollination to generate $BC_4F_2$ population. $BC_4F_2$ were grown as a population segregating for herbicide tolerance trait since $BC_4F_1$ is at heterozygous state for the herbicide tolerance (*Hh*). 25% of the population would be at homozygous state possessing *hh*, which would be killed and eliminated from the population followed by NewPath herbicide application. The remaining 75% of the $BC_4F_2$ population consists 50% of homozygous *HH* and 50% of heterozygous *Hh*, which we would need to identify by allowing the selected plants to self-pollinate again in the absence of molecular marker for the HT gene. The seeds harvested from the selected $BC_4F_2$ were grown into $BC_4F_3$ panicle rows in the field following the application of herbicide to screen for segregation of the tolerance. Panicle rows that had about 50% of the plants killed were not further selected from since the plants survive in the same row consisted of 50% of *HH* and 50% of *Hh*. The panicle rows that had no plants responded to the herbicide application and survived were consisted of homozygous *HH* status for the HT. Finally phenotypic selections were made based upon agronomic characteristics and various floret characteristics such as anther size and pollen exertion rate. The selected plants were crossed with the A1 to generate $F_1$ seeds to grow in the field with the corresponding reference male plants to evaluated the heterosis within the $F_1$ and to assure the sterility of $F_1$ that were restored to at least 85%, a process also known as heterosis testcrosses (HTC). Seeds were then harvested from the selected plant, named CFR7.

3.2.3 Objective 3 - Seed production ability of various treatments in ESP

The A-line seeds for treatments 4 and 5 were produced ahead of the ESP planting by crossing ht/ht A-line (A1) with its isogenic HT/HT B'-line (7019B) to generate hemizygous state of HT/ht A/B'-line (A1/7019B). Hemizygous A1/7019B seeds were first produced in the 2014 ESP field in El Campo, TX to be used as female seeds later in the ESP production; A1 and 7019A were separately annually produced from the maintenance seed production system in 2014 and 2015. R7 and CFR7 were also produced annually from the maintenance seed production system in 2014 and 2015 field in El Campo, Texas.

For each of the ESP block, $GA_3$ was applied at stage when A-line or R-line, whichever was later, reached 10% heading stages to enhance the panicle exertion in each ESP block. Supplementary pollination was carried out four times a day with a leaf blower roughly 20 minutes during the peak of flowering each day. The supplementary pollination lasted until the R-lines finished flowering, which was about 10 days since the 10% heading.

A randomized complete block design (RCBD) was the experimental design for objective 3. Each treatment had its own A-line female plants as pollen receiver and R-line male plants as pollinators. A-lines from treatments 1 and 6 were A1. A-lines from treatments 2 and 3 were 7019A. The A-lines used in treatments 4 and 5 were produced from foundation seed production using A1 as female and 7019B as male to produce female plants with heterozygous at the locus of the herbicide resistance gene (A1/7019B). The R-lines used in the treatments 1, 3, and 5 are homozygous for herbicide resistance gene (CFR7). The R-lines used in the treatments 2, 4, and 6 are homozygous for herbicide resistance gene (R7).

In 2015 and 2016 in El Campo, TX, direct seeding was performed with 30g of seeds / $m^2$ for both female and male plants in the ESP field in normal planting season. There were three replications for each ESP plot. Each ESP plot includes the following five treatments that's

randomly assigned for their order in each ESP plot: treatment 1 includes the female A1 and the male CFR7; treatment 2 included female 7019A and male R7; treatment 3 included female 7019A and male CFR7; treatment 4 included the hemizygous female A1/7019B and male CFR7; treatment 5 included the hemizygous female A1/7019B and male R7; treatment 6 included the parents of the original hybrid, female A1 and male R7.

To ensure the genetic purity of each production field, isolation was established by setting up a tarp barrier in between treatments. The row ratio of A-lines to R-line was 2:4 with the R-line on the first two rows and A-line on the next four rows perpendicular to the wind direction. ESP field setting is demonstrated as in Figure 3 with pink rows representing A-line and purple rows representing R-lines. Each ESP block contains 13 repeats of same ratios of A-lines and R-lines. The total length of each ESP block is 19.9 meter and the width is 2.13 meter.

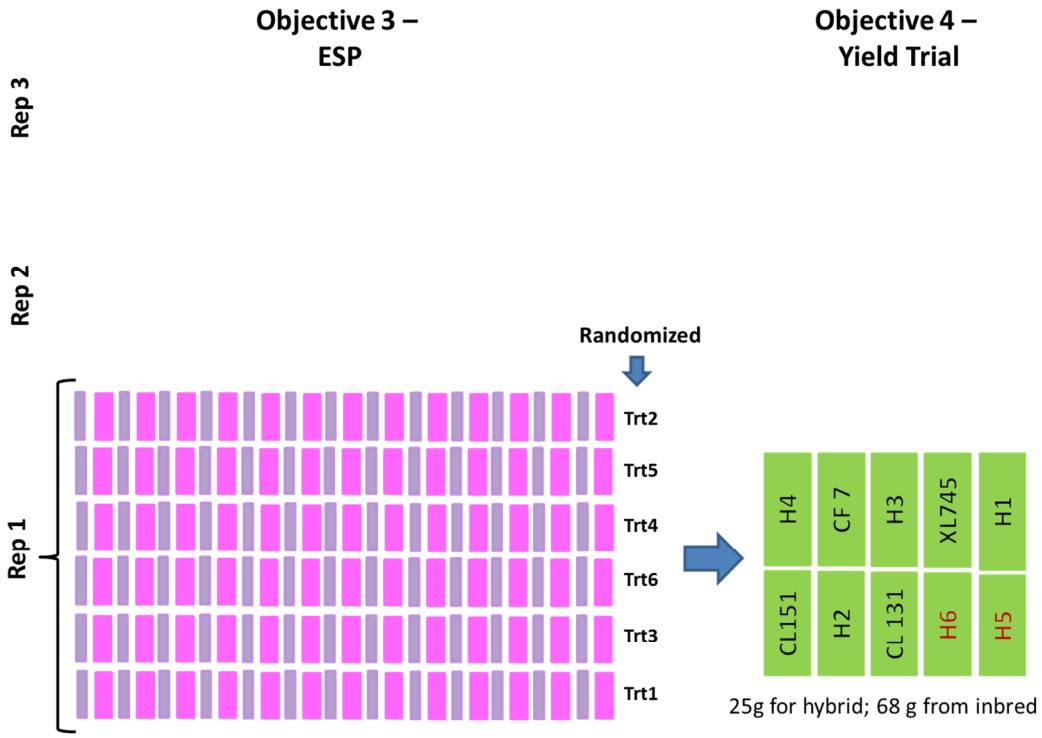


**Figure 3. Demonstration of the field setup for objective 3 and objective 4. In objective 3, pink rows and purple rows represent A-lines and R-lines respectively.**

The application of gibberellic acid ($GA_3$) was at 10% heading at a dose of 30 g/acre (74.1g/ha) of was applied with a backpack sprayer. The purpose of applying $GA_3$ is to stimulate the cell elongation and promote panicle exertion in female lines. It also has the following effects: 1) increases the duration of floret opening, thus ensures pollination; 2) increases stigma exertion and its receptivity; 3) promotes plant height; 4) widens the flag leaf angle which facilitates entry of the pollen grains; 5) influences flowering and thus transplanting in parental lines can be adjusted; 6) promotes panicle exertion and growth rate of secondary and tertiary tillers (Suralta and Robles, 2002).

Besides natural wind, supplementary pollination was carried out using the backpack leaf blower to pollen from male plants to adjacent female plants. This process was done five times a day starting roughly from 11:00 AM depending on the weather with 30 minutes in between sets of blowing. It was continued for 10 days after the first day of $GA_3$ application. The leaf blower is set at approximately 15 mph (24 km/hour) of wind speed.

The following observations were taken for each of the treatments on both A-line and R-line separately in every repeated ESP plot: Plant height (cm) at prior to harvest, tiller number (number of tillers per $m^2$ in the same area) at prior to harvest, days to 50% flowering (days), phenotype rating (1-7, 1: poor, 7: excellent) at prior to harvest, relative moisture (%) of both R-line and $F_1$ seeds on A-line - at harvesting, and major pest or disease responses (1-5, 5: susceptible, 1: tolerant).

The following observations were taken for each of the treatments on only R-line in every repeated ESP plot: anther size (1-5; 1: small, 5: big) and anther exertion (1-5; 1: poor, 5: excellent).

The following observations were taken for each of the treatments on only A-line in every repeated ESP plot: stigma size (1-5; 1: small, 5: big), stigma color, number of filled grains per panicle (spikelet fertility %), seed set rate (%), stigma viability (%), and seed set yield at 12% relative humidity (kg/ha).

The mean of seed set rate (%) was calculated from the main panicles from 30 randomly picked A-line plants in an ESP block to determine stigma viability. For one plant, the seed set rate was the number of fertilized spikelet divided by total number of spikelets on the same panicle, subtracted by the self-pollinated-seeding rate resulting from ratooning, which is normally zero for a sterile WA-CMS A-line. Evaluation of all the traits will be conducted

according to the "Standard Evaluation System for rice (SES) manual" provided by International Rice Research Institute (IRRI) (IRRI, 2002).

3.2.4 Objective 4 - Comparison of yields and quality of various treatments

For the $F_1$ preliminary yield trials as the experiment 2, six hybrids generated from experiment 1 were planted with aforementioned controls (Inbred varieties CL151, CL131, CFR7, and Hybrid XL745) randomly assigned in adjacent plots as a preliminary yield trial. In the statistical analysis of data, hybrid seeds generated from ESP treatment 1 corresponds to entry 3, hybrid seeds generated from ESP treatment 2 corresponds to entry 4, hybrid seeds generated from ESP treatment 3 corresponds to entry 5, hybrid seeds generated from ESP treatment 4 corresponds to entry 1, hybrid seeds generated from ESP treatment 5 corresponds to entry 2, and hybrid seeds generated from ESP treatment 6 corresponds to entry 6. The six hybrids were referred to as iso-hybrids.

CL131 was a conventional inbred variety developed by the LSU AgCenter's Rice Research Station in 2004, and CL151 was another conventional inbred variety developed in 2008. Both of them were registered with BASF in 2011. It has a gene resistant to imazethapyr and therefore can be used with Clearfield production system. The rice grain yield of CL151 was reported to reach 8.3 Mg $ha^{-1}$ compared with 7.5 Mg $ha^{-1}$ for CL131. CL151 was also reported to have higher seedling vigor than CL131. CL151 has a plant height of 94 cm while CL131 has a height of 84 cm, and both of them reached 50% heading around 81 days when grown in Louisiana (Blanche et al., 2011). Hybrid XL745 is a commercial hybrid released by Rice Tec in 2007 that is tolerant to the imidazolinone class of herbicides. It can be used with Clearfield production system. It was reported to possess roughly 739.36 kg yield average over CL151. The XL745 hybrid and the seed production method were patent as US 8153870 B2 (Rice Tec, 2012).

XL745 reached 50% heading around 71 days and has a height of 115 cm according to data collected in Alvin, TX from the patent disclosure.

A randomized complete block design (RCBD) with three replications and ten entries was used for preliminary yield trial. The field was blocked so that each replication had similar soil types, water supply and fertilization.

Plots were 1.83 m x 4.27 m with six rows. Each trial included hybrid seeds from six treatments, a HR commercial hybrid, and three HR commercial inbred varieties as controls. All genotypes had similar maturity habits, and were mechanically direct-seeded with a planting depth of less than 25 mm to facilitate a uniform stand. For each hybrid entry, 25g of hybrid seeds were planted, and 68g of seed for each inbred variety. Trials were managed similarly to commercial rice production to optimize production. Production practices included timely water management, soil fertilization, and insect and weed control.

Yield trials were conducted in El Campo and Pierce, Texas, in 2015 and 2016. Soil at El Campo is a sandy clay loam, while soil at Pierce is heavy clay with a dark color and high organic matter. The trials were managed with standard agronomic procedures. Prior to planting, 62 kg/ha N in the form of ammonium sulfate was applied. Pre-flood nitrogen fertilizer was applied at 62 kg/ha in the form of urea and 17 kg/ha of ammonium sulfate. Fields were flushed twice; the first time was immediately after planting where water was applied for 24 hours and then drained. The second flush was five days following the first drain. The first NewPath herbicide application was made at the 3-4 leaf stag at a rate of 280.2 g/ha. Ten days later, the second application of NewPath at a rate of 280.2 g/ha was applied respectively on corresponding plots. Evaluation of the plant stand, plant vigor, and germination were conducted before the herbicide application. The response to herbicide was conducted five days after the 2$^{nd}$ herbicide application. When individual plots reached maturity at 18-20 % moisture content, they were cut and threshed

with a stationary thresher. Grain yields of each plot was adjusted and converted based on the moisture content prior to analysis. The harvested seeds were dried with a specialized propane heated dryer. After the seeds reach 10-12 % moisture, a sub-sample from each was for grain quality assessment. After harvesting, NewPath herbicide was applied at 3X of the labeled rate (840.64 g/ha) on the ratoons of every plot to review the level of resistance and injuries.

The following observations were recorded from the preliminary yield trial: **g**ermination (1-5, 1: poor, 5: excellent) at three weeks after planting, plant stand establishment (1-5, 1: poor, 5: excellent) at three weeks after planting, seedling vigor (1-5, 1: poor, 5: excellent) at three weeks after planting, plant height (cm) at prior to harvest, tiller number (number of tillers per plant) at prior to harvest, days to 50% flowering (days), lodging (1-5, 1: weak straw, 5: strong straw) at prior to harvest, phenotype rating (1-7, 1: poor, 7: excellent) at prior to harvest, segregation of any noticeable traits (culm color, stigma color, etc), relative moisture (%) at harvesting, grain yield at 12% relative humidity (kg/ha), 1000 grain weight (g), major pest or disease resistance (1-5, 1: susceptible, 5: tolerant), responses to NewPath herbicide (1-5, 1: susceptible, 5: resistant) on seedlings at 3-4 leaf stage and ratoon.

Grain quality of each hybrid combinations were determined by the following evaluations: milled rice yield (%), head rice yield (%), chalkiness (%; 0-10%: little chalk; 11-25%: acceptable; > 25% chalky), translucency (1-5), color (1-5), length: width ratio (L:W).

The middle seven rows from each plot were harvested to reduce potential variation attributable to border row effects. Grain yield was determined by adjusting the total grain yield from each plot to the weights at 12% relative moisture. Milling yields were determined using a 100g sample of paddy rice dehulled and milled with the standard protocols. Total milled rice was weighed, broken kernels removed, and head rice yield was determined. Percent head rice was determined by dividing the weight of the whole grain milled rice by the total milled rice.

*3.3 Statistical analysis*

3.3.1 Yield data adjustment

All of the harvested weights from ESP and yield trials measured in pounds were subjected to adjustment based on the moisture content at harvest to standardize them to the adjusted weight as if at 12% moisture content before further analysis.

**Analysis of yields and other measurable traits**

The adjusted yield data, as well as other measurable traits, were then analyzed using JMP Pro 12 Software from ESP and yield trials. JMP Mixed Model was used for ANOVA to test for the significance of the effects. Different treatments (genotype entries), locations, and years are the three main effects. The ten treatments are the 10 genotype entries; the two years are 2015 and 2016; the two locations are El Campo and Peirce Ranch, TX. All three factors were treated as fixed effects. Blockings within each environment were treated as random effects. Pairwise significant differences between different treatments (genotype entries and locations) in ESP and yield trials were performed using LSMean Student's t-test at 95% confidence with JMP statistics. Values indicated by different letters represent significant differences at $P<0.05$.

Mid-parent heterosis (MPH) in the A1/7019B was measured based on means for the stigma viability from the two A1 and 7019A based on the seed set yield in ESP trial analysis. The following formula was used to estimate the MPH:

MPH (%) = (F1-MP)/MP * 100, while MP is the average means for the stigma viability from the two A1 and 7019A. F1 is the stigma viability of the A1/7019B from the ESP seed set yield analysis data.

**Grain quality**

When the whole batches of grain reached moisture content of 10-12%, five sub-samples of 1000 grains were taken from each genotype as determined by a mechanical seed counter and then weighed to obtain a weight per grain. Samples were collected from only 2016 Pierce Ranch since the length, width, and the ratio of the rice grains is a highly heritable trait, which is relatively stable per variety. Broad sense heritability of L:W of grains in rice was calculated as 0.89 with an F2 population (Rabiei et al., 2004) and the narrow sense heritability of L:W of grains of rice was estimated to be greater than 0.84 with an F2:3 population (Fahliani et al., 2011). Twenty grains were randomly sampled from each entry genotype to measure the length and the width of the caryopsis, and the ratio of the L:W.

**CV% and combined analysis**

A coefficient of variation (CV%) was calculated for each test. Lower CV% values indicate greater lesser inter-plot variation and often times a measure of the quality of the test. For varietal yield trials, CV% values < 10% are generally considered to be extremely reliable due to little variance in the environment and CV% values between 10% and 30% are considered acceptable (Taylor et al., 2008). The LSD and CV% values for yield in these tests are reported in the footnotes of each test. CV% for each individual trial in each year of each location was calculated individually before any data combination from different years or locations. Two outlier data points from 2015 El Campo and two outlier points from 2016 Pierce Ranch were deleted because of either animal damages or severe lodging, which would have resulted in a high standard deviation before further combining analysis.

**GxE interactions**

Genotype-Environment Interaction (GxE interaction) on yield and other traits performances of all genotype entry in different environments (year & location) was determined through ANOVA. In this way, it was ascertained if a genotype had a stable consistency across years and locations or was there is some level of GxE interaction.

# 4. RESULTS AND DISCUSSION

## *4.1 Objective 1 - Development the isogenic HT A'-line/ B'-line*

The converted HT B'-lines, $HT_1$-$BC_4$, was named 7019B because it has an 87% resemblance to the original B1 (molecular data not shown). A small portion of dissimilarity was desired between the B1 and 7019B so that feasibility could be verified for this method of only four backcrosses, which allows breeders to save time and labor in developing isogenic lines. Some degree of heterosis in the A1/7019B $F_1$ may help with hybrid seed production. The acceptance of the conversion is conditioned only if the resultant 7019A has stable sterility when crossed with 7019B, fully restorable by the R7 and CFR7, and with comparable heterosis when crossed with R7 and CFR7 as with the original A1. The positive results would suggest the conversion of the HT B'-lines through four backcrosses is adequate to keep the same level of heterosis in the hybrids and useful in the new hybrid seed production method.

The pollen staining test with $I_2$-KI solution during the CMS conversion confirmed that 7019B was a maintainer line for WA-CMS A1 because the hybrid of the resulting $F_1$ from the HTC crosses between A1 and 7019B was completely sterile. Therefore, the $F_1$ progenies were backcrossed with 7019B to obtain the first generation of backcross ($HT_1$-$BC_1$), which was later used as female in the following generation of backcrossing to obtain $HT_1$-$BC_2$, which was again repeated until the $HT_1$-$BC_4$ was obtained. Final selections in the field were performed among all the $HT_1$-$BC_4$, and the resulting plant was named 7019A. Evaluation between 7019A and 7019B showed there was no significant agronomic difference among them other than pollen sterility.

Moreover, both A1 and 7019A have excellent stigma exertions and big stigma size (Table 2 and Fig 4). The comparable stigma receptivity was proven from the ESP trials

(objective 3) that showed both of them have similar outcrossing rates and final seed set yields under similar field conditions over two years.

Major differences between A1/B1 and 7019A/7019B include plant height, days to 50% heading, and panicle length in the normal growing season (Table 2 and Fig 5).


**Figure 4. Panicles of three females, A1, 7019A, and A1/7019B**

Other than the traits listed in the table 2, the A1/B1 and HT 7019A/7019B lines differ in regards to stigma color (Fig 5), apiculus, and the culm bases. The original A1/B1-lines possess purple culm bases, stigma and apiculus due to the anthocyanin pigmentations accumulation at those plant parts, while the 7019A/7019B lack anthocyanin pigmentations and therefore have a green culm, white stigma, and apiculus. The anthocyanin pigmentation pattern received relatively less attention since they are not considered as critical factors to grain yield or any other

important characters of the rice plants. Anthocyanins are water-soluble vacuolar pigments that may appear red, purple, or blue depending on the pH inside the cells. They belong to a parent class of molecules called flavonoids synthesized via the phenylpropanoid pathway. Localization of the anthocyanin pigments can be used a variety as unique; however, the color is often weakly expressed at young stages or if the plant part is shaded. The expression of anthocyanin color in the stigma is observed only when the apiculus is colored. The coloration of the culm base was also found to be related to the anthocyanin pigmentation pattern of the stigma and apiculus. Although colors are an easily visualized difference between A1/B1 and 7019A/7019B, there is no evidence to indicate that this is a trait is critical to the stigma receptivity. Furthermore, there are published studies that suggest a relationship between color and the stigma receptivity.

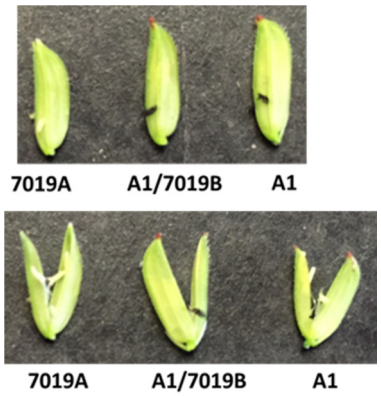


**Figure 5. Florets of three females, A1, 7019A, and A1/7019B.**

| Genotype | Description | Plant Height | 50% Heading | Panicle Length | Tiller Number | Stigma Viability | Grain Shape | Stigma Size |
|---|---|---|---|---|---|---|---|---|
| | | cm | days | cm | No./plant | % | S,M,L | 1-5 |
| A1 | CMS donor | 74.10 c | 64.15 b | 15.47 b | 16.00 a | 45.69 b | L | 5 |
| 7019A | HT donor A'-line | 84.85 a | 82.65 a | 21.44 a | 15.90 a | 44.08 c | L | 5 |
| A1/7019B | HT Hemizygous | 78.65 b | 73.15 b | 22.20 a | 16.30 a | 53.16 a | L | 5 |

**Table 2. The phenotypes, floret characteristics, and grain types descriptions of female plants, A1, 7019A, and A1/7019B. Different letters represent significant differences at P<0.05 with LSMean Student's t-test at 95% confidence with JMP statistics.**

Plant height, days to 50% heading, panicle length, number of tillers, stigma viability and size were measured on 30 randomly selected plants for each genotype. Plant height was measured from the ground up to the tip of tallest panicle just prior to harvest. Plant height is generally an additive trait, so with A1/7019B being about halfway between A1 and 7019A fits that model (Table 2). Days to 50% heading was similar to plant height, in that this trait is supposedly controlled by additive gene action. Therefore with A1/7019B being in between the early maturing A1 and later maturing 7019A, fits that model. There were no significant differences among the genotypes for number of tillers, but for panicle length 7019A and A1/7019B had longer panicles than A1. Although some researchers have reported that panicle length is predominantly controlled by additive gene action, the estimate of inheritance of the panicle length in rice can vary according to experimental designs, materials, and the approaches to measure. Babar et al., (2007) estimated the broad sense heritability of 0.74, while others had a

0.04 broad sense heritability of panicle length (Sabu et al., 2009). An estimate of narrow sense heritability of 0.19 was estimated for panicle length by different population of rice (Fahliani et al., 2010). Results of this study support the notion that dominant action of the genes is controlling panicle length since the A1/7019B was not significantly different than 7019A.

Once the 7019B was developed, the herbicide tolerance trait was introduced into the female line at the last stage of basic seed production (A-line x HT B'-line) to generate female seeds (A1/7019B) for the AxR hybrid seed production in ESP. Therefore, the best use of this proposed 3way$F_1$ hybrid production method is to use the herbicide tolerance that is controlled by a single locus dominant gene encoding tolerance at both homozygous and heterozygous states. The majority of the known herbicide tolerance traits are controlled by a single locus dominant gene.

The most critical and challenging part of the 3-line hybrid breeding program is identification of a female line that possess good general combining ability (GCA) with other desirable traits such as pollen acceptance which allows it to serve as a tester in a breeding program. Other than the necessity of being mostly sterile as a requirement that has to be met by an A-line candidate; according to Hallauer (1975), an ideal tester should maximize the genetic gain when crossed with the R-lines. More detailed criteria of a good tester in hybrid breeding programs was further suggested by Hallauer (1975), including the ease of use, ability to generate information that classifies the potential for the crossings correctly, maximum genetic gain, and elimination of lines with unsatisfactory performance from evaluation of the $F_1$ combinations (Duarte et al. 2003; Elias et al. 2000). Breeders have reported that by using good testers, they can also serve as means of classifying genotypes into different heterotic groups, which critical information in a hybrid breeding program (Nestares et al. 1999). Therefore, the development and identification of an elite A-line is the most important step in a hybrid breeding program that can

be labor-intensive and time-consuming. The A-line is the most valuable germplasm asset that breeders create because that can substantially impact $F_1$ combinations with a wider range of R-lines.

Unlike A-lines, thousands of R-lines can be selected each year from the RxR populations of advanced generations R-lines in a moderate hybrid rice breeding program. The last step of the R-lines selection needs to be test crossed with the existing A-line to evaluate the performance of the respective $F_1$ combinations of their heterosis and the existence of specific combining ability (SCA). SCA is used as the parameter used to assess the value of unique combinations between an A-line and an R-line. This parameter is obtained by the difference between the mean of a specific cross in relation to the overall mean of crosses with a particular tester. GCA is a parameter that describes the value of parents over a large number of progeny combinations. SCA can be interpreted as deviations of hybrid combinations from the expected in the GCA of the parents (Marin et al. 2006). These deviations are usually due to the action of dominance or epistatic effects.

Hence, one of the biggest advantages of this 3way$F_1$ hybrid production method is that it allows plant breeders to make the most effective use of an elite A-line known for its potential to create heterotic $F_1$ progeny with different herbicide tolerance traits into the female lines prior to production of the hybrid instead of breeding and maintenance for individual AxB pairs with different traits of interest (herein tolerance to different herbicides) separately, which can be expensive in terms of time and money. During the same time of selecting for different R-lines to be tested with the A-line, development of isogenic B'-lines can be individually developed by repeatedly backcrossing, which can even be faster with the aid of marker assisted selection. This should increase the versatility to produce unique isogenic B'-lines carrying different traits of

interest. A pool of isogenic B'-lines with different traits of interest could be specifically designed and developed to meet any potential markets can be created and ready to be used.

There can be possible obstacles with this method when the chosen donor of the gene of interest is not a maintainer by itself, but a restorer with *Rf3* or *Rf4* genes. If the gene of interest is closely linked with restorer genes on the same chromosome, it may be difficult to remove by repeatedly backcrossing without the use of molecular markers to assist in breaking genetic linkages. The female seeds A'-line will be partially fertile in its own maintenance breeding process. The number of plants with partially fertile seed set will increase in each step of the basic seed production that causes impurity and requires more labor at rogueing. Moreover, the A-line/B'-line produced from this converted B'-line at the last step of the basic seed production will be partially fertile. This can cause further impurities in hybrid seed production. It is worth noting that some level of partial fertility may be acceptable by the growers if the $F_1$ seeds produced in the AxR hybrid seed fields meet seed purity standards.

*4.2 Objective 2- Development the isogenic HT R'-line*

One of the other solutions incorporating the herbicide resistance or other trait of interest into the hybrid with a relatively quicker and easier way is by introducing the trait into the hybrid via the R'-line developed from the original R'-line. Development of isogenic R'-lines would be similar to the process of developing isogenic B'-lines through repeated backcrossing since both are male fertile. By producing different isogenic R'-lines carrying different traits of interest, a pool of isogenic R'-lines with various traits of interest can be specifically developed to meet anticipated demands in the marketplace. This results in a hemizygous state for the genes or alleles determining the trait of interest in the $F_1$ hybrid plants.

| Geno type | Description | Plant Height | 50% Heading | Panicle Length | Tiller number | Anther size | Grain Shape |
|---|---|---|---|---|---|---|---|
| | | cm | days | cm | No./plant | 1-5 | S,M,L |
| **R7** | R-line | 75.72 a | 72.00 a | 30.82 a | 18.82 a | 5 | L |
| **CF R7** | HT donor R'-line | 75.60 a | 71.15 a | 29.73 a | 18.53 a | 5 | L |

**Table 3. The phenotypes, floret characteristics, and grain types descriptions of male plants, R7 and CFR7. Different letters represent significant differences at P<0.05 with LSMean Student's t-test at 95% confidence with JMP statistics.**

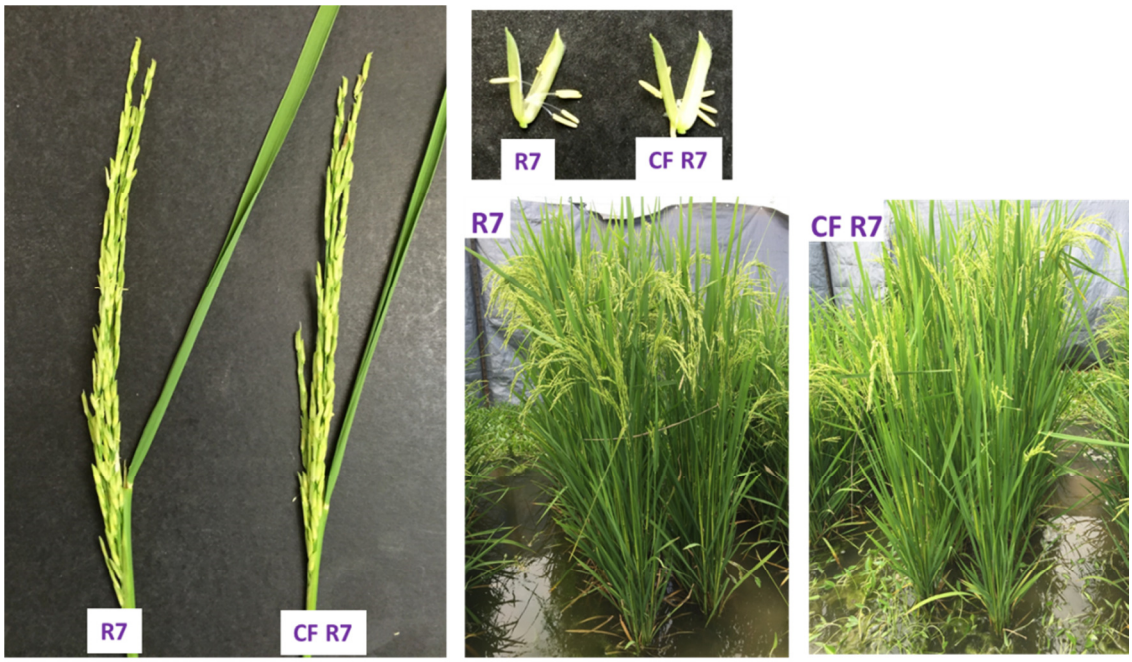


**Figure 6. Panicles, florets, and plant types of the two male plants, R7 and CF R7.**

R7 and CF R7 had no significant differences in the plant height, days to 50% heading, panicle length, the number of tillers, and florets (Table 3 and Fig 6). In addition, the appearances of plant types and grain shapes are similar. Both had anther sizes with the rating of five, similar floret opening time during anthesis, and high restore ability of the A1 making them ideal pollinators in the ESP.

*4.3 Objective 3- Seed produce ability of various treatments in ESP*

There is considerable time saved from not having to fully convert the HT B'-line and HT R'-line with the only minor differences, including the HT gene itself, is conditional. It is acceptable only when the resulting the 3way$F_1$ (A/B, A'/B', and R') hybrid does not possess obvious segregation in regards to some critical traits. The most important traits are related to

grain yield that could affect heterosis if they segregate. The second important trait, which is less obvious during segregation, is heading dates and maturity lengths of the 3way$F_1$ hybrid. Uniformity of heading dates and maturity lengths is a critical factor for growers attempting to harvest the crop at a stage with optimal grain moisture across the whole field, which in turn maximizes yield and grain quality. The third important trait is plant height because a field with heterogeneous heights causes difficulties during harvest, and shorter plants can be shaded by taller plants, which results in lost grain yield. Other traits that could possibly segregate and can be readily seen by growers but have no effects on the heading durations and yield are considered to be inconsequential.

The original A1/B1 lines and converted HT 7019A/7019B lines differ in the stigma color and a few of the traits as mentioned in objective 1. Whether this level of conversion is acceptable depends upon the ESP's ability to produce seed and yield performance compared to the original hybrid and corresponding iso-hybrids.

| | | | Seed Set Yield | | |
|---|---|---|---|---|---|
| | ESP Combination | | (kg/ha) | | |
| **Entry** | **Female** | **Male** | **2015 & 2016 Combined** | **2015** | **2016** |
| **1** | A1/7019B | CFR7 | 1045.04 a | 725.11 a | 1284.99 a |
| **2** | A1/7019B | R7 | 945.92 a | 600.59 b | 1291.25 a |
| **3** | A1 | CFR7 | 430.17 b | 510.12 bc | 370.21 c |
| **4** | 7019A | R7 | 559.51 b | 389.35 d | 729.68 b |
| **5** | 7019A | CFR7 | 542.75 b | 395.16 d | 690.34 b |
| **6** | A1 | R7 | 417.40 b | 430.61 cd | 404.19 c |

**Table 4. Seed set yield (kg/ha) for the six entries analyzed separately in 2015, 2016, and combined analysis from the two years ESP.**

Interaction of year*entry, entry, and year are all significant from the ANOVA F-test (Appendix 1). There are environmental and genetic factors that can influence grain yields in the ESP. Temperature, humidity, wind speed, and precipitation can affect both pollen dispersal and stigma receptivity during pollination. In general, temperature at 24-28 $^{o}$C, a relative humidity 70-80%, a clear sky, and a wind speed at least 19 km/hour during anthesis everyday in the course of heading has been identified as the most favorable environment for efficient pollination in rice (Xu and Li, 1988). The ESP seeds were planted in May in 2015 and 2016 in the same ratios and managed with the same protocol. The temperatures (Max, min, and mean) and the amount of precipitation during the flowering time in August in both years were similar. The most critical environmental difference between years was wind speed, which is the most important pollination supplement after the application of $GA_3$ to achieve a higher seed set yield in a hybrid seed production field. In August of 2015, the maximum and average wind speeds were 24 km/hour and 8 km/hour respectively. In August 2016, the maximum and average wind speeds were 42

km/hour and 16 km/hour respectively. Although artificial pollination was facilitated using leaf blowers every 30 minutes during the floret opening time in both years, natural and continuous blowing wind is more effective than intermittent artificial pollination. Differences in wind might be the primary cause of higher averaged seed set yields in 2016 compared to 2015.

It is desirable for male parent plants to tiller abundantly since it directly results in more panicles. Floret characteristics of the male plants, including anther size and pollen exertions are also important in determining the efficiency of pollination. Recall that R7 and CFR7 had no significant differences among most traits related to plant type or floret characteristics. For the female plant, the amount of tillering determines the number of effective panicles, which is as important as the number of tillers in the male plants in the ESP system. Recall that the females A1, 7019A, and A1/7019B had no significant differences in the number of tillers. Physical differences among florets in female parents and differences of how female plants respond to the environments or $GA_3$ application become the most critical factors determining final seed set on the female plants when grown with the same pollinators (R7 and CFR7). Other genetic factors of female parents that influence seed set yield on each panicle, which is used to calculate seed set rate (%) and overall stigma viability of a specific female variety, includes stigma size, stigma longevity, floret opening time, floret opening angle, and stigma exertion rate. Together these traits determine stigma viability of a specific variety as a female parent.

Synchronization of heading time between male and female plants in the ESP is an important factor affecting pollination efficiency. 7019A and A1/7019B had days to 50% heading closer to R7 and CFR7 than to A1. This inherently gives 7019A and A1/7019B an advantage in setting seed during hybrid seed production.

Because of the different pollination environments that resulted in significant year*entry interaction and year effects as elucidated by the ANOVA F-test, an analysis of seed set yield by

years along with the combined analysis was conducted to more fully explain how female and male plants responded to environmental conditions.

Although the average seed set yields between years are different according to the 2-factorial analysis, the two 3way ESP blocks, entry 1 (A1/7019B//CFR7) and entry 2 (A1/7019B//R7) with A1/7019B as female parents, consistently had the highest seed set yields among the six entries in each of the two years. This seed set advantage of A1/7019B was even more profound in 2016 with environmental conditions that were favorable to pollination, which were almost double the amount of seed set yields from the same ESP in 2015. A similar trend of seed set from the two ESP with 7019A as female parents entry 4 (7019A/R7) and entry 5 (7019A/CFR7) was observed. Seed set yield of the two ESP in 2016 was nearly double the seed set yield in 2015 most likely due to stronger wind speed that favored pollination. The two ESP with A1 as female parents, entry 3 (A1/CFR7) and entry 6 (A1/R7), had similar performances in the two years with different pollination environments. The seed set yield difference between the blocks with A1 and blocks with the other two females may be due to the fact that the A1 reached 50% heading eight days earlier than the other female parents, which was less synchronized with the R-lines. A1, however, had longer stigma longevity and reception ability than 7019A in 2015, which was an environment that was less favorable for cross-pollination. A1 was poorly synchronized with the two R-lines. Nevertheless, entry 3 (A1/CFR7) had a comparable seed set yield with entry 5 (7019A). In 2016, with favorable pollination conditions, A1 still had relatively stable performance in the seed set yield, while the seed set yields on 7019A was lower than seed set yields on A1 in 2015.

To determine the performance of A1/7019B as a female in the ESP, it is more appropriate to compare A1/7019B with 7019A as females since both headed around the same time as the two male parents in both years (Table 4). 7019A as a female was more susceptible

to poor weather in 2015, which resulted in the lowest seed set among the six entries. A1/7019B was consistently the female parent with the highest seed set yields compared to 7019A with either R7 or CFR7 as the pollinators.

Lastly, from both of the combined analysis and the analysis by year, in the same ESP trial, the same females were not significantly different in terms of seed set from the male parents, suggesting there is no significant negative effect on pollination attributed to the incorporation of HT gene in R-lines.


**Figure 7. Main panicles from three female plants from three different ESP blocks with R7. Brown spikelets are the fully developed seeds, and the green spikelets are the spikelets that were not fertilized by pollens from the R-lines, and therefore didn't develop into seeds.**

The seed set rate of a single female plant was determined based upon the number of fully developed seeds per number of total spikelets on the main panicle due to outcrossing prior to harvest (Fig 7). The seed set rate of the female line in a a particular ESP was determined by averaging the seed set rate of the main panicles from 30 randomly selected female plants from the same ESP plots. The data indicates A1 had comparable seed set rates with 7019A despite

being less synchronized with the R-lines, which is consistent with the hypothesis that A1 had more stigma longevity. The significantly longer panicle length of 7019A and the better synchronization with the R-lines could be the main factors contributing to the higher seed set yields of the ESP with 7019A as female compared to A1. The seed set rates on A1/7019B were significantly higher than 7019A and A1, which is consistent with the results from the seed set yield analysis. The seed set rates of the ESP blocks consisting the same female variety were averaged to determine the stigma viability of that female variety. A1/7019B had significantly higher stigma viability than 7019A and A1, while A1 had significantly higher stigma viability than 7019A.

Based on the stigma viability of A1, 7019A, and the A1/7019B from the ESP trials, mid-parent heterosis (MPF) was calculated using the formula:, MPH (%) = ($F_1$-MP)/MP * 100 = (0.532 – 0.449) /0.449 * 100 = 18.49%

MP is the average of the calculated stigma viabilities from the A1 (0.457) and 7019A (0.441). $F_1$ is the calculated stigma viability of the A1/7019B (0.532).

These results suggest a 18.49% advantage of the stigma in A1/7019B over the mean of the stigma viability of A1 and 7019A.

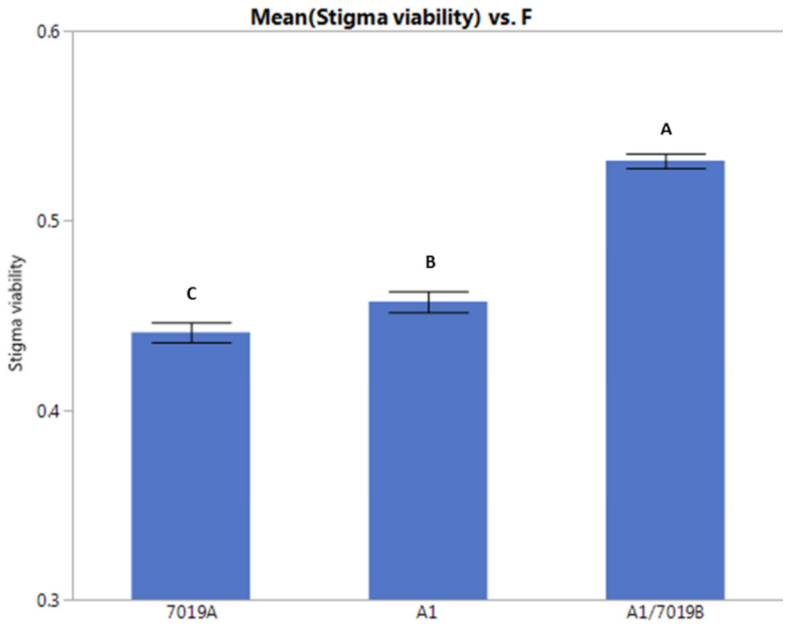


**Figure 8. Combined analysis from 2015 and 2016 of the stigma viability calculated from the average the seed set rates (%) of the main panicles of 30 randomly picked female plants in a same ESP block. Different letters represent significant differences at P<0.05 with LSMean Student's t-test at 95% confidence with JMP statistics. Each error bar is constructed using 1 standard error from the mean.**

This new seed production method appears to be feasible with the possible advantage of this method generating a positive heterosis effect in the A1/7019B $F_1$ which may have contributed to higher stigma viability in florets compared to the corresponding parents 7019A and A1, since 7019B is not fully converted to A1. The higher stigma viability could further result in a better response to the $GA_3$ application and thus significantly improve pollen receptivity in favorable and unfavorable climate conditions (Fig 8).

*4.4 Objective 4- Comparison of yields and quality of various treatments*

Adequate numbers of backcrosses when developing B'-line is not only necessary to preserve heterosis in the final 3way$F_1$ hybrid plants, but also to make sure segregation of genes not fully converted resemble the B-line will not significantly affect important traits in the 3way$F_1$ hybrid, such as plant height, growth durations, grain yield and qualities, etc. Other traits that may not be directly related to yield, but can affect the uniformity of a population, are needed to be assessed. In the end, the most important evaluations of the feasibility of 3way$F_1$ hybrid production system is to compare yield with iso-hybrid lines.

For traits of interest in B'-lines that are not fully converted to the status of the original B-lines, they will segregate as if they were an $F_2$ population. For instance, the pubescent/glabrous characteristics on leaves and hulls, controlled by a single dominant gene, will fit the expected 3:1 segregation ratio. The 3way$F_1$ hybrid plants generated from treatment 5 (entry 2) are supposed to be segregating for herbicide tolerance with the ratio of 1:1. The 3way$F_1$ hybrid plants generated from treatment 4 (entry 1) are all supposed to be tolerant to the herbicide with the homozygous or heterozygous of dominance HT genes.

Plantings were done by direct mechanical seeding both years. The planting at Pierce Ranch was almost 2 months later than the planting in El Campo both years. At both locations, the field was flushed twice, once was immediately after planting and the second flushing was five days after the first flush was drained. It was followed by fertilization of ammonium sulphate. The first application of NewPath herbicide was applied on the plots with the herbicide tolerance gene (homozygous or heterozygous) as well as herbicide resistant controls at the 3-4 leaf stage to verify the effectiveness of the tolerance gene at the rate of 280.2 g/ha using a $CO_2$ pressured backpack. This helped to eliminate possible weeds or contamination of any foreign

seeds. Responses to the herbicide of the applied plots were reviewed fivedays later, and no obvious symptoms were observed between the plots treated with a herbicide and untreated plots. The second application of NewPath was applied ten days after the first application at a concentration of 280.2 g/ha. Responses to the second application of the NewPath herbicide were reviewed and scored. No obvious differences were observed between treated and untreated plots. An extra plot adjacent to the trials with (A1/R7) hybrids were sprayed with same dosage of NewPath were killed ten days after the 2nd application, while the plots that were not sprayed show no symptoms of herbicide damage. This evidence suggested the dosage was sufficient to kill susceptible plants without HT genes (Fig 9). No obvious differences were observed between trials possessing HT genes in the homozygous or heterozygous state, suggesting the zygosity of HT genes are completely dominant and do not affect the level of tolerance to the NewPath herbicides.


**Figure 9. Responses to NewPath herbicide 10 days after the 1st and the 2nd applications of A1/R7 and A1/7019B//CFR7 plots.**

A key point to the successful use of this 3way hybrid production system is to have a uniform 3way$F_1$ hybrid field. Traits could segregate as if they were an $F_2$ population because of only a partial conversion to the HT-B lines from the original A-line. In our yield trials, the color of culm bases, stigma, and apiculus segregated within hybrids as expected since the original A1/B1 has purple culm bases, stigma, and apiculus, and the converted 7019A/7019B has white culm bases, stigma, and apiculus, similar to the HT donor, Puita-Inta-CL. The color differences of culm bases, stigma, and apiculus are due to the anthocyanin pigmentation patterns of different plant parts. No other obvious segregation related to the yield component can be observed in both of the 3way$F_1$ hybrid plots.

Germination rates, plant stand rating, and seedling vigor was recorded at the 3-4 leaf stage in each plot as responses of plants to the NewPath herbicide were recorded. There were no significant differences among the ten entries for germination and plant stand ratings. However, the two 3way$F_1$ hybrids (entry 1 and entry 2) and the other three out of the iso-hybrids (entry 3, entry 4, and entry 6) had higher seedling vigor than the rest of the controls, inbred or hybrid (Fig 10).

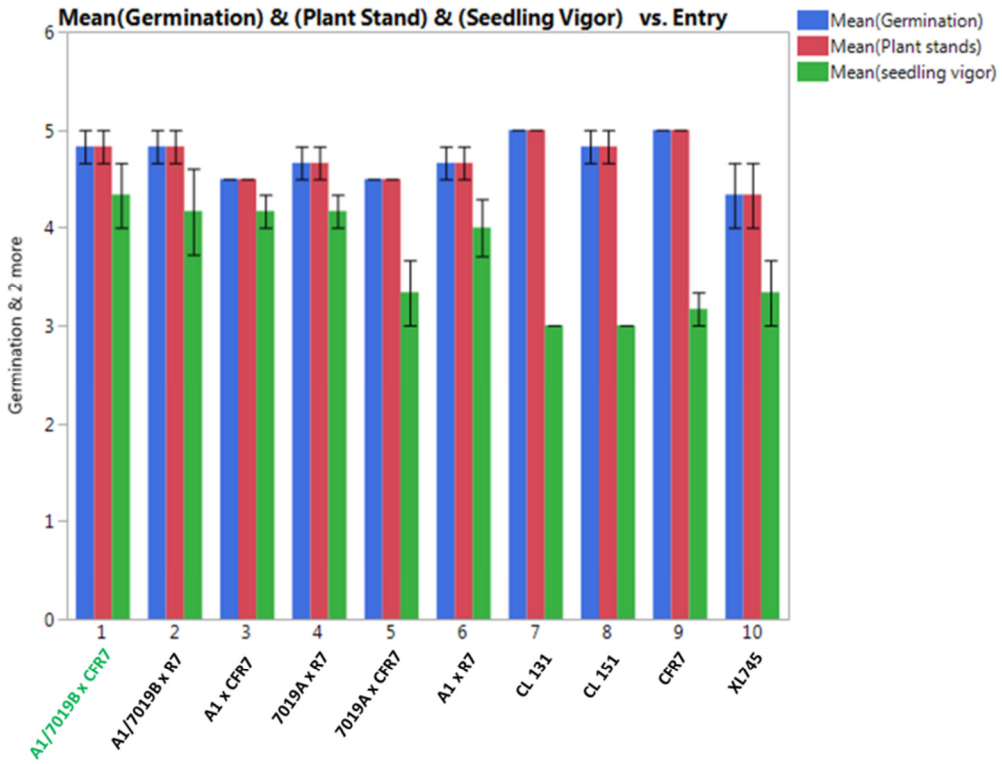


**Figure 10. Combined analysis of the germination, plant stand, and seedling vigor of the ten genotype entries from the yield trials from two years and two locations. Different letters represent significant differences at P<0.05 with LSMean Student's t-test at 95% confidence with JMP statistics. Each error bar is constructed using 1 standard error from the mean.**

Height is one of the most important factors for a uniform field. As expected, the inbred varieties (entries 7, 8, 9) were significantly shorter than the hybrids. XL745 is significantly taller than all others. Entries 1, 2, 4, and 5 were not significantly different from one another. Entries 3 and 6 were not significantly different from each other, and slightly shorter than other hybrids, entries 1, 2, 4, and 5. This might be due to the height of A1 being shorter than the other two females, 7019A and A1/7019B. However, differences among these lines were not easily visualized in the field (Fig 11).

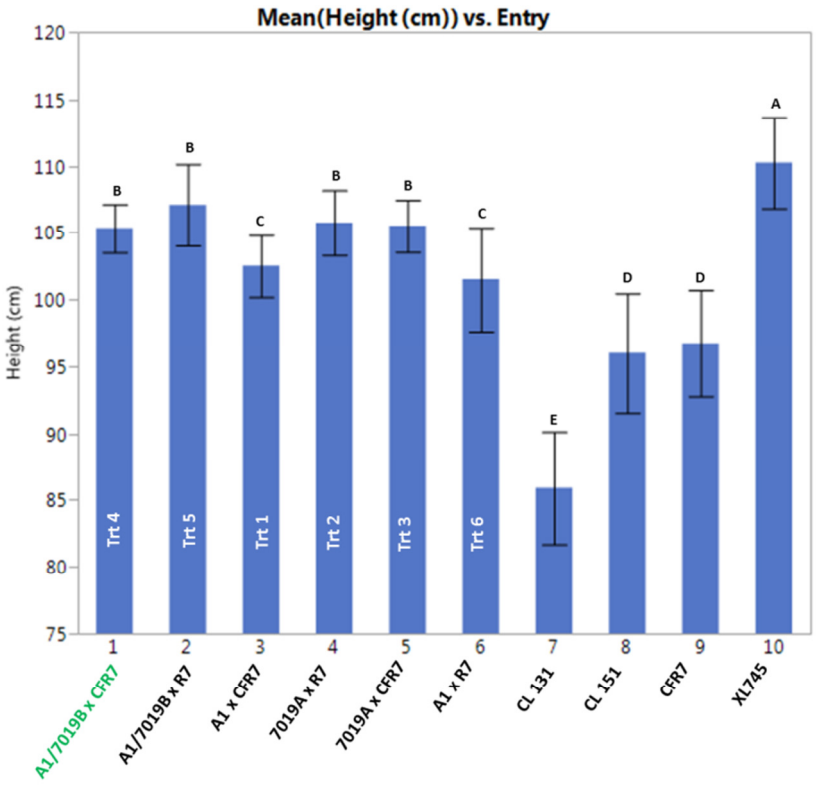


**Figure 11. Combined analysis of the height of the ten genotype entries from the yield trial from two years and two locations. Different letters represent significant differences at P<0.05 with LSMean Student's t-test at 95% confidence with JMP statistics. Each error bar is constructed using 1 standard error from the mean.**

Heading and maturity are other important factors determining uniformity and ease of harvesting. Heading and maturity can be affected by planting methods and soil fertilization. Direct seeded rice plants usually flower and mature a few days earlier than transplanted seedlings. Deficiency in nitrogen often stresses rice plants and hastens maturity, whereas heavy fertilization can delay crop maturity. Maturity is also known to be strongly affected by air temperature, and to a lesser extent, water temperature, especially when the crop is direct seeded. In this study, all treatments received the same management from seeding to harvesting except for the herbicide treatment on HT genotypes, which should have had no effect on plant performance. The maturity period is known to be regulated by polygenes, therefore selections for a similar

maturity during the backcrossing process of the converted B'-line is important so that transgressive segregation of the maturity habit in the 3way$F_1$ hybrids (Fig 12).

Recording the date of 50% heading of a population in a yield trial is a standard method to represent crop maturity. The time between flowering, ripening to grain maturity is relatively constant among lines, which is roughly 30-35 days in normal growing season (Vergara and Chang, 1985).

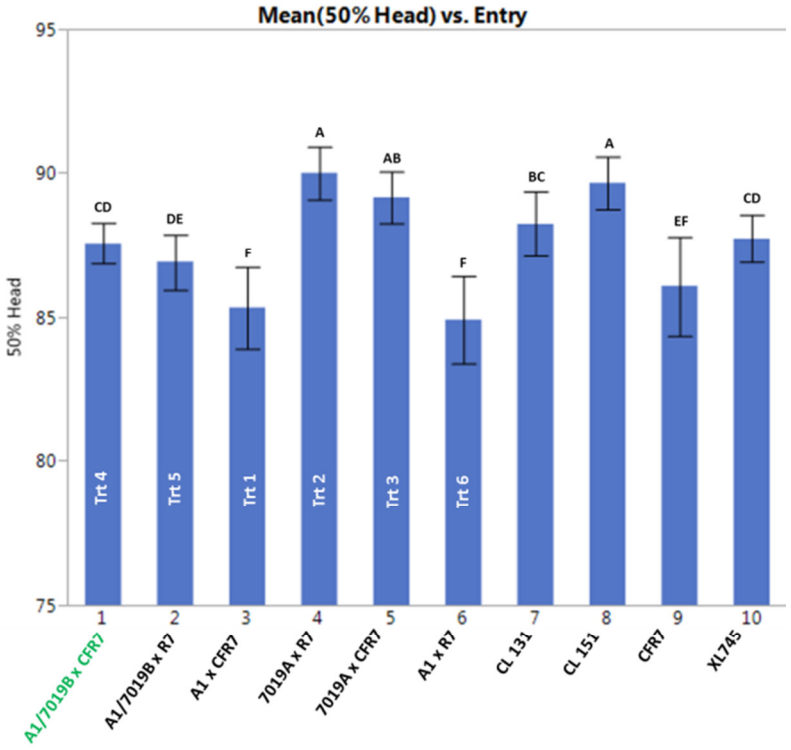


**Figure 12. Combined analysis of the days to 50% heading of the ten genotype entries from the yield trial from two years and two locations. Different letters represent significant differences at P<0.05 with LSMean Student's t-test at 95% confidence with JMP statistics. Each error bar is constructed using 1 standard error from the mean.**

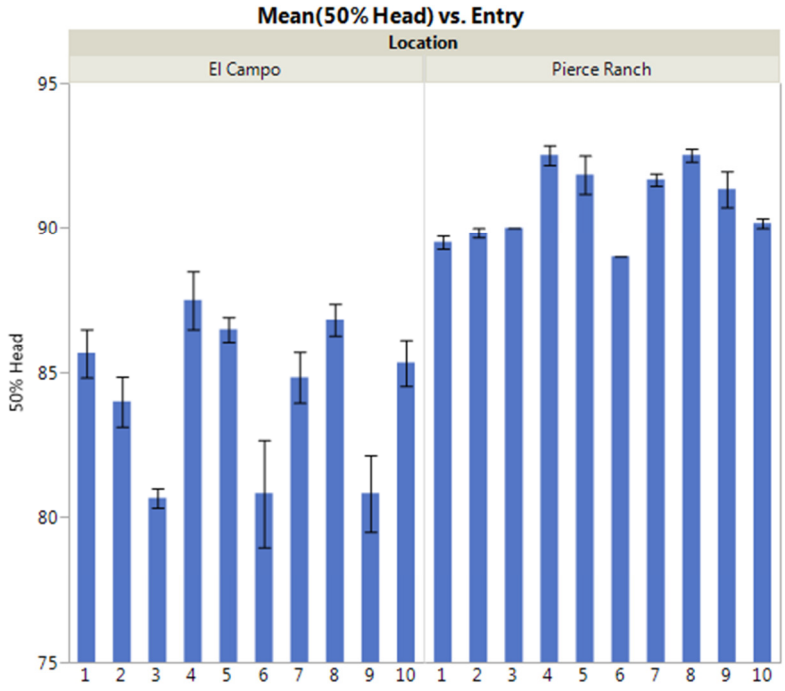


**Figure 13. Combined analysis of the days to 50% heading of the ten genotype entries from the yield trial by locations, combining two years. Each error bar is constructed using 1 standard error from the mean. Entry numbers represents the same genotype as in the Fig 20.**

Among all the effects in ANOVA for days to of 50% heading, only the effects of year and year*location were not significant (Appendix 2). There are significant differences between locations, most likely due to the trials at Pierce Ranch being planted almost three months later than the trials at El Campo. Due to the significant interaction, the day to 50% heading was analyzed by location (Fig 13). Rice at Pierce Ranch had a relatively longer growing period until they reached 50% heading compared to trials at El Campo in both years. Differences among genotypes for days to 50% heading were relatively smaller at Pierce Ranch than in the trials at El Campo. Given that planting at Pierce Ranch was almost three months later than the planting in El Campo, day lengths, air termperature differnces, water termperature differnces, and day/night temperature difference are assumed to have combined influenced the duration of plants of all genotypes. However, the degree of reaction to changes in day length was unique to each genotype. There was no consistent pattern of how 3way$F_1$ hybrids responded compared with the corresponding iso-hybrids. Variation in response to day length resulted in differences in the three-way interaction, year*entry, and location*entry of days to 50% heading.

| | | Yield | Milled Rice yield | | 1000 Grain Weight | L : W ratio | Chalkiness | Translucency | Color |
|---|---|---|---|---|---|---|---|---|---|
| **Entry** | **Pedigree** | **(kg/ha)** | **Milled (%)** | **Head (%)** | **(g)** | | **(%)** | **(1-5)** | **(1-5)** |
| **1** | A1/7019B//CFR7 | 7273 b | 65.8 cd | 54.3 e | 27.26 a | 3.33 | 2.33 cd | 5 | 1 |
| **2** | A1/7019B//R7 | 6753 c | 66.1 bcd | 56.3 de | 26.2 ab | 3.33 | 3.00 bc | 5 | 1 |
| **3** | A1/CFR7 | 6787 c | 65.8 cd | 58.5 bcd | 25.34 b | 3.00 | 4.33 b | 5 | 1 |
| **4** | 7019A/R7 | 6844 bc | 66.2 bcd | 56.9 cde | 25.00 b | 3.33 | 2.13 bc | 5 | 1 |
| **5** | 7019A/CFR7 | 6746 c | 65.8 cd | 59.1 abcd | 26.3 ab | 3.33 | 2.33 cd | 5 | 1 |
| **6** | A1/R7 | 6012 d | 67.4 ab | 61.9 ab | 25.64 b | 3.00 | 2.23 bcd | 5 | 1 |
| **7** | CL 131 | 4962 e | 67.6 ab | 62.1 ab | 22.60 c | 2.50 | 3.67 bc | 5 | 1 |
| **8** | CL 151 | 4945 e | 68.4 a | 62.8 a | 22.84 c | 2.50 | 11.00 a | 5 | 1 |
| **9** | CFR7 | 3684 f | 66.7 bc | 60.6 abc | 22.40 c | 4.00 | 1.00 d | 5 | 1 |
| **10** | XL745 | 8348 a | 66.7 bc | 60.6 abc | 26.18 ab | 3.33 | 10.20 a | 5 | 1 |

**Table 5. Combined analysis of the grain yields (kg/ha) and grain quality data of the ten genotype entries from the yield trial. Different letters represent significant differences at P<0.05 with LSMean Student's t-test at 95% confidence with JMP statistics.**

There were no significant interactions among years, locations, and entries for grain yields (Table 5). Among the two way interactions, only entry*location was significant, suggesting genotypes performed differently in reaction to the location. For both locations in both years, XL745 was consistently the highest yielding hybrid among entries. CL131, CL151, and CFR7 were consistently the lowest yielding inbred varieties at both locations in both years. Stability in the yield performance of these controls in all environments suggested that these lines were good candidates to be included in yield trials to test against other genotypes. The inbred control, CFR7, was the lowest yielding entry at both locations. Yield performance of the other hybrids, both 3way$F_1$s and 2way$F_1$s, did not considerably vary across locations. CL131 and CL151 were lines that had the most extreme differences in performance by location. These lines contributed most of the mean squares to the entry*location and location effects in the combined analysis. Grain yields in both years were not significantly different. Yields among entries were different.

Although 7019A and CFR7 were supposedly closely related to the original parents A1 and R7, they are classified as different lines with minor differences, including the trait of interest. These minor differences in the genetic backgrounds might contribute to levels of heterosis which affected grain yield or other traits in hybrids as a result of genetic linkage. This might be the reason the three iso-hybrids, entry 3 (A1/CFR7), entry 4 (7019A/R7), and entry 5 (7019A/CFR7) had higher yields than the original hybrid, entry 6 (A1/R7). Another possible reason is that the three iso-hybrid plots were treated with NewPath herbicide which confirmed tolerance to the corresponding herbicide and to control the weeds at the early stages of crop development, which is usually the most critical stage for the weed control in rice. Entry 6 might have suffered some degree of biological stress from weed competition compared to the other entries.

Entry 2 (A/7019B//R7) had a lower grain yield compared to its iso-hybrid, entry 1 (A/7019B//CFR7). The differences could be the result of varying degrees of heterosis from the combination of 7019B with CFR7 or from the combination of A1 with CFR7 because that female is hemizygous as a result of the hybridization between A1 and 7019B. Entry 1 (A1/7019B//CFR7) may have encountered little biological stress due to weed competition, which could have provided an advantage for grain yielding compared to entry 2 (A1/7019B//R7) because entry 2 did not receive any NewPath herbicide treatment at the seedling stage since half of the population would be killed by the herbicide since the male parent, R7, was not tolerant to the herbicide.

To assess the possibility of higher heterosis from acquiring the 3rd parent in the 3way$F_1$ compared with its iso-hybrids with only two parents in the $F_1$, yield performance of the 3way$F_1$ was compared to the other entries having the same male parent in the hybrid combination. It is more appropriate to compare entry 1 (A/7019B//CFR7) to entry 3 (A1/CFR7) and 5 (7019A/CFR7) than to compare entry 2 (A1/7019B//R7) to entry 4 (7019A/R7) and 6 (A1/R7). Because entries 1, 3, and 5 had all been treated with a herbicide, and entries 2 and 6 were not treated with herbicide, while entry 4 was treated. The yield of entry 1 (A/7019B//CFR7) is higher than both entries 3 (A1/CFR7) and 5 (7019A/CFR7), suggesting there is a chance of more heterosis in the 3way$F_1$ because it has three parents in the hybrid.

Although entry 1 (A1/7019B//CFR7) had a higher yield than entry 2 (A/7019B//R7), 3 (A1/CFR7), and 5 (7019A/CFR7), the differences were economically inconsequential. To assess the effects of copy number of HT genes in hybrids, entry 3 (A1/CFR7) was compared to entry 4 (7019A/R7), which had only one copy of the HT gene from the male parent and female parent respectively with entry 5 (7019A/CFR7), having two copies of the HT genes from both parents. Entry 5 (7019A/CFR7) was not different than entry 3 (A1/CFR7) and 4 (7019A/R7), suggesting

that the copy number of the HT gene in the hybrid did not have any significant effects on yield. This is consistent with the observation that all entries with HT gene, homozygous or heterozygous, did not have any injuries from the herbicide application.

Yield performance of the 3way$F_1$ hybrid and its stability across environments was the most important determining factor of its viability as a useful process of creating hybrid seed. These results showed that yield is not negatively affected by introducing the 3$^{rd}$ parent into the 3way$F_1$ hybrids. There is even possible existence of additional heterosis from incorporating the 3$^{rd}$ parent into the 3way$F_1$ hybrid compared to a more traditional two-parent hybrid system.

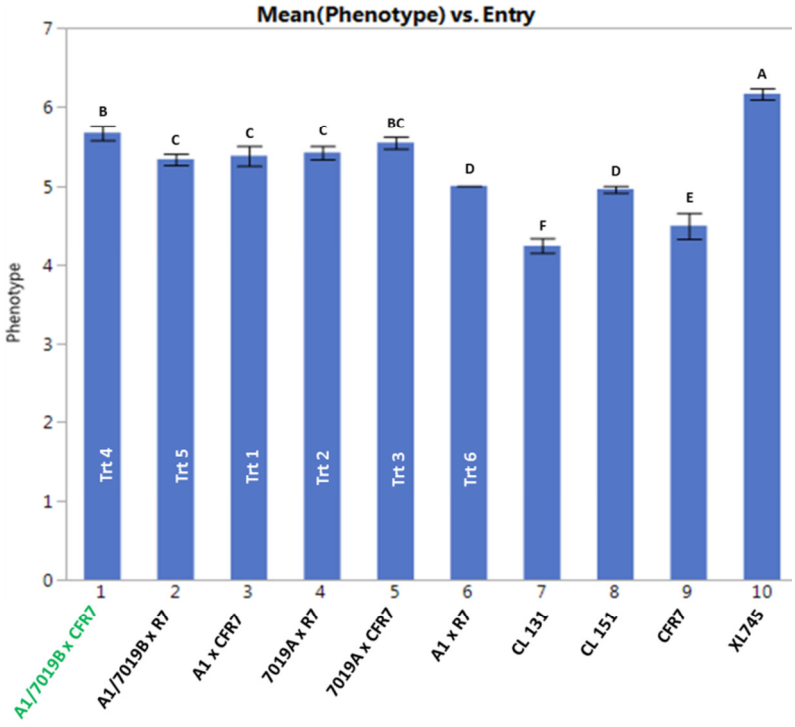


**Figure 14. Combined analysis of the phenotype rating of the ten genotype entries from the yield trial from 2 years and 2 locations. Different letters represent significant differences at P<0.05 with LSMean Student's t-test at 95% confidence with JMP statistics. Each error bar is constructed using 1 standard error from the mean.**

The phenotype rating is an overall evaluation of the plant type. XL745 has a significantly higher rating of phenotype, which is consistent with its consistent highest yield and its general appeal of the plant type across years and locations (Fig 14).

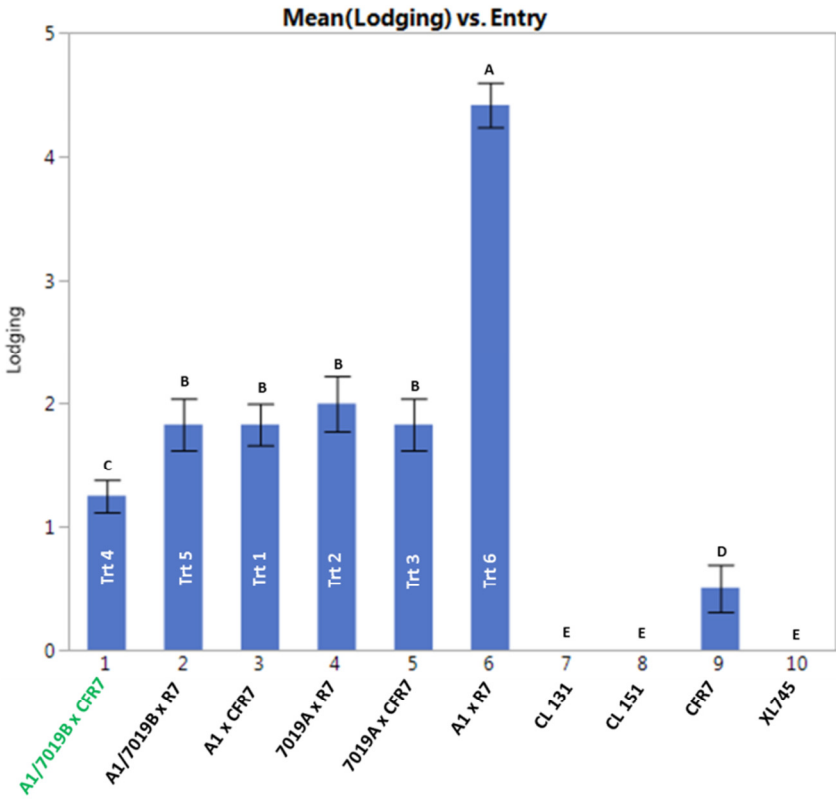


**Figure 15. Combined analysis of the lodging rating of the ten genotype entries from the yield trial from 2 years and 2 locations. Different letters represent significant differences at P<0.05 with LSMean Student's t-test at 95% confidence with JMP statistics. Each error bar is constructed using 1 standard error from the mean.**

Lodging was rated on a scale of 0 to 5, with 0 equal to all plants being completely upright and five being all plants lying on the ground. CL131, CL151, and XL745 were consistently the least lodged entries across years and locations (Fig 15). CL131 and CL151 had shorter plant stature and smaller panicles, while XL745 had stronger straw which primary contributor to lodging resistance. The original hybrid entry 6 (A1/R7) had the most severe lodging issue across locations and years, which could have resulted in the loss of yield. Entries 2, 3, 4, and 5 were not different in lodging tendency among each other, but entry 1 (A1/7019B//CFR7) was less likely to lodge. There were not differences in plant height among

the six iso-hybrids, so this does not explain the differences in lodging scores within entry 6 (A1/R7). Other genetic factors related to lodging resistance include the accumulation of the carbohydrates in the culm at fully ripe stage, culm components (lignin content), and length and diameter of the internodes. There have been studies on the inheritance of straw strength, and it has been generally agreed upon that hybrids show more resistance to lodging although they are often 5-10 cm taller than the inbred parents. More studies are still needed to answer whether the observations within this study are the result of significant improvement of straw strength attributable to heterosis in the iso-hybrids compared to the original hybrid entry 6 (A1/R7). However, if heterosis could be a contributor to decreased lodging, it is consistent with this study's hypothesis that the 3way$F_1$ results in progeny with greater heterosis from having three parents.

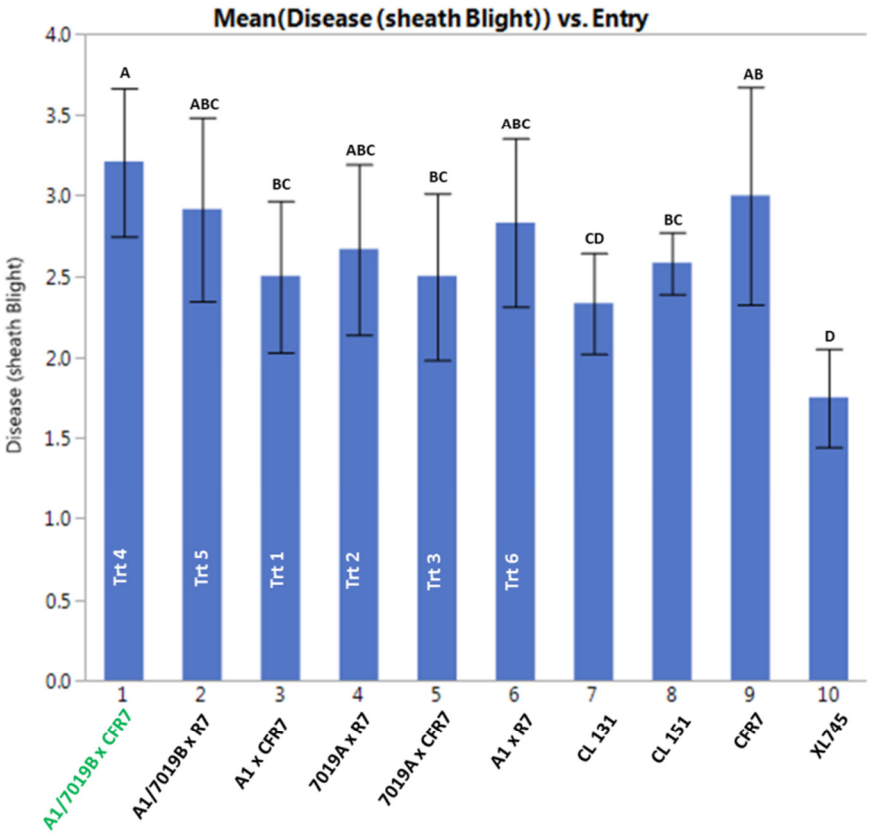


**Figure 16. Combined analysis of the disease responses rating to sheath blight of the ten genotype entries from the yield trial from two years and two locations. Different letters represent significant differences at P<0.05 with LSMean Student's t-test at 95% confidence with JMP statistics. Each error bar is constructed using 1 standard error from the mean.**

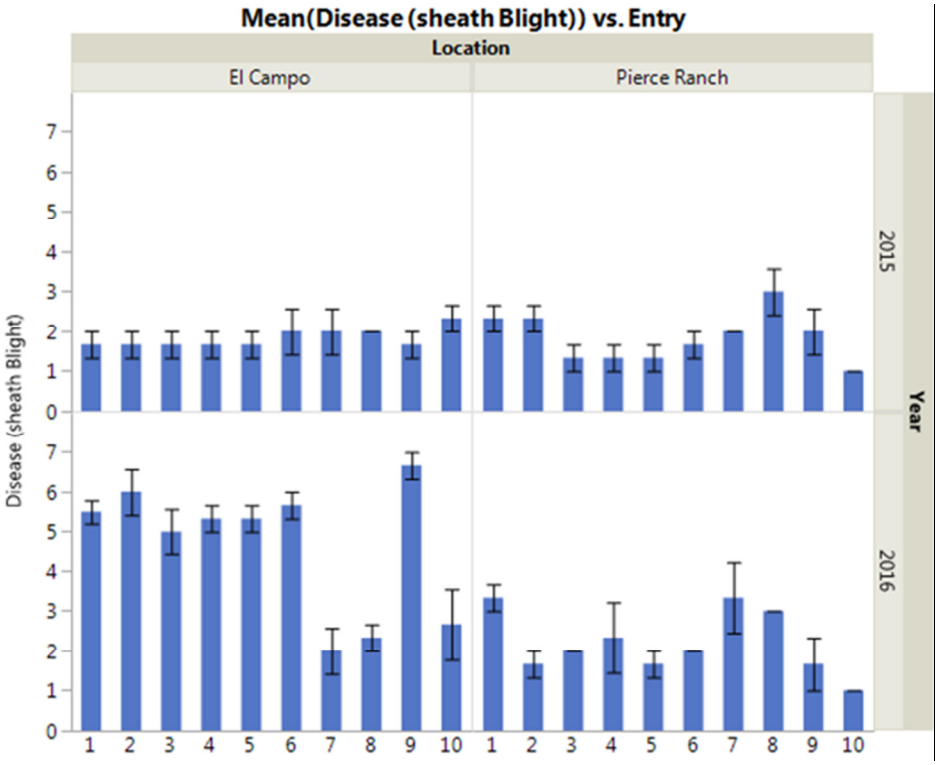


**Figure 17. Analysis of the disease responses rating to sheath blight of the ten genotype entries from the yield trial by years and locations. Different letters represent significant differences at P<0.05 with LSMean Student's t-test at 95% confidence with JMP statistics. Each error bar is constructed using 1 standard error from the mean. Entry numbers represents the same genotype as in the Fig 16.**

Rating plants for infestation severity to sheath blight, a fungal disease caused by *Rhizoctonia solani*, is critical because damage could result in leaf area or tiller senescence and eventual yield reduction (Fig 16). Sheath blight is most severe in areas with high temperatures, high relative crop canopy humidity, and high levels of nitrogen fertilizer. Plants can be most vulnerable during the seed maturation period after heading during heavy rainfall. At El Campo and Peirce Ranch, 2016 had a higher rate of precipitation, and a higher max/min temperature compared to 2015. The Pierce Ranch was planted almost three months later than El Campo, and therefore the temperature was lower at Pierce Ranch during the grain maturation stage.

Therefore, it is more appropriate to analyze responses to sheath blight disease by year and location separately instead of doing the three-way factorial combining analysis (Fig 17). In 2015 that had moderate rainfall at both locations, there were no differences among hybrid entries and control varieties. However, in 2016, especially at El Campo, all the iso-hybrids were more severely infected than the conventional inbred control lines, CL131 and CL151, most likely because there were no major QTLs for resistance in the pedigrees. Moreover, the hybrids had a higher number of tillers compared to the inbred varieties, which may have resulted in a denser canopy which is a favorable environment for the fungal disease to develop. Whereas in the rest of the three less favorable environments for the disease to develop, there is no significant differences in response to sheath blight disease between the hybrids and the inbred varieties.

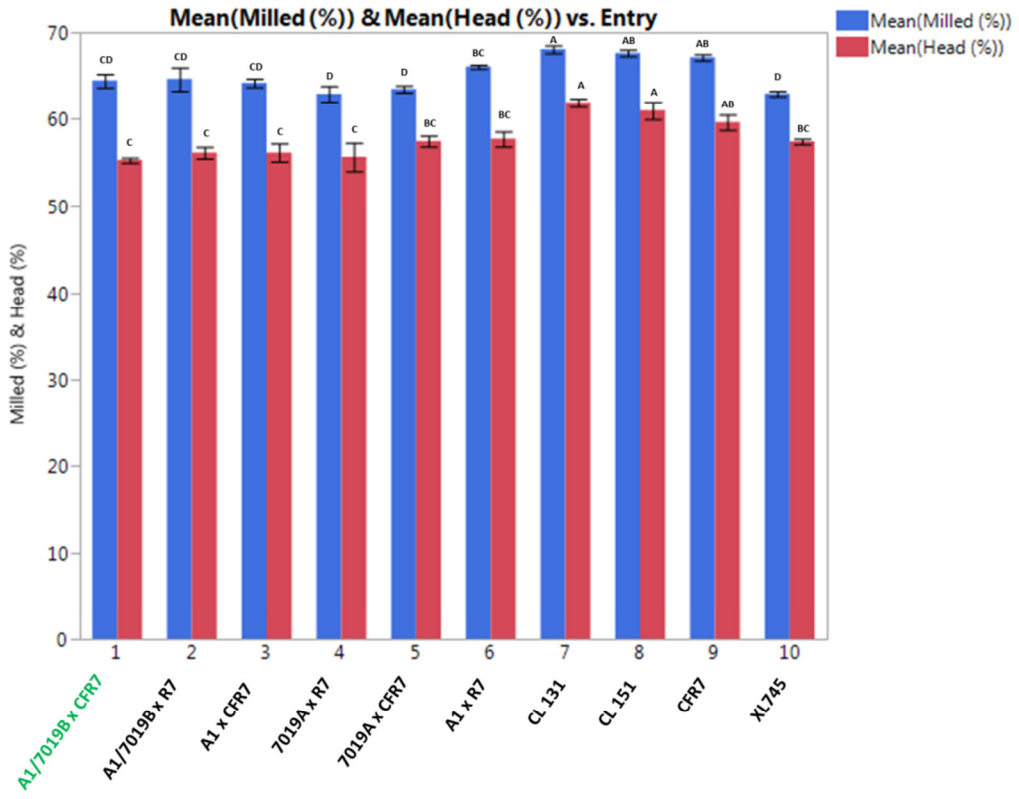


**Figure 18. Analysis of the percentages of milled rice and head rice of the ten genotype entries from the yield trial from two years and two locations. Different letters represent significant differences at P<0.05 with LSMean Student's t-test at 95% confidence with JMP statistics. Each error bar is constructed using 1 standard error from the mean.**

In general, the inbred varieties had a slightly higher percentage of milled rice and head rice compared to the hybrids. However, there were no differences among the hybrid entries, nor among inbred lines.

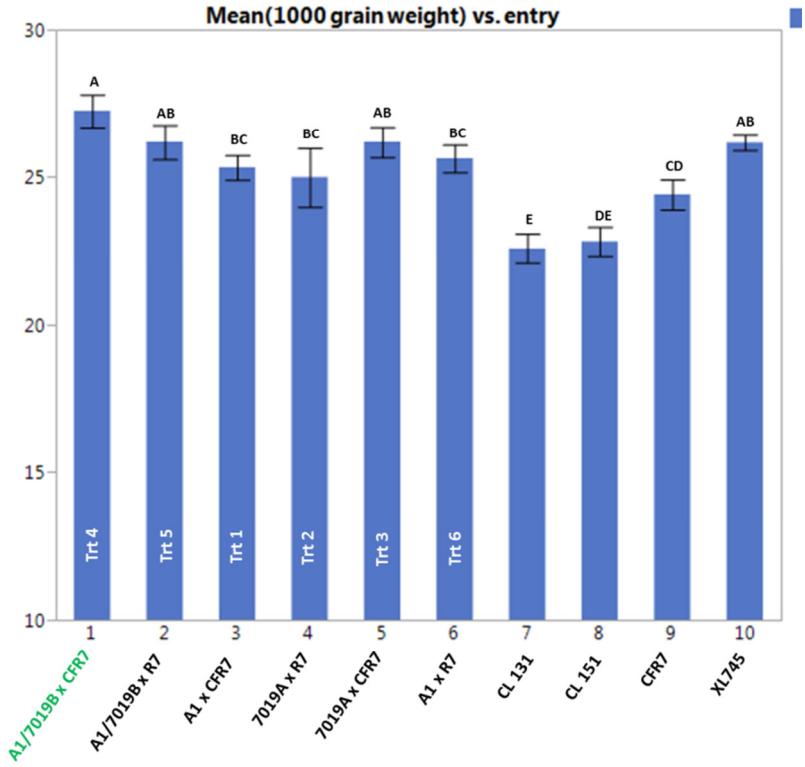


**Figure 19. Analysis of the 1000 grain weight measured in grams of the ten genotype entries from the yield trial from two years and two locations. Different letters represent significant differences at P<0.05 with LSMean Student's t-test at 95% confidence with JMP statistics. Each error bar is constructed using 1 standard error from the mean.**

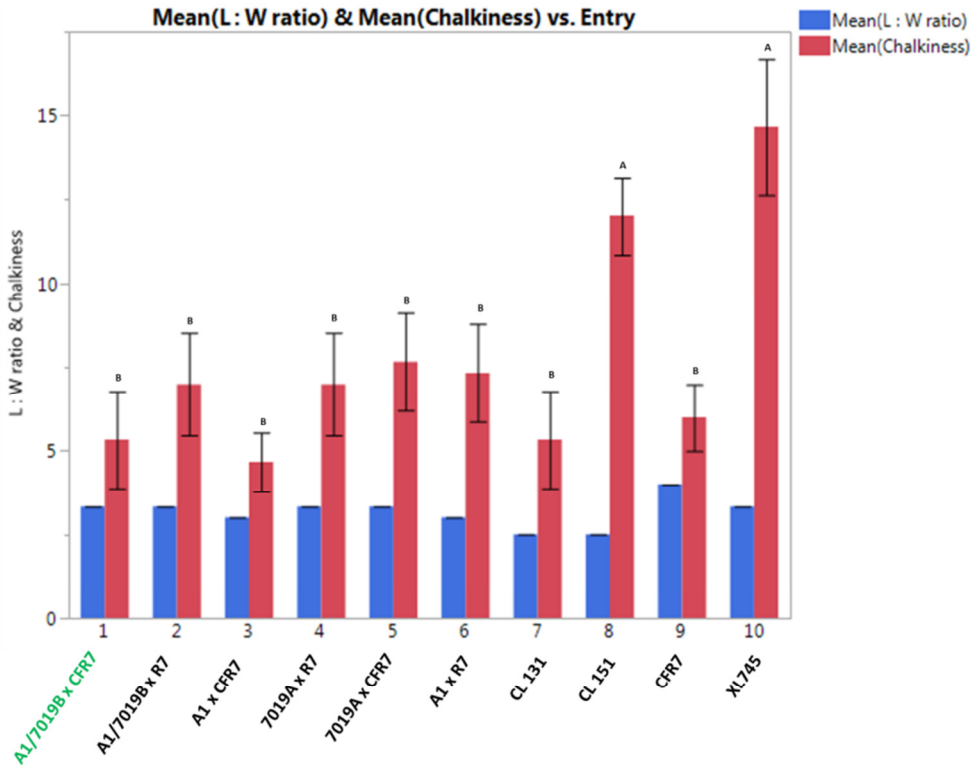


**Fig 20. Analysis of the percentages of the chalkiness and length : width (L:W) ratios of the ten genotype entries from the yield from two years and two locations. Different letters represent significant differences at P<0.05 with LSMean Student's t-test at 95% confidence with JMP statistics. Each error bar is constructed using 1 standard error from the mean.**

The grain quality of the ratio of the length and width of the grains is used internationally to classify varieties into long, medium, or short grains. The consistency of the ratio also serves as a criterion to describe the uniformity and purity of the grains. 1000 grain weight gives more information about the density of the grains when the grains are standardized for moisture (Fig 19). Means and standard errors were calculated from the five sub-samples of randomly selected 1000 grain weights. Five out of the six iso-hybrids did not have differences for grain weight. Only the 3way$F_1$ entry 1 (A1/7019B//CFR7) was heavier than entries 3 (A1/CFR7), 4 (7019A/R7), and 6 (A1/R7). CL131 and CL151 had the lightest grain weight, which was consistent with its L:W ratio. It was smaller than all other entries (Fig. 20).

Chalkiness is one of the most important ratings when evaluating the quality of rice grains. The chalk refers to the opaque area in the rice grain and is an undesirable trait in rice. Chalk occurs most commonly when high night time temperatures occur during grain development. Chalky grains tend to break during the milling process more frequently than translucent grains, which result in lower percentages of whole grains and devaluation of the head rice. A percentage of chalkiness of < 10% is generally considered to be high quality and 10-25% is acceptable, but the selling price might be affected by this (IRRI, http://www.knowledgebank.irri.org/). Data suggests that the six iso-hybrids and the inbred male parents of the three hybrids did not differ in the amount of chalkiness, which all could be classified into the high quality category. XL151 and XL745 had a relatively higher percentage of chalkiness, but was still acceptable. The ratings of translucency (1-5) and the color (1-5) of the head rice are the other two general grain appearance evaluation of a variety, which did not differ among entries (Fig 20).

*4.5 Objective 5- Patent application*

We have successfully obtained the patent through World Intellectual Property Organization (WIPO) (Frank et al., 2014).

# 5. CONCLUSION

This new production method is a viable option for the following reasons:

1. There were no seed set yield differences among iso-hybrids from the EPS fields suggesting the introduction of a third line carrying a gene of interest into the original three line hybrid production system did not negatively affects pollen receptivity of female plants.

2. Introducing the HT gene into the R-line did not have negative effects upon the pollination and fertility restoration ability of female plants.

3. Utilization of the heterosis, if it exists, in the A1/7019B as female resulted in a higher stigma viability, which leads to a higher seed set yield in hybrid seed production.

4. There were no grain yield differences among the six iso-hybrids from the yield trials, suggesting the introduction of a third line carrying a gene of interest into the original three line hybrid production system does not cause any negatively impact the heterosis influence on yield performance.

5. There were no differences in the response to diseases, especially sheath blight, among the iso-hybrids.

6. There were no negative effects on germination, plant stand, seedling vigor, and straw strength from the 3way$F_1$ system compared to other iso-hybrids.

7. There were no differences in the milled rice (%), head rice (%), and other grain quality ratings among the iso-hybrids.

8. There was no segregation of traits related to yield or yield components in the 3way$F_1$ hybrids.

9. This method allows testing of a new herbicide system earlier than using the current hybrid combination and enables breeders to make decisions later in the process on whether to do a conversion to A'-line. Thisit adds flexibility and diversity in herbicide usage.

10. Makes the most use of the current elite A-line.

11. Since B-lines are male fertile like R-lines, conversion of such lines into isogenic B'-lines containing one gene of interest can be conveniently achieved by repeated backcrossing. Breeders can develop various isogenic B-'lines to the same A-line that is used in a promising hybrid combination with few differences including the gene of interest responsible for resistance to different herbicides. The promising hybrid can thus be modified by incorporating different converted B'-lines that result in hybrids with known heterosis, but possessing resistance to different herbicides. The types of herbicide resistance B lines can be developed based upon market needs.

12. The converted B'-lines do not need to be fully converted to the corresponding A'-line, which would take more time and labor due to possible linkage genes causing partial fertility. This would take more generations to backcross to make sure it meets the requirements for a stable male sterility and hence be classified as a female line.

13. Incorporation of herbicide tolerance into the A-line only needs to take place at the last step of producing female seeds for the hybrid seed production. By crossing A x B' to generate the A-line seeds with hemizygous state at that specific locus increases the versatility of the commercial hybrid containing the specific gene of interest that confers a specific trait.

14. There is no need to multiply A-lines with each of the different HT B'-lines to maintain the different B'-lines. The B'-lines are multiplied by simply self-pollinating plants, which requires

minimal labor and time. The amount of each B'-line to be produced can be based upon the market demands. Although glyphosate and glufosinate are the most popular HT traits, market demand for different herbicides will increase over time due to evolving weed spectrums in different countries and the needs for rotating different HT systems to combat HT weeds, especially glyphosate-resistant weeds.

15. In the ESP production field, treatment 5 was a restorer line that was a non-HT R-line, therefore the producers can mix planting the hemizygous A/B' seeds and the R-line seeds instead of strip planting to increase the outcrossing rate. The pollinator R-lines can be removed by spraying the corresponding herbicide after pollination. Seeds set can thus be harvested from all the remaining plants as pure hybrid seeds without possible contamination from the R-line seed mixture.

16. All of the non-herbicide tolerance plants, including genetic variants, volunteers, or red rice, will be removed by herbicide application in the hybrid seed production field and therefore increase the purity of the hybrid seeds produced and allows for better profits.

17. Herbicide rogueing greatly reduced the cost for labor.

18. The CMS line is generally believed to be more important than two other lines (maintainer and restorer lines) in the three-line hybrid system, because it takes more effort to develop and crossed with two other lines resulting in a greater genetic contribution to the hybrid. The intellectual property protection can be ensured with this proposed method because the original female and it's maintainer lines (A1/B1) used in the promising hybrid are the most valuable genotypes in this combination, whereas the chances of unintentionally mixing A1 and B1 seeds in the hybrid seeds for sale will be minimized in the step of producing hybrid seeds where growers can spray herbicides and kill any B1 carried over from the previous steps. The A1xB1

small acreage increase will receive the highest level of protection among all the foundation seed production.

19. There is a possibility of improving synchronization between A-lines and R-lines in the ESP. However, this applies only when the female parent and the male parent of the original hybrid are more than seven days apart in days to 50% of heading. Breeding and selection for the isogenic A'-line that is closer to the anthesis of the R-lines will result in a hemizygous A/B' line closer to the R-line or R'-line in terms of maturity, which facilitates pollination and thus seed set yield on the female plants in the ESP.

This proposed technology incorporating herbicide tolerance gene in the hybrid seed production can be extended to the resistance to other herbicides resistance allele, including sulfonylurea, glyphosate, glufosinate, benzonitrile, cyclohexanedione, phenoxy proprionic acid and L-phosphinothricin. Other genes of interest, such as cold tolerance, insect resistance, fungus tolerance, disease tolerance, drought tolerance, salinity tolerance, submergence tolerance, or even some quality traits can also be considered as future application as long as it is nuclear-inherited and acting in a dominant expression pattern, monogenic resistance.

The herein method are not limited to the use or hybrid rice, but can be extended to other hybrid crops utilizing three-line production systems, including wheat (*Triticum aestivum*), corn (*Zea mays*), cotton (*Gosssypium hirsutum* or *Gosssypium barbadense*), soybean (*Glycine max*), sorghum (*Sorghum bicolor*), rapeseed (*Brassica napus*), mustard seed (*Brassica juncea*), barley (*Hordeum vulgare*), oat (*Avena sativa*), rye (*Secale cereale*), pearl millet (*Pennisetum typhoides*), alfafa (*Medicago sativa*), tomato (*Lycopersicon esculentum*), sugar beet (*Beta vulgaris*), sunflower (*Helianthus annuus*), onion (*Allium cepa*), petunia (*Petunia hybrid*), or carrot (*Daucus carota*).

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## APPENDIX

## TABLES

| SOV | DF | F ratio | Prob > F |
|---|---|---|---|
| Year | 1 | 36.5660 | 0.0038 * |
| Entry | 5 | 28.8427 | <0.0001 * |
| Year*Entry | 5 | 11.3940 | <0.0001 * |

**Table A.1. F-test of Entry, Year and Entry*Year interaction effects for seed set yield from ESP. ns indicates non-significant factor and * indicates significant at 99% confidence with JMP statistics.**

| SOV | DF | F ratio | Prob > F |
|---|---|---|---|
| Year | 1 | 0.5122 | 0.4946 $^{ns}$ |
| Location | 1 | 196.7529 | <0.0001 * |
| Year*Location | 1 | 0.0205 | 0.8897 $^{ns}$ |
| Entry | 9 | 20.7060 | <0.0001 * |
| Year*Entry | 9 | 4.2381 | 0.0002 * |
| Location*Entry | 9 | 7.6737 | <0.0001 * |
| Year*Location*Entry | 9 | 4.1683 | 0.0002 * |

**Table A.2. F-test of Entry, Year, Location, and all possible interactions effects for 50% heading from yield trial. ns indicates non-significant factor and * indicates significant at 99% confidence with JMP statistics.**