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Characterization of Maize Sex-Determination Gene Orthologs in Rice (*Oryza Sativa L. Japonica Cv. Nipponbare*)

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CHARACTERIZATION OF MAIZE SEX-DETERMINATION GENE
ORTHOLOGS IN RICE (*ORYZA SATIVA L. JAPONICA CV. NIPPONBARE*)

BY

JOHN VENTURA

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
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OF

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2012

ABSTRACT

Ensuring food security will be a crucial problem in the coming decades. Heterosis, the phenomenon where hybrid progeny exhibit increased vigor in comparison to their inbred parents, has been exploited to increase agricultural yield in maize (*Zea mays*) for decades due to its monoecious reproductive state. Unisexuality in maize is genetically controlled due to the concerted action of the *Tassel seed1* (*ts1*), *Tassel seed2* (*ts2*), and *Silkless1* (*sk1*) genes. Orthologs of the *Tassel seed* genes and *sk1* can be found in the rice genome even though rice maintains an ancestral cosexual floral state. This study identified maize sex determination (SD) orthologs in rice, and included subsequent characterization of these orthologs in wild-type and transgenic rice tissues. Rice *ts1* (*rTs1*) was confirmed to be japonica rice MSU-TIGR gene locus LOC_Os04g37430, rice *ts2* (*rTs2*) was confirmed to be locus LOC_Os03g18740, and rice *sk1* (*rSk1*) was confirmed to be LOC_Os04g44354 based on bioinformatic analysis. RT-PCR and multiplexed Taqman qRT-PCR experiments showed that these genes were broadly expressed throughout the plant during vegetative growth in leaves, shoots, and roots, and the *rTs1* and *rTs2* genes are expressed in inflorescences. Transgenic analysis, using *GUS* reporter translational fusions, revealed that *rTs1* and *rTs2* are expressed in leaf vasculature and that *rTs2* is expressed in spikelet vasculature, developing anthers, and emerging roots. These results suggest that SD orthologs in rice may be involved in development, specifically with programmed cell death processes, which may involve jasmonic acid signaling.

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CHAPTER 1

INTRODUCTION

Hybrid Vigor and Food Security

“Heterosis” is the phenomenon where heterozygous F1 hybrids, derived from homozygous inbred parents, demonstrate superior phenotypic performance. Heterosis, also called “hybrid vigor”, was first observed in the 19th century, most notably by Charles Darwin (Darwin, 1876), and famously demonstrated in corn by George H. Shull and Edward M. East using hybrid corn varieties developed by William James Beal. By 1917, Donald F. Jones successfully introduced one of the first hybrid corn breeding programs, and this allowed large-scale farming of high-yielding hybrid corn varieties. Heterosis has also been demonstrated in animal breeding programs, examples being the “Black Baldy” hybrid between the Black Angus and Hereford cattle breeds and numerous breeds of domesticated dog (Z. J. Chen, 2010). The concept of superior performance is relative to the trait observed, as heterosis can be considered either positive or negative. For instance, positive heterosis is associated with increase yield or stress tolerance, whereas negative heterosis may be used to describe for decreasing time to seed maturation (Alam et al., 2004).

The genetic basis of heterosis has been debated for many years, and both Mendelian and non-Mendelian factors have been implicated. One such suggestion claims that heterosis may occur due to the heterozygotic advantage of possessing

dominant alleles as protection against possibly deleterious recessive traits. This is known as the “dominance hypothesis,” first proposed by Charles Davenport (Davenport, 1908). An alternative theory proposed that overdominance, when a heterozygous condition has a more pronounced effect on phenotype than either homozygous condition for a gene with contrasting alleles, may provide a possible explanation for the causes of heterosis. This “overdominance” theory has been advocated on several fronts due to the fact that overdominance has been established in single genes with possible links to heterosis. Possible evidence for overdominance in polygenic traits has been born out from Quantitative Trait Locus (QTL) mapping (Krieger, Lippman, & Zamir, 2010). This proposes a possible third “pseudo-overdominance” theory, where tightly linked gene loci (such as a dominant beneficial allele linked to a unrelated deleterious allele of another gene) can skew the actual measure of overdominance (Birchler, Yao, Chudalayandi, Vaiman, & Veitia, 2010). It is apparent that the genetic underpinnings of heterosis are intricate and complex, due to the fact that heterosis is inextricably linked to the inheritance of quantitative traits, which involves a complex array of possible genetic interactions.

Heterosis may be influential in addressing problems facing global food security. It is expected that the human global population will continue to grow at an exponential rate, until gradually leveling to approximately 9 billion in about fifty years (as reviewed by Godfray et al., 2010). Although previous green revolutions allowed for the distribution of elevated volumes of cereal food crops to many areas around the world, it is estimated that 1 in 7 people still fail to meet their necessary daily allotment of nutrition. Global nutritional deficiencies will only be exacerbated with the increase

in demands for meat, dairy, and agricultural goods as the end of the current century approaches. The need for food is a priority for policy makers in today's geopolitical climate and therefore provides a critical avenue of research for biologists, agriculturists, and farmers who must work in concert to seriously address these upcoming threats to the wellbeing and survival of the human race.

Maize (*Zea mays*) and cultivated rice (*Oryza sativa*) are members of the grass family (Poaceae) of monocotyledonous plants with great agronomic importance due to their essential contributions to global food systems. Maize is a monoecious plant, producing a male terminal inflorescence, the tassel, and female lateral inflorescences, the ears (as reviewed by Bortiri & Hake, 2007), making the plant easily amenable to crossbreeding between varieties to produce agriculturally superior cultivars. Due to its monoecious state, maize can generate hybrid varieties expressing certain agriculturally favorable phenotypes via outcrossing. Therefore, heterosis can be easily exploited in maize due to the fact that maize F1 hybrids display superior yield and fertility compared to the F0 parental generation (discussed by Lippman & Zamir, 2007). An estimated 15% more crop yield has resulted from the selective breeding of maize hybrids and this percentage has steadily increased over time (as reviewed by Duvick, 2001).

Rice is estimated to feed at least half of the world's population. Rice, however, has perfect inflorescences and is naturally self-pollinating. This reproductive strategy poses a challenge in developing hybrid varieties, which must be overcome if the routine production of hybrid rice is to become a commercial reality. Heterotic response has been observed in rice for agriculturally significant traits, such as

panicle length and grain weight and gains per panicle (Virmani, 1994). These observations suggest that if rice hybrids could be generated more efficiently, then heterosis would be a possibility for increasing yield in this essential food crop for future generations.

Sexual Reproduction in Flowering Plants

Flowering in plants (angiosperms) begins with an indeterminate vegetative shoot apical meristem transitioning to a floral meristem, which ultimately produces the primary sight of sexual reproduction, the flower. Floral meristems can produce a number of floral organs such as sepal, petals, carpels and stamens. The gametophyte generations are housed in the stamens and carpels; stamens harbor the male gametophyte (pollen grains) and carpels harbor the female gametophyte (the embryo sac). Flowers can be classified as either bisexual or unisexual, the former containing mature carpels and stamens in a single flower; the latter exhibiting either functional carpels or stamens but not both, a condition termed dicliny. Some 20-30% of flowering plants display some form of diclinous sexual state, with male and female flowers segregated on different plants (dioecious) or on the same plant (monoecious) (Tanurdzic & Banks, 2004). Nevertheless, the predominant sexual mode in plants is a hermaphroditic floral state in which the flowers are bisexual and contain both stamens and carpels.

Bisexual flowers function in both cross-pollination (allogamy) and self-pollination (autogamy). Cross-pollination, or outcrossing, minimizes the likelihood of

inbreeding depression while maximizing heterozygosity, increasing genetic variability and genetic exchange (reviewed by Dellaporta & Calderon-Urrea, 1993). Allogamy can be promoted by the intercession of the wind, water, or an insect vector, the spatial arrangement and morphological variation of floral sex organs (herkogamy), or temporal differences in sex organ development (dichogamy), where stamens and carpels mature at different times. This variation also allows for a diversity of sexual polymorphisms to occur within populations in individual plant species, where each sexual morph can display one or a subset of differing heterostylous conditions (reviewed by Barrett, 2002). Plant species with bisexual flowers also engage in self-pollination strategies, which can also confer some evolutionary advantages. By eliminating the need for another conveyer of genetic material, autogamous plant species save on the energy cost of segregating individuals into two sexes (discussed in Dellaporta & Calderon-Urrea, 1993). Selfing can also preserve beneficial genotypes in rapidly changing environments that encourage quick reproduction cycles and can maintain the option of putatively outcrossing to another individual in the selfing population (Niklas, 1997).

The Molecular Basis and Regulation of Flower Development

The development of flowers is under the control of both extrinsic and intrinsic factors. A cascade of regulatory factors and signaling molecules interact to promote and regulate meristematic transitioning as well as floral organ maturation. This pathway is best characterized in *Arabidopsis* and *Antirrhinum* systems (reviewed by

Sablowski, 2007). Early steps in floral development require well-coordinated signaling events that promote the transition from indeterminate vegetative meristems to flowers. This signaling pathway is directed by the activity of a suite of regulatory transcription factors acting upstream of well-characterized floral meristem transcription factors. In *Arabidopsis*, for instance, these early signaling steps require the activity of the *LEAFY* (*LFY*) and *FT* genes, which act in concert with phytohormones, such as gibberellin, to disrupt the *WUSCHEL* / *CLAVATA* signaling pathway necessary for the maintenance of vegetative meristem indeterminacy (Blazquez, Green, Nilsson, Sussman, & Weigel, 1998; Weigel, Alvarez, Smyth, Yanofsky, & Meyerowitz, 1992) (Figure 1). Following the conversion of a vegetative meristem to an inflorescence meristem, the synchronized activity of A, B, and C class MADS-box transcription factors control the ontological fate of each whorl in the newly developing floral meristem. Downstream targets of A class genes specify sepal development in the first whorl, the combined activity of A and B class genes and B and C class genes determine petal development in the second whorl and stamen development in the third whorl respectively, and the action of C class genes alone promote carpel development in the fourth whorl (Coen & Meyerowitz, 1991).

This so-called ABC model (illustrated in Figure 1) is well conserved in flowering plants. Homologs have been identified in tomato (Pnueli et al., 1991), tobacco (Hansen, Estruch, Sommer, & Spena, 1993), and in the representative monocotyledonous species maize (Schmidt et al., 1993) and rice (Gao et al., 2010). Rice, for example, has been found to contain orthologs to many well-characterized MADS-box transcription factors (Arora et al., 2007) as well as the floral time

regulator *CONSTANS (CO)* which acts upstream of FT in *Arabidopsis* flowering determinacy (Yano et al., 2000).

Maize and Rice Floral Development

Maize and rice belong to a group of plants called cereals. Cereals are grasses, members of the monocot family Poaceae, which all have the same general floral morphology. The basic unit of the grass inflorescence is called a “spikelet.” Each spikelet consists of two leaf-like structures, called glumes, and one or two flowers, called “florets.” The floret contains a solitary pistil and multiple stamens enclosed by two bracts, the lemma and the palea. Rice (genus *Oryza*) spikelets consist of sepal like organs, the lemma and palea, surrounding a single hermaphroditic fertile floret with a fused central carpel and six proximal stamens (reviewed by Itoh et al., 2005) (Figure 3).

Inflorescence development in rice follows a transition of vegetative to reproductive growth stages, analogous to the model systems *Arabidopsis* and *Antirrhinum*. The process can be delineated in the following progression: 1) bract primordia arise from the shoot apical meristem indicating inflorescence initiation, 2) emergence of the primary branches from bract primordia, 3) emergence of secondary branches from the base of primary branch primordia, and 4) formation of spikelet meristems on the primary and secondary branches with subsequent differentiation of the spikelet primordia ultimately resulting in spikelets on the ends of primary and secondary branches of the panicle (reviewed by Y. Wang & Li, 2005). As mentioned

previously, spikelet meristems in rice produce single cosexual fertile florets subtended by a double pair of small bracts called empty glumes and rudimentary glumes.

Inflorescence development in maize follows a similar developmental sequence of events with one important difference, the generation of spikelet pair meristems, derived from the shoot apical meristem for tassels and from auxiliary shoot meristems in ears, generate two staminate florets in the tassel and two florets in the ear, with only one floret containing a functional pistil (reviewed by Bommert, Satoh-Nagasawa, Jackson, & Hirano, 2005). Floral development in maize is discussed in more detail below.

Sex Determination in Plants

Plant sex determination (SD) is the process by which plants generate unisexual flowers. Two dominant modes of unisexual flower development have been documented. Type I unisexuality is characterized by the initiation of a bisexual floral meristem containing both stamens and pistils followed by a developmental arrest or abortion of one sex with only the stamens or the carpels attaining functional maturity. The step impeding the development of floral sex organs is at an immature stage well prior to reaching sexual maturity (reviewed by Kinney, Columbus, & Friar, 2008). A type I SD mechanism has been demonstrated in maize (Kinney et al., 2008). In contrast to Type I, the Type II SD mechanism involves the emergence of only one type of sex organ in each flower bypassing the bisexual stage of floral development (Kinney et al., 2008).

The sex determination process can vary between species but appears to remain consistent within species. Natural selection in theory can act on any intermediate step during the development of the male and female gametophyte from a vegetative meristem. This can include inhibiting the initiation of floral organ primordia, as in *Cannabis*, abortion of pistil primordia and arrest of stamen primordia, as in maize, inhibition of megasporogenesis or microsporogenesis, as in *Asparagus* (Strittmatter, Hickey, & Negron-Ortiz, 2008), or during megagameteogenesis or microgameteogenesis, as in *Vitis* species (reviewed by Dellaporta & Calderon-Urrea, 1993).

Sex Determination in Maize

Maize is a monoecious plant bearing staminate florets in the terminal inflorescence, the tassel, and pistillate florets in one or more axillary “ear” inflorescences. Florets in both inflorescences undergo a conversion from a nascent bisexual state to a mature unisexual state (reviewed by Bortiri & Hake, 2007). In pistillate ear inflorescences, branch initials bifurcate to form two paired spikelets. Each spikelet contains two bisexual floral meristems that mature to form spikelets with solitary pistils. Stamens in all ear florets undergo cell cycle arrest at the time of style development a process that involves the phytohormone gibberellic acid (GA) (Kim et al., 2007). Mutations in GA biosynthesis, collectively called *dwarf* mutations, are stunted plants with stamens in ear spikelets that fail to arrest (Rood & Pharis, 1980).

Staminate florets in maize are found in the terminal tassel inflorescence. As in the ear, tassel spikelets are paired and contain two florets that are initially bisexual. During the SD process, tassel spikelets become staminate via the selective abortion of preformed pistil primordia, a process under both genetic and hormonal control (reviewed by H. M. Wu & Cheun, 2000). Mutants of the recessive *Tassel seed* phenotype fail to undergo pistil abortion in the tassels and display additional pistils in ear spikelets (reviewed in Dellaporta & Calderon-Urrea, 1993). Maize sex determination is illustrated in Figure 4.

The molecular and biochemical basis of maize sex determination is beginning to be understood. Pistil abortion in the tassel and in secondary florets of the ear spikelets require the action of certain *Tassel seed* genes. The first gene cloned, *Tassel seed2* (*ts2*) encodes a short-chain alcohol dehydrogenase/reductase (SDR) expressed in developing pistil primordia (DeLong, Calderon-Urrea, & Dellaporta, 1993). The *Tassel seed1* (*ts1*) gene was shown to encode a lipoxygenase required for the synthesis of the phytohormone jasmonic acid (JA) (Acosta et al., 2009). JA levels in *ts1* mutants are also about 10-fold lower when compared to wild-type tassels (Acosta et al., 2009), suggesting that JA is involved in the early abortion of pistils through a nucleated cell death process in developing flowers within the tassel. Both *ts1* and *ts2* mutant plants can be rescued by exogenous application of JA, a result consistent with JA involvement in the pistil abortion process (Acosta et al., 2009). These results lend credence to the hypothesis that failure in proper JA signaling during staminate floral development leads to ectopic feminization of the maize tassel and the maturation of pistils in ear florets that are normally aborted in the wild-type condition. JA

biosynthesis and the putative biochemical roles of *ts1* and *ts2* are illustrated in Figure 5.

JA is a well-known plant growth regulator that influences many plant developmental processes. JA is derived from the 18-carbon fatty acid linolenic acid, which is synthesized into JA by enzymes associated with chloroplasts and β -oxidation in peroxisomes (Vick & Zimmerman, 1984). JA signaling has been most commonly implicated in defense responses against pathogens, where JA has been found at the sight of wounding and is later distributed throughout the plant (reviewed by Ryan & Moura, 2002). JA signaling has also been connected with differentiation of vasculature tissue in tomato (Wasternack et al., 2006), and lateral root development in both *Arabidopsis* and rice (Raya-Gonzalez, Pelagio-Flores, & Lopez-Bucio, 2012). In *Arabidopsis*; JA biosynthetic genes are highly expressed in the neighboring anther filament and directs signal-mediated development of the anther in both the filament and in the anther itself (Ishiguro, Kawai-Oda, Ueda, Nishida, & Okada, 2001). The active form of JA (Jas-Ile) has recently been found to interact with CORONATINE-INSENSITIVE 1 (COI1) F-box protein, part of an E3 ubiquitin-ligase complex SCF^{COI1} involved in the polyubiquitination and subsequent 26S proteasome-mediated degradation of JAZ transcriptional repressors, thereby freeing various jasmonic acid-regulated genes for expression (Thines et al., 2007).

Expression profile studies of *ts1* and *ts2* in maize show broad tissue-specific expression patterns for both genes. Both *ts1* and *ts2* are expressed during vegetative growth in roots, stems and leaves as well as in inflorescences, while *ts2* levels in stems appear to be comparatively lower than *ts1* (Acosta et al., 2009). *In-situ* hybridization

studies demonstrated that *ts1* is actively transcribed in cells subtending the developing spikelet base at the spikelet-inflorescence axis junction of both the staminate flowers of the tassel and the pistillate flowers of the ears (Acosta et al., 2009), whereas *ts2* expression has been observed in the subepidermal layer of developing pistils in tassel inflorescences just prior to their abortion (Calderon-Urrea & Dellaporta, 1999). Surprisingly, *ts2* was also expressed in ear pistils including the functional pistil in the upper floret. It is believed that *ts1* may act upstream of *ts2* due to the fact that *ts2* expression is down-regulated in *ts1* mutants (Calderon-Urrea & Dellaporta, 1999). In pistillate spikelets in the ear, abortion in the upper florets blocked by the action of the *Silkless1* (*sk1*) gene. In *sk1* mutant plants, all pistils undergo abortion leaving the ear inflorescence sterile; the tassel inflorescence remains staminate. The SK1 protein appears to protect the upper floret from *tasselseed*-mediated cell death. This result suggests that *sk1* may negatively suppress the pistil abortion activity of *ts1* or *ts2* in the upper floret in ear spikelets (Veit et al., 1991). Although the precise mechanism is currently unknown, the *sk1* gene has been recently cloned (Dellaporta laboratory, unpublished results) and functional characterization of *sk1* will be important for full unraveling of the maize sex determination process.

Current Hybrid Rice Breeding Technologies

The need for hybrid breeding technologies in essential cereal crops has become more urgent as the demand for food increases. Rice hybrids, for instance, have the potential of generating 15-20 % more yield than inbred varieties (Viraktamath &

Virmani, 2001). As rice breeding programs progress in both the United States and China, methods have emerged to exploit preexisting variation in cultivated rice to generate male-sterile lines and produce hybrid seed. Since rice is a self-pollinating crop, male-sterile varieties that contain defects in microsporogenesis are necessary to produce F1 hybrids. Current strategies used to develop male-sterility are cytoplasmic male sterility (reviewed by Ivanov & Dymshits, 2007), chemically inducible sterility, and environment-sensitive genetic male sterility, a system which includes photoperiod-sensitive sterility and thermo-sensitive genetic male sterility (discussed in Virmani, 1994). The different strategies for developing hybrid rice are illustrated in Figure 2.

The cytoplasmic male-sterility system (CMS) can be found naturally in an assortment of higher plants and have been exploited in the production of hybrid seed in several self-pollinating cereal crop lines, most notably in rice (Ivanov & Dymshits, 2007; Z. Wang et al., 2006). CMS involves interactions between nuclear genetic factors and cytoplasmic genetic factors that encode unique open reading frames within mitochondrial DNA. Sterility is caused by expression of mitochondrial genetic factors that ultimately cause cytoplasmic dysfunction in cells engaged in pollen development. Fertility restoration can be accomplished through the action of nuclear-encoded *Restorer (Rf)* genes (Komori et al., 2004; Schnable & Wise, 1998). Since sterility is induced via action by the mitochondria, CMS is inherited maternally.

CMS systems require the use of three separate breeding lines in order to perpetuate the male-sterile condition. Under conditions where *Rf* genes are homozygous recessive (*rf/rf*), plants will persist in a male-sterile state if the

mitochondrial-derived cytoplasmic sterility factor (S) is also present (Vedel et al., 1994). These plants can be considered the A-line. The use of *Rf* homozygous recessive *Maintainer* line (or B-line) allows for A-line sterile plants to be propagated because B-line plants possess an innocuous cytoplasmic factor (N). B-line maintainers can perform two functions: they maintain the male-sterile A-line (with nuclear genotype *rf/rf* and cytoplasmic genotype S) by restoring pollen fertility, and they self-propagate due to the fact that they lack the male-sterile cytoplasmic factor (N) and are therefore fertile. F1 hybrid seed is generated by crossing the maintained A-line with pollen from a *Restorer* line (or R-line), which is either homozygous dominant (*Rf/Rf*) or heterozygous (*Rf/rf*) for the nuclear *Rf* gene. Since active expression of *Rf* circumvents the sterility phenotype conferred by the cytoplasmic sterility factor (S), F1 hybrids produced by a cross between A-line and R-line plants are completely fertile.

Although widely implemented, some disadvantages exist for CMS breeding strategies; for instance, generating the three lines and making the hybrid crosses can be costly and time-consuming. Successful breeding requires fastidious planting regimes and synchronous flowering times between lines. Lastly, CMS is restricted to only those rice species with available *Rf* genes, which excludes many agronomically important japonica lines (Viraktamath & Virmani, 2001).

Chemically-inducible male sterility involves the use of gametocides that are applied to rice with the intention to inducing male-sterility. This strategy has not been pursued with as much interest as other hybrid breeding programs due to several factors: it can be difficult to efficiently generate and maintain male sterility; some

chemicals must be applied during specific developmental stages; the genotypic background of some rice varieties can affect the efficacy of some compounds; and many gametocide chemicals are putative carcinogens (arsenic compounds) (Ali, 1993). Chemically-induced sterility has the potential to produce impure seeds if the compound is less than 100% effective in inducing male sterility, and it is very possible that some compounds can impart serious health hazards if improperly handled. If an ideal gametocide agent can be found (one that is economical, harmless to animals and humans, has a low environmental impact, and affects stamen function while leaving ovule function unimpeded), an advantage of chemically induced sterility would be generating hybrid seed without the use of a three line breeding system (such as in CMS).

Environment-sensitive male sterility comes in two prominent modes, photoperiod-sensitive genetic male sterility (PGMS) and thermo-sensitive genetic male sterility (TGMS). Both require an interaction between specific environmental conditions and the concomitant expression of environmentally-induced genes that confer the male-sterility phenotype. This method for hybrid rice breeding can be advantageous due to the fact that both PGMS and TGMS are facilitated by dominant genes and can therefore be inherited by many different genetic backgrounds producing favorable genetic variability within the hybrid population through an efficient two-line breeding system (Zhou et al., 2012). Also, TGMS lines can be developed in rice varieties that cannot undergo CMS hybrid breeding methods due to their lack of endogenous *Rf* restorer genes (e.g. japonica rice) (Viraktamath & Virmani, 2001).

Most PGMS and TGMS two-line breeding systems of this type are male sterile under long day lengths and fertile under short day lengths (Peng et al., 2008) and most TGMS lines exhibit sterility when plants are grown in temperature conditions above 25°C (Liu, Li, Zhang, & Wang, 2010). PGMS is controlled by at least three genes, *pms 1*, *pms 2*, and *pms 3*, which have been mapped to the rice genome and very little is currently known about their function (Peng et al., 2008). TGMS is controlled by recessive genes that differ between rice varieties, including *tms 1*, *tms 2*, *tms 3*, *tms 4*, *tms 5*, *ms-h*, and *rtms1* that have also been mapped to the rice genome (Liu et al., 2010). Disadvantages include the high sensitivity PGMS and TGMS have to environmental light availability and temperature fluctuations respectively; any sudden shifts in photoperiod or ambient temperature can severely impact the production of hybrid seed.

It is apparent that although hybrid rice breeding technologies are already implemented, each strategy contains its own particular disadvantages and drawbacks. A more reliable agricultural hybrid-breeding program, which can also be extended to cosexual cereal crops other than rice, is required to assure global food security in the coming decades.

Rationale and Justification for Studies

Exploiting the maize SD pathway offers a viable solution to future global food insecurity. World-wide food supplies can be greatly increased by extending this pathway and hybrid technologies to closely related cosexual cereal crops. To realize

this goal, however, several fundamental questions remained unanswered. For instance, why has maize evolved a SD mechanism but closely related cereals, such as rice, has not? As described herein, clear orthologs of the maize SD genes exist in related cosexual cereals, yet most maintain their hermaphroditic sexual mode of development.

It will be necessary to identify orthologs of maize SD genes, such as *ts1*, *ts2* and *sk1* in order to address differences in function between rice and maize. In the proposed 52 million years since the divergence of the maize and rice lineages (Bremer, 2002), large homologous regions between the maize and rice genomes have been preserved (Salse, Piegu, Cooke, & Delseny, 2004). Maize SD orthologs would therefore be conserved within these syntenic genomic blocks, and once identified, these SD gene orthologs can provide a wealth of information concerning basic genetic underpinnings of controlled cell death, phytohormone regulation processes, and mechanisms influencing cell cycle arrest in related cereals.

On a more utilitarian level, elucidating endogenous gene expression patterns of *Tassel seed* and other SD orthologs in rice could provide some of the groundwork needed to develop novel hybrid breeding technologies for agriculture in the future. Extending unisexuality to rice could facilitate the generation of hybrid rice above current hybrid rice breeding programs, increasing yield via hybrid vigor. Using gene expression studies (in association with future investigations in the genetic basis for monoecy in wild relatives, such as *Zizania* species, as a foundation), the answer to the question as to why SD gene orthologs in rice function differently than in maize and whether the maize sex-determination system can be transferred to cereals

(encouraging the development of unisexuality in these cereals) can be found. As mentioned previously, heterosis entails increasing the amount of food material from a particular cereal crop species by exploiting the phenotypic superiority of F1 hybrid offspring to their parents for many agronomic traits including increased yields and higher resistances to disease (Z. X. Wu, 2003). From these facts, a breeding regime for agriculturally significant cereal crops with an ultimate objective of producing hybrid seeds could be devised to increase crop yield and generate more food for the world, a powerful strategy for enhancing long-term food security. It is important to note that one advantage of using hybrid breeding technologies would be the reduction of required land area for use in agriculture while maintaining increased yield; the loss of arable land available for food production being one of the many pressing problems of global food security in the next half century (as reviewed by Godfray et al., 2010). What is needed, therefore, is an effective and stable strategy for generating unisexuality in cosexual cereal crop systems for the purposes of engendering hybrid seed production. The genetically determined sex-determination pathway that underlies the monoecious condition of maize can be used as a model to study how this goal can be accomplished.

Summary

The studies conducted in this thesis were designed to identify and characterize orthologs of the sex determination genes *ts1*, *ts2* and *sk1* of *Zea mays* (maize) in the cosexual cereal crop *Oryza sativa* (rice). Chapter 2 describes the methods used in

these studies and materials used in all cloning and gene expression studies performed. Chapter 3 outlines the results of experiments that clearly show the existence of rice orthologs of the maize SD genes *ts1*, *ts2*, and *sk1*. Also in Chapter 3, endogenous spatial-temporal rice SD ortholog gene expression patterns using RT-PCR, Taqman qRT-PCR gene expression assays, and a transgenic approach with *GUS* gene reporter constructs to further characterize endogenous gene expression patterns of these orthologs, were determined. Chapter 4 discusses these results with an emphasis on cell death signaling and JA biosynthesis in rice, including possible implications these results may have for future hybrid breeding programs centered around cosexual cereals.

Figure 1: Representative flowering time and floral-organ developmental genes involved in flower development in flowering plants including the ABC model of flower development. A, B, and C class MADS-Box transcription factors associate in the coordination of floral organ identity in developing floral meristems. Adapted from (Goff et al., 2002). Gene names are from *Arabidopsis*.

Figure 2: Hybrid rice breeding strategies. CMS: Cytoplasmic male sterility, ESGMS: Environment-sensitive genetic male sterility, TGMS: thermo-sensitive genetic male sterility, and PGMS: photoperiod-sensitive genetic male sterility. Commercially adapted strategies , such as the three-line hybrid rice breeding strategy for cytoplasmic male sterility and environment-sensitive genetic male sterility is outlined in pink and blue respectively. Non-commercially adapted strategies are shaded in grey.

Figure 3: Floral structure of the rice and maize spikelet. Depicted is a mature rice spikelet compared to an immature maize spikelet prior to undergoing sex determination. The rice spikelet contains a single fertile floret whereas maize contains spikelets that form two staminate florets in the tassel and a single pistillate floret in the ear.

Figure 4: Maize sex determination pathway. Each spikelet begins as two cosexual florets (left) that subsequently undergo a sex determination process to form unisexual florets. In tassels, pistils abort to form two staminate florets, a process mediated by JA signaling involving the *Tassel seed* genes. In ears, all stamens arrest in both the upper and lower florets whereas in the upper floret, *Silkless1* protects the upper floret from *tasselseed*-mediated abortion.

Figure 5: The role of *Tassel seed* genes in jasmonic acid (JA) biosynthesis. The primary dedicated step in JA synthesis is the peroxidation of α -linolenic acid to produce (13S)-hydroperoxyoctadecatrienoic acid (13-HPOT). TS1 may be involved in this process. Allene oxide synthase (AOS) and allene oxide cyclase (AOC) participate in converting 13-HPOT to (13S)-12,13-epoxy-octadecatrienoic acid (12,13-EOT) and then to cis-(+)-12-oxophytodienoic acid (OPDA) respectively. Cis-(+)-OPDA is transported to peroxisomes where OPDA reductase (OPDAR) reduces cis-(+)-OPDA to form 12-oxophytodienoic acid (OPC-8:0). Sequential cycles of β -oxidation are carried out, generating JA as one of the products. The first step in β -oxidation is an oxidation step carried out by a dehydrogenase enzyme, which may putatively be TS2. Adapted from (Acosta et al., 2009)

CHAPTER 2

MATERIALS AND METHODS

Ortholog Identification

BLASTp searches were performed using the National Center for Biotechnology Information (NCBI) BLAST suite (<http://blast.ncbi.nlm.nih.gov/>) using the RefSeq and SWISS-PROT protein databases limited to the *Oryza sativa japonica* rice reference entries. Maize TS2 and SK1 protein sequences were run using BLASTp against the RefSeq protein database; TS1 was run using BLASTp against the SWISS-PROT rice protein database due to the removal of the original RefSeq protein entry containing the highest maize TS1 sequence homology. This omission occurred following a new scaffolding of the japonica rice genome in NCBI. The SWISS-PROT protein database retained the rice protein entry that was removed in the RefSeq database. The rice protein entry with the highest similarity to maize was exported.

Each top rice protein entry was then analyzed a second time using the *Gramene* comparative genomics platform (<http://www.gramene.org/> release 34b) and the BLASTp algorithm against the *Oryza sativa japonica* reference genome. The *Gramene* web-based comparative genomics toolset provides predictive orthology identification and synteny visualization for many genes found among cereal crops. This analysis also returns the MSU-TIGR gene locus identifiers that were used in these studies. Genomic regions and sequence annotations encoding each protein were

exported in the GenBank format from *Gramene*. This region included 2,000 bp of sequence upstream of the predicted transcription start site (+1) and 1,000 bp downstream of the predicted translation stop site. The GenBank files were imported into the Seqbuilder module of the Lasergene DNA analysis suite (version 8, DNASTAR, Inc.)

Chromosomal synteny was determined by the comparative genomics toolkit found in the Genome Evolution (GEvo) software found in the University of California Berkeley Genome Evolution web-based platform (<http://genomeevolution.org/CoGe/GEvo.pl>). GEvo performs a BLASTZ search against any two fully sequenced genomes found in the GEvo database and identifies high scoring pairs (HSPs) between regions sharing extensive sequence colinearity. GEvo also illustrates each syntenic region by connecting HSPs between genomes to visualize the extent of synteny for each region. A cutoff was assigned to HSPs scoring below 10000.

Phylogenetic Analysis

Maize *ts1*, *ts2*, and *sk1* protein sequences underwent BLASTp searches in the RefSeq (for *ts1*, *ts2*, and *sk1*) and SWISS-PROT (for *ts1*) NCBI protein sequence databases. For each putative ortholog the top 100 most homologous protein sequences in the RefSeq database was downloaded in the fasta format and analyzed by Bayesian inference using MrBayes (version 3.2.0) using the Louise high performance computer

cluster (HPC) at Yale University. For *tsI*, the dataset included the rice ortholog Q7XV13.2 omitted from the RefSeq database.

Fasta sequences were aligned using CLUSTALW (version 2.1) via default parameters. After the initial alignment, one or more proteins showing low homology and large indels were removed from each dataset and the sequences realigned and exported in the aligned fasta format. The aligned fasta files were edited using Gblocks (version 0.90) using the following parameters: for *rTsI*: minimum number for conserved position (51), minimum number of sequences for flank position (85), maximum number of contiguous nonconserved positions (8), minimum length of block (10), and allowed gap positions (none); for *rTs2*: minimum number for conserved position (51), minimum number of sequences for flank position (85), maximum number of contiguous nonconserved positions (8), minimum length of block (10), and allowed gap positions (none); and for *rSkI*: minimum number for conserved position (51), minimum number of sequences for flank position (85), maximum number of contiguous nonconserved positions (8), minimum length of block (10), and allowed gap positions (with-half).

The Gblock output file (nexus file format) for each alignment was imported and analyzed with MrBayes (version 3.2.0) (Ronquist et al., 2012) with the following parameters: a Poisson fixed amino acid substitution model with four independent chains; 10,000,000 generations with a sample frequency of 100; a print frequency of 100; and a diagnostic frequency of 1000. The analysis was stopped when the standard deviation of split frequencies approached values below 0.01 indicating that trees were converging. Output parameters, branch length, and tree files were summarized using

the MrBayes built-in sump and sumt commands, respectively. Resulting output consensus trees containing posterior probabilities for each node were visualized using Figtree (version 1.3.1) (<http://tree.bio.ed.ac.uk/software/figtree/>).

Taqman[®] Gene-Expression Analysis

Tissue was harvested from the shoots and roots of eleven- days after planting (DAP) seedlings by quick freezing tissue in liquid N₂. Three biological replicates were collected for each tissue from three independent plants. Two biological replicates of leaf tissue was collected from 26 DAP seedlings. Leaf tissue samples were separated into three zones that represented the basal, middle, and apical sections of the leaf.

Total RNA was extracted from each tissue sample using the Ambion TRIzol[®] PureLink RNA Extraction Kit following the manufacturer's instructions. The total RNA sample was treated with Roche RNase-free DNase I (approximately 1 unit per μ g RNA) in 1X DNase I buffer supplied by manufacturer. The DNase I-treated total RNA was then extracted with an equal volume of phenol-chloroform (1:1 v:v), and the aqueous phase was precipitated with two volumes of 100% ethanol. The precipitated RNA was collected by centrifugation for 30 min at 12,000 x g, and the pellet was washed with 80% ethanol, air dried and redissolved in 10 mM Tris, 0.5M EDTA pH 8 (TE) buffer. Approximately 100 ng of total RNA was synthesized into first-strand cDNA in a 20 mL reaction using Invitrogen Superscript III-Reverse Transcriptase cDNA synthesis kit according to the manufacturer's instructions.

All Taqman primers and probes were designed by Applied Biosystems to bridge exons in the target genes. The Taqman gene expression assay was designed as a multiplex real-time PCR assay with an internal control gene, elongation factor 1a (eEF-1a) labeled with VIC, and the appropriate target gene (rice ortholog of *ts1*, *ts2* or *sk1*) labeled with FAM, according to standard Applied Biosystems protocols. The sequences of all primers and probes are shown in Table 1. Twenty μ l multiplexed qRT-PCR reactions were run in triplicate on the Eppendorf Mastercycler® ep Realplex thermocycler under the following conditions: 95°C initial denaturation for 10 minutes, 95°C for 30 seconds and 60°C for 1 minute for 45 cycles, and a 4°C final hold. 1 μ l of cDNA reaction was as a template.

Raw Ct values were adjusted via a multiplex efficiency correction factor (see Table 10 in appendix). This correction accounted for differences in amplification efficiency between the Ct value of the target gene in the singleplex verses the multiplex reaction conditions. The corrected Ct value for each replicate was then normalized to each corresponding adjusted internal control Ct value to produce corrected Δ Ct values. Standard errors were calculated for the mean corrected Δ Ct values from technical and biological replicates for each tissue type. Two-way ANOVA analysis were conducted on the corrected Δ Ct means to identify statistically significant differences between target genes and tissues. Two-way ANOVA was performed via PASW Statistics 19, Release Version 19.0.0 (SPSS, Inc., 2009, Chicago, IL, www.spss.com).

Relative expression was determined using the comparative Δ Ct method (Livak & Schmittgen, 2001). The $\Delta\Delta$ Ct values were generated by subtracting corrected Δ Ct

mean of a target gene from the corrected ΔCt mean of a given calibrator (the highest ΔCt value per comparison). Error was determined from calculating the standard error of the mean (SEM) from corrected ΔCt mean values. The equation $2^{-(\Delta\Delta\text{Ct} \pm \text{SEM})}$ gave relative quantification (RQ) values for gene expression levels.

Transgene Construction

Several reporter constructs were obtained by fusing the promoter and terminator regions of the target gene (*rTs1* and *rTs2*) with the coding sequence from the reporter gene β -glucuronidase (*GUS*) (Jefferson, Kavanagh, & Bevan, 1987). The promoter fragment included a 1-1.5 kb promoter region upstream of the transcription start site as well as the 5' UTR sequences of *rTs2* (75 bp) or *rTs1* (unknown size) fused to the reporter gene *GUS* at the translation start site (ATG). The *rTs1::GUS* vector pYU2885 was generated using ligation-independent cloning (LIC) technology as described below. The *rTs2::GUS* vector pYU2749 was constructed using an isothermal DNA assembly method (Gibson et al., 2009) coupled with LIC directional cloning techniques as described. Each methodology is illustrated in Figure 6.

All LIC methods used in this study followed the same general method: 5 μl of purified PCR amplicon was included into a 10 μl T4 reaction mix (1.5 μl distilled water, 1 μl 10X NEBuffer 2, 1 μl 25 mM dCTP, 0.5 μl 100 mM dithiothreitol, 0.5 μl bovine serum albumin, and 0.5 T4 Polymerase (NEB Cat. No. M0203) which was incubated at 22°C for 30 minutes. Five μg of a selected T-DNA plasmid vector, with added *Bsa*I restriction site, was added to a 100 μl *Bsa*I restriction digest and was

incubated at 50°C for 2-3 hours and heat inactivated at 65°C for 15 minutes. Fifty µl of BsaI-digested plasmid DNA was incubated a 100 µl T4 reaction mix (10 µl NEBuffer 2, 10 µl 25 mM dGTP, 1 µl bovine serum albumin, 0.5 µl dithiothreitol (DTT), 5 µl NEB T4 Polymerase, 23.5 µl distilled water) for 30 min at 22°C. One µl of BsaI-T4 treated plasmid vector was mixed with 2 µl T4-treated PCR amplicon, incubated at 22°C for 10 minutes and transformed into Invitrogen TOP10 *E. coli* (Life Technologies Cat. No. C4040) following the manufacturer's instructions.

To construct the *rTs1::GUS* fusion gene, a fragment from the *rTs1* regulatory regions that included 1398 bp upstream of the translation start signal was amplified using primers P4102 and P4387 in 50 mL PCR reactions. A 507 bp terminator region that included the translation stop signal and 3' UTR region was amplified using primers P4390 and P4058 in 50 mL PCR reactions. The forward promoter primer and the reverse terminator primer incorporated LIC sites for subsequent vector cloning as described above. Assembly of the *rTs1::GUS* translational fusion construct followed the one-step "enzymatic isothermal DNA assembly" protocol (Gibson et al., 2009).

rTs2 promoter regulatory regions 1033 base pairs upstream of a translation start signal 550 base pairs downstream from a translation stop signal were amplified with 5' and 3' LIC adaptors and 3' and 5' SmaI site adaptors using NEB Phusion Hot Start II Polymerase. Primers P4043 and P4044 were used to amplify the *rTs2* promoter and primers P4045 and P4046 were used to amplify the *rTs2* terminator. Promoter and terminator fragments were gel purified using the Qiagen QIAquick Gel Extraction Kit and then ligated using NEB quick ligase according to the manufacturer's instructions. Promoter-terminator fusions were cloned via LIC into

vector pYU2745 and then digested with SmaI restriction enzyme (NEB Cat. No. R0141) in a 50 µl restriction digest for 30 minutes at 25°C. A *SacB* LIC cloning cassette was amplified using Phusion HotStat II Polymerase and primers P3380 and P3381 from pYU2392, which was then phosphorylated via NEB T4 Polynucleotide Kinase (NEB Cat No. M0201) according to the manufacturer's instructions, and finally ligated into SmaI digested pYU2745 via NEB Quick ligase (NEB Cat. No. M2200), generating pYU2746. Entire *rTs2* expression cassettes were amplified using primer P4043 and P4046 from pYU2746, gel purified using the Qiagen QIAquick Gel Extraction Kit (Qiagen Cat. No. 28704) and then cloned via LIC into the pYU2724 T-DNA delivery vector, generating pYU2747. *GUS* reporter gene cassettes were amplified from pYU529 via PCR using Phusion Hot Start II Polymerase using primers MB08F014 and MB08R014 with anti-LIC5 and anti-LIC3 5' and 3' adaptors. T4 DNA polymerase-treated *GUS* inserts were then cloned into BsaI digested-T4 DNA Polymerase treated pYU2747 via LIC generating *rTs2::GUS*.

***Agrobacterium*-Mediated Transformation**

Agrobacterium-mediated rice transformation followed a standardized protocol developed by the Iowa State Plant Transformation Facility (<http://www.agron.iastate.edu/ptf/>). This protocol was adapted from an earlier protocol (Toki, 1997) and was used in all rice transformation experiments. The rice transformation process is illustrated in Figure 7. *Oryza sativa japonica* cv.

Nipponbare seeds were acquired from the Dale Bumpers' National Rice Resource Center germplasm library (<http://www.ars.usda.gov/>).

Transformation experiments were performed using *Agrobacterium tumefaciens* strain EHA101 transformed with both *rTs1::GUS* and *rTs2::GUS* reporter constructs via electroporation and allowed to grow at 19°C for three days on YEP medium (5 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl₂, 15 g/L Bacto-agar. pH to 6.8 with NaOH) supplemented with 100 mg/ml spectinomycin, 50 mg/ml kanamycin, 10 mg/ml rifampicin, and 100 mg/ml chloramphenicol. Regenerated transgenic plants were transplanted to greenhouse conditions (16:8 photoperiod with supplemental lighting at 150 W/m², 1:1 calcite clay:soilless mix, under constant sub-irrigation supplemental with 15-5-15 peter's excel cal/mag fertilizer [Everris Cat. No. 99140]). Independent T-DNA insertion events were cataloged and each plant regenerated from each event was assigned individual plant numbers.

Screening of Transgenic Events

Genomic DNA was extracted from each transgenic plant via a urea-based phenol-chloroform extraction protocol (J. Chen, Dellaporta, S. L. , 1994). Genomic DNA was subjected to PCR amplification to assay for the *bar* herbicide resistance gene and the *GUS* reporter gene. Positive controls used in this analysis were as follows: construct pYU121 at 1 ng/μl containing a copy of the *bar* herbicide resistance cassette, and pYU2885 at 5 ng/μl containing the *GUS* reporter gene.

PCR confirmation of the *bar* herbicide cassette was performed in the same fashion for both *rTs1::GUS* and *rTs2::GUS* transgenic plants. Reactions were prepared in 25 µl volumes using primers P994 and P995 at 25 µM concentrations with Phusion Hot Start II DNA polymerase and 1 µl of extracted genomic DNA (25 ng/µl) as a template. Thermocycle conditions were 98°C for 30 seconds, 98°C for 10 seconds, 58°C for 30 seconds, and 72°C for 1 minute repeated for 35 cycles, 72°C for 5 minutes with a final 4°C hold.

PCR confirmation for the *GUS* reporter gene in *rTs1::GUS* transgenic plants used a *GUS* Taqman gene-specific primer and probe and thermocycle conditions: 95°C for 3 minutes, 95°C for 10 seconds and 60°C for 1 minute with a final 4°C hold. The reactions were prepared in 20 µl volumes using 2X Gene Expression Master Mix from Applied Biosystems and 1 µl of extracted genomic DNA (25 ng/µl) was used as a template. PCR confirmation for the *GUS* reporter gene in *rTs2::GUS* transgenic plants used primers P4644 and P4645 and thermocycle conditions: 98°C for 30 seconds, 98°C for 10 seconds, 64°C for 30 seconds, and 72°C for 2 minutes repeated for 35 cycles, 72°C for 5 minutes with a final 4°C hold. These reactions were prepared in 25 µl volumes using Phusion Hot Start II DNA polymerase and 1 µl of extracted genomic DNA at 25 ng/µl was used as a template.

Histochemical GUS Staining Assay

The histochemical GUS assay used throughout this study was adapted from Jefferson et. al. (Jefferson et al., 1987). Tissue was submerged in a GUS staining

solution (50 nM Potassium ferricyanide, 50 nM Potassium ferrocyanide, 0.2 M Sodium Phosphate Buffer pH=7, 0.5 M Sodium EDTA, 10% Triton-X, and 25 mg/ml X-Gluc [Rose Biotechnology Cat. No. ES1007] dissolved in N,N Dimethylformamide) in 6, 12, or 48 well microtiter plates, depending on the experiment. Samples were vacuum infiltrated for 3-5 minutes and incubated at 37°C overnight. GUS staining solution was removed and samples were incubated in 70% ethanol with 37°C incubation for up to one week to clear chlorophyll from leaf cells. During this time, the ethanol solution was changed several times to facilitate the clearing process.

Microscopy

Tissue samples were mounted onto clean glass slides and covered with plastic cover slips. Images were obtained on a Zeiss Discovery V12 SteREO dissecting scope with a Plan AopS 1.5X optical lens. Pictures were taken using the Zeiss AxioCam microscope camera with compatible AxioVision software release 4.8 under standard configuration settings.

(A)

Figure 6: Cloning methodologies used in this study. (A) Ligation independent cloning (LIC) method employing the use of a *SacB* expression cassette such as the strategy used to construct *rTs2::GUS* and (B) one-step isothermal DNA assembly technique used to construct *rTs1::GUS*.

(B)

Figure 6: Cloning methodologies used in this study. Continued.

Figure 7: Flowchart of the rice transformation process used in this study. Embryogenic callus is induced from mature seed and is used as an explant for T-DNA delivery by *Agrobacterium*-mediated transformation. Transformed callus is selected on media supplemented with the herbicide bialaphos. Transformed callus tissue resistant to biaplaphos is then subsequently regenerated into vegetative plants. Images taken from (Nishimura, Aichi, & Matsuoka, 2006).

Primer	Gene	Purpose	Sequence 5'3'
P994	<i>bar</i> forward	genotyping	TGCACCATCGTCAACCACTACATC
P995	<i>bar</i> reverse	genotyping	CTGAAGTCCAGCTGCCAGAAACC
P4644	<i>GUS</i> forward	genotyping	GTCTGGATCGCGAAACTGTG
P4645	<i>GUS</i> reverse	genotyping	AATATCTGCATCGGCGAACT
P4102	<i>rTs1</i> promoter forward	cloning	TACTTCCAATCCATGCAACCAGACAACCACTCCT
P4387	<i>rTs1</i> promoter reverse	cloning	TTGGGGTTTCTACAGGACGGACCATCGCCGCCGCCGATCCCCCTC
P4390	<i>rTs1</i> terminator forward	cloning	AGCAGGGAGGCAAACAATGATGCGCCGCTGGTGCCCGCCGA
P4058	<i>rTs1</i> terminator reverse	cloning	TATCCACCTTTACTGAGCCGAAAACCTTAGCAATCGA
P4043	<i>rTs2</i> promoter forward	cloning	TACTTCCAATCCCTGCAAGCGTGCCATAAAA
P4044	<i>rTs2</i> promoter reverse	cloning	TCCCCCGGGTACGACCCTGTCTAGCAAGT
P4045	<i>rTs2</i> terminator forward	cloning	TCCCCCGGGCTCTTCTCTCCCTCTAGATGAAT
P4046	<i>rTs2</i> terminator reverse	cloning	TATCCACCTTTACTGGCATTGCGTGTTGAGTATGT
P4388	<i>rTs1 GUS</i> forward	cloning	GAGGGGGATCGGCGGCGCATGGTCCGTCCTGTAGAAACCCCAA
P4389	<i>rTs1 GUS</i> reverse	cloning	TCGGCGGGCACCAGGCGGCGCATCATTGTTGCCTCCCTGCT
P3380	<i>SacB</i> forward	cloning	GTACTTCCAATCCATGGAGACC
P3381	<i>SacB</i> reverse	cloning	GTATCCACCTTTACTGGAGACCG
MB08F014	<i>GUS</i> forward	cloning	ATGGTCCGTCCTGTAGAAACCCCAA
MB08R014	<i>GUS</i> reverse	cloning	TCATTGTTTGCCTCCCTGCT
<i>GUS</i> taqman	<i>GUS</i> taqman forward	genotyping	GGTGGGAAAGCGCGTTAC
	<i>GUS</i> taqman probe	genotyping	AATTGCCCCGCTTTCT
	<i>GUS</i> taqman reverse	genotyping	CGGCGAACTGATCGTTAAACTG
<i>rTs1</i> taqman	<i>rTs1</i> taqman forward	qRT-PCR	GAGCTCGTCAGCACGGA
	<i>rTs1</i> taqman probe	qRT-PCR	FAM-ACCGACCCAAGGAAGGGG-NFQ
	<i>rTs1</i> taqman reverse	qRT-PCR	CGACGGCTTGCTCTTCTTC
<i>rTs2</i> taqman	<i>rTs2</i> taqman forward	qRT-PCR	CTGGGACTCCCCCAATGG
	<i>rTs2</i> taqman probe	qRT-PCR	FAM-CCAAGAGGCTGGAAGGG-NFQ
	<i>rTs2</i> taqman reverse	qRT-PCR	CCCCGCCGGTGACAAT
<i>rSk1</i> taqman	<i>rSk1</i> taqman forward	qRT-PCR	GCAAGGGCGTCATCTCAGA
	<i>rSk1</i> taqman probe	qRT-PCR	FAM-TCCGGTACTCCCGCTGTC-NFQ
	<i>rSk1</i> taqman reverse	qRT-PCR	CTTGTCGTCGAGCTGATTCG
<i>eEF-1a</i> taqman	<i>eEF-1a</i> taqman forward	qRT-PCR	CTCTGGTGTAAGCAGATGATCT
	<i>eEF-1a</i> taqman probe	qRT-PCR	VIC-CAAGATGGATGCCACCACTC-NFQ
	<i>eEF-1a</i> taqman reverse	qRT-PCR	TAACGGGCCTTGGAGTACTTG

Table 1: Primers used in this study. FAM: 6-carboxyfluorescein florescent dye. VIC: VIC florescent dye.

NFQ: Nonflorescent quencher.

CHAPTER 3

RESULTS

Identifying Sex Determination (SD) Gene Orthologs in *Oryza sativa japonica*

Maize SD gene orthologs in japonica rice were identified using four main criteria: protein sequence homology, synteny between the maize and rice genomes, conserved gene architecture and biochemical functionality, and Bayesian phylogenetic analysis. BLAST searches were conducted to find japonica rice proteins with the highest sequence homology with maize SD genes *ts1*, *ts2*, and *sk1*. BLASTp searches of maize TS1, TS2, and SK1 protein sequences were performed against the National Center for Biotechnology Information (NCBI) *Oryza sativa japonica*-specific complete genome sequence in the SWISS-PROT (for TS1) and the RefSeq (for TS2 and SK1) protein databases (<http://blast.ncbi.nlm.nih.gov/>).

The 25 top scoring hits for a BLASTp search are reported for each gene. The top scoring hits for each gene were tentatively assigned as putative orthologs of maize SD genes. Japonica rice proteins Q7XV13.2, NP001049848.1, and NP001053356.1, scored highest for maize TS1, TS2, and SK1 respectively (see Tables 2-4). Q7XV13.2 shares 87.99 % amino acid identity and 92.55 % similarity with maize TS1 protein (Table 2), NP001049848.1 shares 82.65 % identity and 88.53 % similarity with maize TS2 protein (Table 3), and NP001053356.1 shares 65.37 % identity and 75.07 % similarity with maize SK1 protein (Table 4). Cross-referencing with *Gramene*

provided MSU-TIGR gene locus identifications for each rice protein sequence: Q7XV13.2 matched *LOC_Os04g37430*, NP001049848.1 matched *LOC_Os03g18740*, and NP001053356.1 matched *LOC_Os04g44354* (Table 5). Gene loci were assigned descriptive loci designations *rTs1*, *rTs2*, and *rSk1* respectively.

To further investigate orthology of the putative rice SD genes, chromosomal synteny was analyzed by identifying colinear genomic stretches between maize and japonica rice genomes using the comparative genomics toolkit found in the Genome Evolution (GEvo) Genome Evolution web-based platform (<http://genomeevolution.org/CoGe/GEvo.pl>.) GEvo performs BLAST searches against any two fully sequenced genomes found in the GEvo database using a BLASTZ algorithm (Harris, 2007) that uses high scoring pair (HSP) matches to visualize colinear homologous genomic stretches. Maize SD protein sequences were subjected to BLASTZ searches against the reference japonica rice genome found in the GEvo database, and outputs for each rice gene are illustrated in Figure 8.

HSP matches from *in silico* GEvo analysis showed that maize *ts1* on maize chromosome 2 not only shares strong homology with the *rTs1* gene on japonica rice chromosome 4 directly, but also exhibited colinearity with adjacent neighboring regions between the two chromosomes, indicating strong synteny (see Figure 8A). The *rTs2* gene also displayed strong colinearity between *ts2* found on maize chromosome 1 (see Figure 8B). The *rSk1* gene showed similar colinearity, having homology with *sk1* and adjacent regions on maize chromosome 4 (see Figure 8C). This data supported the assignment of orthology for the rice genes to their corresponding maize SD genes.

The gene architectures of *rTs1*, *rTs2*, and *rSk1* were compared to the structure of their corresponding maize genes. This analysis was done by comparing conserved exon-intron structure between both japonica rice and maize following BLASTp searches in *Gramene*. BLAST results indicated that all three rice SD orthologs exhibited a conserved gene architecture (Figure 9). The *rTs1* gene shares very similar exon-intron structure with *ts1*, where *ts1* has two exons that share homology with one exon in *rTs1* (Figure 9A). The *rTs2* and *ts2* genes both have two exons flanking one intron (see Figure 9B), and the *rSk1* gene and the *sk1* gene both have two exons with one very large intron (Figure 9C).

The encoded rice SD proteins were found to share predicted biochemical functionality with the maize SD proteins. This analysis was done by comparing conserved domains (CD) found in the maize and rice proteins with the *Comparative Domain Database* (CDD) function in NCBI (Marchler-Bauer et al., 2011). These results are listed in Table 6. The results for the encoded *rTs1* protein indicated that it belonged to the lipoxygenase family of enzymes and contained the conserved PLAT/LH2 and the PLN02337 lipoxygenase superfamily domains that it shared with the maize TS1 protein. The rice *rTs2* and maize *ts2* genes encoded short-chain alcohol dehydrogenases with shared conserved Rossmann-fold NAD(P)(+)-binding and PLN02253 domains. The *rSk1* and maize *sk1* genes were both found to encode UDP-glycosyl transferases with a shared Glycosyltransferase_GTB_type superfamily domain. These results provided additional support of orthology of the identified rice genes with the corresponding maize SD genes.

To ascertain phylogenetic relationships between the *rTs1*, *rTs2*, and *rSk1* genes, a Bayesian analysis of the rice and maize encoded proteins, together with other closely related proteins found in NCBI databases, was performed. The TS1, TS2, and SK1 protein consensus trees all showed the corresponding japonica rice protein falling within the same clade as their corresponding maize ortholog in well-supported nodes with posterior probabilities ≥ 0.95 . The *rTs1* protein falls within a strongly supported clade (posterior probability = 1.00) with two maize lipoxygenases, *ts1* and its duplicate gene *ts1b*, and with putative lipoxygenases from both *Sorghum bicolor* and *Brachypodium distachyon* (Figure 10, insert). The *rTs2* protein is also located in a strongly supported clade (posterior probability = 0.97) with the maize TS2 protein and short-chain alcohol dehydrogenases from *Sorghum* and *Brachypodium* (Figure 11, insert). The *rSk1* protein is contained within a strongly supported clade (posterior probability = 1.00) that includes the maize SK1 protein as well as maize putative orthologs from *Sorghum* and *Brachypodium* (Figure 12, insert). These results further supported the assignment of these rice genes as true orthologs of the maize SD genes.

Functional Analysis of Rice SD Gene Orthologs

A functional characterization of rice SD orthologs was performed to examine tissue-specific expression patterns of these genes in wild-type japonica rice. These studies included quantitative real-time PCR (qRT-PCR) analysis and reporter gene expression analysis, using promoter fusions to the *GUS* gene in transgenic rice plants.

The qRT-PCR assays were used to examine the tissue-specific expression pattern for each rice ortholog. These experiments employed a multiplexed Taqman assay with a probe for the target rice ortholog (labeled with FAM) and a common VIC-labeled internal control probe for elongation factor 1 α (*eEF-1 α*). The main advantage of the multiplex Taqman design was the ability to amplify the target gene and the internal control in a single reaction as opposed to comparing target gene amplification in a separate reaction. This allowed the target gene to be normalized to an internal control within and between reactions.

Relative gene expression for each gene-specific Taqman assay used a comparative Ct approach (Livak & Schmittgen, 2001). When calibrated to expression in eleven days after plating (DAP) shoots, *rTs1* exhibits expression in all tissues analyzed with the highest expression in the base of leaves (Figure 13A). Previous RT-PCR tissue profiling also demonstrated that *rTs1* is expressed in inflorescence tissues approximately four-month-old (Figure 14). Expression for *rTs2* was also seen in every tissue-type analyzed when calibrated to expression in apex leaf tissue, with the highest expression level in eleven DAP shoots (Figure 13B). Similarly to *rTs1*, *rTs2* has also been shown to be expressed in inflorescence tissue approximately four months old (Figure 14). All tissue-types exhibited expression of *rSk1* when *rSk1* expression levels were calibrated to leaf apex tissue, with the highest expression seen in eleven DAP root tissue (Figure 13C). Significant differences in expression, based on normalized Δ Ct values, were found between *rSk1* and both *rTs1* and *rTs2* in roots, *rTs2* and both *rTs1* and *rSk1* in shoots, *rTs1* and both *rTs2* and *rSk1* in leaf base tissue, and between all genes in leaf middle and leaf apex subsections (see Table 7).

Results gleaned from qRT-PCR experiments demonstrated that *rTs1*, *rTs2*, and *rSk1* were expressed in all selected tissue types studied: one-week-old vegetative shoot and root tissue and twenty six day old leaf base, middle, and apex subsections. This indicates a broad plant-wide expression profile (Figures 13, 14, and 15). *rTs1* and *rTs2* leaf tissues exhibit a strong expression gradient progressing from higher expression in younger basal leaf tissue to lower expression in older apex tissue for each gene (Figure 15B). *rTs1* displays the highest expression in leaf subsections relative to *rTs2* and *rSk1* (Figure 15B).

To further characterize tissue-specific expression of *rTs1*, *rTs2*, and *rSk1* in japonica rice, a reporter gene assay study was conducted in transgenic rice plants. The basic strategy was to fuse the promoter region for each target rice gene to the reporter gene *β -glucuronidase* (*GUS*) as a translational fusion construct. These translational fusions, with the plant selectable marker, are shown in Figure 16. Each construct was placed into the T-DNA vector pPZP200b (Hajdukiewicz, Svab, & Maliga, 1994) with the plant selectable marker *bar* gene, and transformed into rice plants via *Agrobacterium*-mediated transformation.

Thirteen independent T-DNA insertion events were generated after transforming the *rTs2::GUS* construct into japonica rice, and three independent T-DNA events were generated following transformation of the *rTs1::GUS* construct into japonica rice (Table 8). Multiple plants were regenerated from each independent transformation event (Table 8) and each plant was tested for the presence of the *GUS* reporter gene and selectable marker (*bar* gene) by PCR (Figures 17 and 18).

Representative plants scored as positive for both *GUS* and *bar* gene were then assayed for GUS expression using a leaf disk GUS histochemical assay. For the *rTs1::GUS* transgenic line, one event (Event 17) , and in *rTs2::GUS* transgenic lines, two independent events (Events 3 and 26), exhibited a positive GUS signal in leaf disks (Figure 19). GUS results from individual plants derived from a single transformation event gave a consistent staining pattern. The GUS signal, a blue precipitate, was seen in vascular tissue of each positive stained leaf disk sample. These results indicated that the rice orthologs of TS1 and TS2 are expressed in the rice leaf but restricted to the vasculature cells.

Further characterization of tissue-specific GUS expression included an examination of various tissues in *rTs1::GUS* Event 17 Plant 1 and *rTs2::GUS* Event 26 Plant 2. These plants had previously tested positive in the leaf disk assays. This expanded analysis showed that *rTs1* expression was seen in leaf vasculature cells (Figure 20). However, for *rTs1::GUS* transgenic plants, GUS staining in inflorescent tissue was not possible due to the fact that *rTs1::GUS* plants did not flower during the course of this study. For *rTs2*, expression was detected in the vascular tissue of developing leaves, the vasculature tissue of developing and mature spikelets, emerging roots, and late-stage developing anthers prior to panicle emergence (Figure 21). These results suggested that *rTs1* and *rTs2* expression is present in leaf vasculature and *rTs2* expression is also present in the vasculature of developing leaf-like floral organs, anthers, and emerging roots.

NCBI subject ID	% identity	% similarity	E value	bit score
Q7XV13.2	87.99	92.55	0.00	1542.0
Q8H016.2	66.59	78.25	0.00	1215.0
P38419.2	48.07	64.58	0.00	758.0
Q84YK8.1	47.91	64.40	0.00	751.0
Q7G794.1	44.31	59.20	0.00	706.0
Q76I22.2	43.18	59.53	0.00	699.0
Q53RB0.1	43.69	61.07	0.00	696.0
P29250.2	42.84	58.79	0.00	667.0
Q7XPY2.1	39.13	56.52	0.07	29.3
Q5NBJ3.1	28.00	48.00	0.08	29.3
Q7XTS4.2	46.43	67.86	0.09	28.9
Q0DV28.2	31.75	47.62	0.46	26.9
Q657C0.1	22.35	41.18	0.50	26.6
Q6ZBH9.1	26.02	34.96	0.61	26.6
A2XUN8.2	21.28	40.43	0.67	26.2
P49661.2	34.29	51.43	1.40	25.0
Q7XU31.3	56.25	68.75	1.40	25.0
Q67WJ2.1	39.13	69.57	1.40	25.0
Q0JEQ2.1	37.93	58.62	1.50	25.0
Q9AV50.1	30.30	60.61	1.60	25.0
Q94CU5.1	31.11	53.33	1.60	25.0
Q851R2.1	24.53	47.17	1.80	25.0
Q6EUG4.1	30.23	53.49	2.00	24.3
Q0IZZ8.2	23.08	40.38	2.20	24.6
Q6ZIK0.1	31.25	46.88	2.50	24.3

Table 2: List of top 25 BLAST hits following a BLASTp search using the *Zea mays* TS1 protein sequence against the NCBI SWISS-PROT protein sequence database specific for *Oryza sativa japonica*.

NCBI subject ID	% identity	% similarity	E value	bit score
NP_001049848.1	82.65	88.53	4.00E-177	493
NP_001060718.1	74.91	82.58	9.00E-131	374
NP_001051666.1	53.19	65.96	2.00E-88	265
NP_001060559.1	46.83	64.44	3.00E-78	239
NP_001060558.2	49.30	64.08	5.00E-77	236
NP_001051806.2	51.61	61.65	2.00E-69	215
NP_001060550.2	46.10	63.83	3.00E-69	216
NP_001052173.1	48.45	60.14	5.00E-69	214
NP_001052174.1	48.97	60.96	8.00E-69	214
NP_001052709.1	50.18	61.21	9.00E-69	214
NP_001060562.2	47.02	63.16	8.00E-68	213
NP_001176566.1	46.62	60.14	2.00E-67	211
NP_001060552.1	41.64	59.43	1.00E-65	206
NP_001060160.1	41.70	61.48	1.00E-65	206
NP_001060553.1	41.61	57.69	6.00E-64	201
NP_001060551.1	43.97	60.64	8.00E-62	197
NP_001060557.1	45.58	62.19	4.00E-61	195
NP_001060560.1	43.86	60.70	7.00E-60	192
NP_001060555.2	42.70	59.43	7.00E-59	189
NP_001066519.2	42.55	57.80	2.00E-58	188
NP_001060561.1	50.88	67.25	2.00E-41	140
NP_001054600.1	28.44	41.59	3.00E-27	103
NP_001049675.1	29.41	44.64	4.00E-27	102
NP_001060556.2	52.69	69.89	7.00E-26	95.5
NP_001066481.1	29.97	44.95	7.00E-26	99.4

Table 3: List of top 25 BLAST hits following a BLASTp search using the *Zea mays* TS2 protein sequence against the NCBI RefSeq protein sequence database specific for *Oryza sativa japonica*.

NCBI subject ID	% identity	% similarity	E value	bit score
NP_001053356.1	65.37	75.07	4.00E-152	437
NP_001051322.1	31.72	47.39	4.00E-71	231
NP_001176085.1	33.33	48.36	1.00E-69	229
NP_001048163.1	32.02	49.27	1.00E-65	218
NP_001053357.1	66.84	71.12	2.00E-63	204
NP_001051321.2	33.63	47.22	2.00E-63	211
NP_001051326.1	28.54	46.82	3.00E-63	210
NP_001061084.2	31.84	45.12	2.00E-60	203
NP_001048164.1	30.77	47.57	4.00E-60	203
NP_001048165.1	29.13	44.69	3.00E-59	200
NP_001051324.1	30.60	48.06	4.00E-59	199
NP_001173869.1	30.88	46.02	5.00E-57	194
NP_001052456.1	32.36	46.35	2.00E-56	193
NP_001052946.1	30.79	45.66	3.00E-56	192
NP_001047223.2	31.69	44.54	5.00E-55	189
NP_001059267.1	30.04	45.88	6.00E-55	189
NP_001064343.1	33.01	49.51	6.00E-55	189
NP_001067815.1	31.01	46.12	7.00E-54	186
NP_001047225.1	31.20	44.83	1.00E-53	185
NP_001061763.1	30.41	44.25	1.00E-51	180
NP_001052470.1	29.90	44.65	3.00E-51	179
NP_001052455.1	30.56	45.44	5.00E-50	175
NP_001057170.1	33.75	46.17	2.00E-49	174
NP_001050964.1	33.41	45.15	6.00E-48	169
NP_001173866.1	29.55	44.33	2.00E-47	167

Table 4: List of top 25 BLAST hits following a BLASTp search using the *Zea mays* SK1 protein sequence against the NCBI RefSeq protein sequence database specific for *Oryza sativa japonica*.

gene name	NCBI subject ID	top 10 <i>Gramene</i> BLASTp hits	bit score
<i>rTs1</i>	Q7XV13.2	LOC_Os04g37430.1	4957
		LOC_Os03g08220.1	4347
		LOC_Os08g39840.1	2794
		LOC_Os08g39850.1	2789
		LOC_Os03g49380.1	2526
		LOC_Os03g49260.2	2504
		LOC_Os03g52860.1	2459
		LOC_Os03g49350.1	2310
		LOC_Os05g23880.1	2307
		LOC_Os11g36719.1	2037
<i>rTs2</i>	NP001049848.1	LOC_Os03g18740.1	1490
		LOC_Os07g49120.1	1195
		LOC_Os11g32030.1	518
		LOC_Os04g10010.1	460
		LOC_Os04g33240.1	456
		LOC_Os04g10000.1	447
		LOC_Os03g59610.1	433
		LOC_Os03g61740.1	432
		LOC_Os07g46920.1	426
		LOC_Os07g46930.1	418
<i>rSk1</i>	NP001053356.1	LOC_Os04g44354.1	2820
		LOC_Os02g42280.1	1128
		LOC_Os03g55030.1	673
		LOC_Os10g17489.1	667
		LOC_Os10g18510.1	663
		LOC_Os10g18480.1	641
		LOC_Os07g13800.1	632
		LOC_Os07g13810.1	628
		LOC_Os08g07200.1	626
		LOC_Os07g13770.1	616

Table 5: List of top *Gramene* BLASTp hits using top japonica rice hits from NCBI BLASTp searches using *Zea mays* TS1, TS2, and SK1 protein sequences. NCBI Subject IDs are displayed adjacent to the MSU annotated gene from *Gramene*.

maize protein	rice gene ortholog protein ID	conserved protein domains
TS1	Q7XV13.2	PLAT/ LH2 PLN02337
TS2	NP001049848.1	Rossmann-fold NAD(P)(+)-binding PLN02253
SK1	NP001053356.1	Glycosyltransferase_GTB_typesuperfamily

Table 6: Protein domain conservation between maize SD proteins and corresponding japonica rice orthologs. Protein domain comparisons were performed via the *Conserved Domain Database* (CDD) search function in NCBI under concise search conditions.

tissue	gene 1	gene 2	significance (p Value < 0.05)
Root	<i>rTs1</i>	<i>rTs2</i>	0.986
		<i>rSk1</i>	0.003
	<i>rTs2</i>	<i>rTs1</i>	0.986
		<i>rSk1</i>	0.002
	<i>rSk1</i>	<i>rTs1</i>	0.003
		<i>rTs2</i>	0.002
Shoot	<i>rTs1</i>	<i>rTs2</i>	0.001
		<i>rSk1</i>	0.953
	<i>rTs2</i>	<i>rTs1</i>	0.001
		<i>rSk1</i>	0.002
	<i>rSk1</i>	<i>rTs1</i>	0.953
		<i>rTs2</i>	0.002
Leaf Base	<i>rTs1</i>	<i>rTs2</i>	0
		<i>rSk1</i>	0
	<i>rTs2</i>	<i>rTs1</i>	0
		<i>rSk1</i>	0.134
	<i>rSk1</i>	<i>rTs1</i>	0
		<i>rTs2</i>	0.134
Leaf Middle	<i>rTs1</i>	<i>rTs2</i>	0
		<i>rSk1</i>	0
	<i>rTs2</i>	<i>rTs1</i>	0
		<i>rSk1</i>	0.032
	<i>rSk1</i>	<i>rTs1</i>	0
		<i>rTs2</i>	0.032
Leaf Apex	<i>rTs1</i>	<i>rTs2</i>	0
		<i>rSk1</i>	0
	<i>rTs2</i>	<i>rTs1</i>	0
		<i>rSk1</i>	0.001
	<i>rSk1</i>	<i>rTs1</i>	0
		<i>rTs2</i>	0.001

Table 7: Pairwise ΔCt mean comparisons of genes within tissue type. P values were generated using two-way ANOVA analysis with IBM SPSS (version 8) to measure possible significant differences between mean ΔCt values. Significant interactions were determined for p values < 0.05.

transgene	transgenic event	plants / event	GUS expression
<i>rTs2::GUS</i>	E01	1	-
	E03	1	+
	E12	2	-
	E16	1	-
	E17	1	-
	E20	2	-
	E22	5	-
	E24	1	-
	E26	6	+
	E27	4	-
	E28	2	-
	E29	7	-
	E30	5	-
	E14	1	-
	E16	3	-
	E17	1	+

Table 8: List of T-DNA insertion events, number of regenerated plants per event, and stable *GUS* expressing events using a leaf disk screening assay. (+) positive for *GUS* expression, (-) negative for *GUS* expression.

(A)

(B)

(C)

Figure 8: Synteny between maize SD genes and rice SD orthologs. Local synteny between (A) maize *ts1* and rice chromosome 4, (B) maize *ts2* and the rice chromosome 3, and (C) maize *sk1* and rice chromosome 4. Syntenic relationships are visualized via the Genome Evolution Analysis platform (<http://genomeevolution.org/CoGe//GEvo.pl>). BLASTZ score cutoff was assigned to 10000. High Scoring Pairs are depicted as red boxes and synteny as pink connecting lines.

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Figure 10: Bayesian phylogenetic analysis using top BLASTp hits derived from maize *ts1* against the NCBI RefSeq protein sequence database. This tree includes japonica rice Q7XV13.2 protein sequence from the NCBI SWISS-PROT protein sequence database. Displayed is a portion of the consensus tree generated after 10,000,000 generations. The boxed region shows the clade containing maize TS1 with the rice ortholog shaded in red. Zm TS1, *Zea mays* TS1; Zm TS1b, *Zea mays* TS1b, Sb TS1, *Sorghum bicolor* TS1; Bd TS1, *Brachypodium distachyon* TS1; r Ts1, *Oryza sativa japonica* TS1 (Os04g37430 as described in the text). Posterior probabilities are displayed within nodes.

Figure 11: Bayesian analysis using top BLASTp hits derived from maize *ts2* against the NCBI RefSeq protein sequence database. Proteins are depicted as NCBI IDs. Displayed is a portion of a consensus tree generated after 10,000,000 generations. The boxed region shows the clade containing maize TS2 with the rice ortholog shaded red. Zm TS2, *Zea mays* TS2; Sb TS2, *Sorghum bicolor* TS2; Bd TS2, *Brachypodium distachyon* TS2; r TS2, *Oryza sativa japonica* TS2 (Os03g18740 as described in the text). Posterior probabilities are displayed within nodes.

Figure 12: Bayesian analysis using top BLASTp hits derived from maize *skl* against the NCBI RefSeq protein sequence database. Proteins are depicted as NCBI IDs. Displayed is a portion of a consensus tree generated after 10,000,000 generations. The boxed region shows the clade containing maize SK1 with the rice ortholog shaded red. Zm SK1, *Zea mays* SK1; Sb SK1, *Sorghum bicolor* SK1; Bd SK1, *Brachypodium distachyon* SK1; r Sk1, *Oryza sativa japonica* SK1 (Os04g44354 as described in the text). Posterior probabilities are displayed within nodes.

(A)

(B)

Figure 13: Taqman qRT-PCR relative gene expression analysis of rice SD orthologs in wild type japonica rice tissues: shoot, root, basal, middle, and apical leaf subsections. (A) *rTs1* expression calibrated to expression in shoots, (B) *rTs2* expression calibrated to leaf apex, and (C) *rSk1* expression calibrated to leaf apex. Relative quantification was determined using the comparative Ct method ($2^{-(\Delta\Delta C_t \pm \text{SEM})}$).

Figure 13: Taqman qRT-PCR relative gene expression analysis of rice SD orthologs in wild type japonica rice tissues: shoot, root, basal, middle, and apical leaf subsections. Continued.

Figure 14: RT-PCR analysis of *rTs1* and *rTs2* expression in wild-type japonica rice tissues. *GAPDH* was used as a positive cDNA control. (-) Non-template control.

(A)

(B)

Figure 15: Combined Taqman qRT-PCR relative gene expression analysis of rice SD orthologs in wild type japonica rice tissues: shoot, root, basal, middle, and apical leaf subsections. (A) Relative gene expression in shoots and roots calibrated to *rSk1* expression in roots, and (B) relative gene expression in leaf tissue subsections calibrated to *rSk1* expression in leaf apex. Relative quantification values were determined using the comparative Ct method using formula ($2^{-(\Delta\Delta Ct \pm SEM)}$).

(A)

(B)

Figure 16: Diagrammatic illustration of the T-DNA region of plant transformation vectors used in this study. (A) *rTs1::GUS* and (B) *rTs2::GUS* translational fusion reporter gene constructs. RB, right border; LB, left border; rTs2r, rice *Tassel seed2* promoter; GUS, β -glucuronidase coding sequence; rTs2Tr, rice *tassel seed2* terminator; TR7, TR7 terminator; Bar, *phosphinothricin acetyltransferase* coding sequence; TE, translational enhancer; 35S, Cauliflower Mosaic Virus 35S promoter; rTs1Pr, rice *Tassel seed1* promoter; rTs1Tr, rice *Tassel seed1* terminator. Figures not drawn to scale.

(A)

(B)

Figure 17: PCR assay for the presence of the *bar* gene in transgenic plants of used in this study. (A) *rTs1::GUS* transgenic plants (lanes 1-5). (B) *rTs2::GUS* transgenic plants (lanes 1-38). Samples were run on a 0.8 % agarose gel. Positive amplicon is 445 bp in length. M: 1 kb molecular marker.

(A)

(B)

Figure 18: *GUS* reporter gene confirmation of transgenic plants used in this study. (A) PCR confirmation of *rTs1::GUS* transgene insertion using Applied Biosystems 2 X Gene Expression Master Mix and *GUS*-specific taqman primers and probe (lanes 1-5). Positive amplicon is 69 bp in length. (B) PCR confirmation of *rTs2::GUS* transgene insertion using Phusion Hot Start II DNA Polymerase (lanes 1-38). Positive amplicon is 136 bp in length. Both were run on a 2% agarose gel. M: 100 bp molecular marker.

(A)

(B)

Figure 19: Histochemical leaf disk assay for stable *GUS* expression. (A) Leaf-disk *GUS* assay for scoring *GUS* expression in *rTs1::GUS* transgenic plants. A *GUS* expressing disk can be seen in well A5 corresponding to transgenic Event 17 Plant 1. Wild-type negative control is in well A6 (B) Leaf-disk *GUS* assay for scoring *GUS* expression in *rTs2::GUS* transgenic plants. *GUS* expressing disks were seen in wells A2, B7, B8, C1, C2, C3, and C4 corresponding to transgenic Event 3 Plant 1, and transgenic Event 26 plants 1-6 respectively. Wild-type negative control is in well E7. Separate T-DNA insertion events are grouped together in boxes.

Figure 20: *rTsI* expression in vegetative leaf tissue from Plant 1 of *rTsI::GUS* transgenic Event 17. (A) wild-type negative control, (B) *rTsI* expression in vascular tissue of vegetative leaves. Red scale bars indicate 1 mm.

Figure 21: GUS staining in various rice tissues from Plant 2 from *rTs2::GUS* transgenic Event 26, displaying tissue-specific *rTs2* expression. (A-E) Wild-type negative controls. (F) *GUS* expression in leaf vasculature, (G) interior of spikelet, (H) anthers, (I) outside of spikelet, (J) young emerging roots. Red scale bars indicate 1 mm.

CHAPTER 4

DISCUSSION

Maize sex determination (SD) is controlled by a genetic pathway that results in abortion of pistils in tassel spikelets and the arrest of stamen initials in ear spikelets to generate a monoecious condition (Figure 4). Pistil abortion results from the production of the phytohormone jasmonic acid (JA) through the action of the *Tassel seed1* and 2 genes (Acosta et al., 2009; DeLong et al., 1993); stamen arrest is controlled by the phytohormone gibberellin (Kim et al., 2007). A third process is active in the maize SD process, the protection of the functional ear pistil by the action of the *Silkless1* gene by an unknown mechanism. In maize, as in other plant SD systems, these genes function to produce unisexuality from an previously bisexual floral meristem suggesting that in most cases, SD mechanisms occur after the beginning stages of flowering. Moreover, unisexuality has arisen multiple independent times in the grass family (Poaceae), suggesting that multiple routes may lead to similar sex determination phenotypes (Malcomber & Kellogg, 2006).

Several important questions remain to be addressed about the origins of the SD pathway in maize. Most importantly, what are the origins of essential SD genes such as *ts1*, *ts2* and *sk1*? Do these represent novel genes found only in maize and its close unisexual relatives, or are they found in cosexual grasses as well? If the latter is true, then what are the functional differences in genes found in cosexual relatives as opposed to their role in unisexuality? To begin to address these questions, this thesis

focused on testing whether orthologs of maize SD genes existed in the cosexual species *Oryza sativa japonica* (rice), and if they do exist, what functional roles might they play in rice. The results reported herein answer the first question and begin to shed some light on the second one.

***Os04g37430*, *Os03g18740*, and *Os04g44354* are Rice Orthologs of Maize Sex Determination (SD) Genes**

First, to test whether maize SD orthologs exist in rice, a multi-pronged approach was used that attempted to cover various criteria necessary to establish orthology. The first approach was to search protein sequence databases in NCBI for rice proteins with the high homology (sequence identity and similarity) to each maize protein. This analysis tentatively identified *Os04g37430* as the homolog of the maize TS1 protein, *Os03g18740* as the homolog of the maize TS2 protein, and *Os04g44354* as the homolog of the maize SK1 protein. Next, chromosomal synteny analysis was performed to show that these homologs were indeed orthologs of their respective maize genes. Each gene was found in a chromosomal region of extensive synteny with their maize counterpart (Figure 8). Rice genes were also analyzed for conservation in gene architecture (exon-intron structure) and conserved protein domains with their maize homologs (Figure 9). Finally, using Bayesian analysis, each ortholog was shown to reside in a well-supported monophyletic clade with their respective maize protein, suggesting ancestry from a common source (Figures 10-12). Therefore, based on conserved amino acid sequence, genomic synteny analysis, gene structure and

biochemical conservation, and phylogenetics, the data overwhelmingly support the *Oryza sativa japonica* gene loci *Os04g37430*, *Os03g18740*, and *Os04g44354* to be true orthologs of the maize *ts1*, *ts2*, and *sk1* SD genes, respectively.

My analysis of maize SD orthologs in rice indicate that (1) maize SD genes have orthologs in rice, a cosexual cereal crop, (2) SD gene function arose from pre-existing genes encoding similar biochemical functions, (3) no significant structural variation in exon-intron structure or significant differences in protein architecture are apparent between maize and rice SD orthologs, and (4) the one structural difference noted is the duplication of the *ts1* gene in maize which is absent from rice. This duplication is consistent with the ancestral tetraploid origin of the extant maize genome (reviewed by Swigonova et al., 2004). Interestingly, the *ts2* and *sk1* genes do not seem to be duplicated in maize by phylogenetic analysis, and maize paralogs to *ts2* and *sk1* are found outside the monophyletic clade that includes the rice orthologs. These results imply that the rice *ts2* and *sk1* orthologs are more closely related to their respective maize SD genes than the maize genes are related to other paralogs in maize.

***rTs1*, *rTs2*, and *rSk1* Display Broad Expression Patterns in Rice**

Results from these studies show that true orthologs of the maize SD genes exist in rice. The next question to ask was: do functional differences between the two species exist? One possibility is that the maize and rice orthologs are differentially expressed, suggesting that recruitment of gene expression into the inflorescence may be the origin of unisexuality. In maize, *ts1* and *ts2* genes are expressed throughout the

vegetative plant and in the developing inflorescence at the time of sex determination (Acosta et al., 2009; DeLong et al., 1993). Previous results show that the excision of the transposable element *Ac* from the *ts2-m1* allele generates wild-type staminate sectors of spikelets in the tassel (DeLong et al., 1993). Likewise, *ts1* expression is thought to be responsible for unisexuality since this gene controls the production of the phytohormone jasmonic acid (JA), and its application to developing inflorescence can restore the wild-type phenotype in mutant *ts1* plants (Acosta et al., 2009).

Therefore, the question arises whether the sexual polymorphism between these species might be due to differences in gene expression between maize and rice inflorescences, where gene expression may be recruited to inflorescences in one species but not in the other.

To test this hypothesis, expression patterns of the rice *ts1*, *ts2* and *sk1* orthologs using reverse transcription PCR (RT-PCR) and quantitative reverse transcription PCR (qRT-PCR) assays were examined. Expression of these genes was examined in the roots and shoots of young seedlings, and vegetative leaf tissues, and developing inflorescences. RT-PCR analysis clearly showed expression of *rTs1* and *rTs2* in seedling tissue, vegetative tissue, and inflorescences (Figure 14). To quantify gene expression levels of the rice orthologs, Taqman assays were performed on the *rTs1*, *rTs2*, and *rSk1* genes in vegetative tissues (for these studies, it is important to note that during the progression of this experiment, inflorescences were unavailable). The qRT-PCR results demonstrated that each SD ortholog was broadly expressed throughout the japonica rice plant during vegetative development. The qRT-PCR analysis confirmed the presence of *rTs1*, *rTs2*, and *rSk1* gene expression in eleven

DAP shoots and roots as well as in twenty-six DAP subsections of leaf tissues chosen to correspond with different stages of leaf vasculature development. Overall, the *rTs1* gene was expressed at a relatively higher level than both *rTs2* and *rSk1* in eleven DAP shoots, and in all leaf subsections (Figure 15). The *rTs2* gene was expressed at slightly higher levels than both *rTs1* and *rSk1* in eleven DAP roots, and slightly higher than *rSk1* in all leaf subsections (Figure 15). The expression of each gene appeared to gradually decrease in expression as leaf tissue matured.

A possible explanation for elevated gene expression in leaf base subsections may involve the presence of intercalary meristems within leaf base tissue subsections collected during the study. Intercalary meristematic tissue is found at the base of leaves in monocotyledonous plants and allows for rapid regrowth of leaves at stem nodes. Leaf base tissue may contain intercalary meristems undergoing cell division and subsequent vasculature tissue differentiation, which could explain the high gene expression levels.

RT-PCR and qRT-PCR results appear to be consistent with vegetative and inflorescence expression patterns of the maize genes (Acosta et al., 2009; DeLong et al., 1993). Therefore, the recruitment hypothesis is inconsistent with the results. In lieu of recruitment of gene expression, differences in the maize and rice orthologs in their temporal-spatial distribution of expression in specific cell types may account for the sexual polymorphisms observed between these two species. This remains an open possibility that needs to be examined in the future.

To further characterize expression of the rice SD orthologs, transgenic *rTs1* and *rTs2* reporter gene experiments were conducted. These studies demonstrated that

rTs1 and *rTs2* were expressed in the vasculature of leaves and in developing roots, results consistent with RT-PCR and qRT-PCR results (transgenic *rSk1* plants were unavailable at the time of this analysis). According to GUS assays, *rTs2* was also expressed in inflorescences, more specifically in developing anthers and in the maturing vasculature of the lemma and palea. These results are consistent with inflorescence expression seen in the wild type japonica rice RT-PCR experiments.

It is important to note possible shortcomings of the evidence gleaned from *GUS* reporter expression. Transgenic populations used in this study, transformed with both *rTs1::GUS* and *rTs2::GUS* translational fusions, were generated via an *Agrobacterium*-mediated transformation procedure. This procedure inserts T-DNA into random locations of the genome of individual rice callus tissue cells. There exists the possibility that expression patterns of reporter genes are influenced by several factors such as multiple transgene insertions and chromosomal positional effects that cause silencing or enhancing transgene expression due to the local genomic environment surrounding the insertion. For example, the transgenic *rTs2::GUS* population, containing only two T-DNA insertion events out of a total of thirteen independent events, was positive for *GUS* expression (Events 3 and 26). Lack of expression in other insertions may be due to transgene copy number, gene silencing or chromosomal position effects. It may be possible that *GUS* expression within plants of Events 3 and 26 was enhanced in comparison to wild-type expression levels due to positional effects such the presence of upstream enhancer elements or high transgene copy number. The variability in reporter gene expression will need to be addressed in future studies.

Jasmonic Acid Biosynthesis and Cell Death Signaling

One interesting observation was that expression of *rTs1* and *rTs2*, based on *GUS* reporter expression, was confined to vascular cells of the leaf, and in the case of *rTs2*, in developing anthers and the vasculature of leaf-like floral organs and emerging roots. Importantly, cell death processes are occurring in each of these tissues during the normal course of development. Leaf vasculature includes xylem tissue, which allows for the transport of water between plant tissues. Xylem includes differentiated cell types called tracheids and tracheary elements that undergo cell death at maturation (reviewed by Turner, Gallois, & Brown, 2007). Anther dehiscence also requires the programmed cell death of specific cell types (including degeneration of tapetal tissue, and rupture of the anther at the stomium), which ultimately results in pollen release (reviewed by Goldberg, Beals, & Sanders, 1993). In maize, the *ts1* and *ts2* genes are associated with pistil cell death during formation of staminate florets. The connection to plant cell death processes may be a shared feature between the maize and rice orthologs.

The biochemical relationship of these maize and rice genes may be explained by the association with cell death signaling and JA signaling. Maize *Tassel seed* genes play a role in JA biosynthesis, and jasmonic acid signaling in various programmed cell death events have been demonstrated in the past. JA signaling is required for anther dehiscence in *Arabidopsis* (McConn & Browse, 1996), wound responses and differentiation of vasculature tissue in tomato (Wasternack et al., 2006), and lateral root development in both *Arabidopsis* and rice (Raya-Gonzalez et al.,

2012). These results, as well as previously published results, suggest that the rice SD orthologs may mimic maize SD gene biochemical function, perhaps by participating in JA biosynthesis and subsequent JA cell death signaling. One interesting possibility is that recruitment of JA signaling into the developing pistils was an essential step in the evolution of sex determination pathway in maize.

Conclusion

Studying the maize SD pathway is interesting from both both a scientific and a practical standpoint. In a scientific perspective, *tasselseed*-mediated cell death to induce monoecy, the role of jasmonic acid in cell death signaling, and *silkless*-mediated protection of the upper ear floret in maize are all important areas for research in plant biology. Practically, research in maize SD may provide new avenues to explore in agricultural biotechnology, which could lead to a new generation of hybrid breeding technologies. Heterosis represents a major strategy in combating growing food insecurity, and extending unisexual traits to cosexual cereals, a crucial step in developing novel hybrid varieties, would require analysis and characterization of important maize SD orthologs within these food crop systems.

This study involved identifying *ts1*, *ts2*, and *sk1* orthologs in rice, as well as a preliminary investigation of tissue-specific expression profiles of these orthologs with the hope of establishing a firm foundation for future research in this area. The results clearly showed that orthologs of maize SD genes exists in rice, and that these orthologs are expressed broadly throughout the rice plant. The results also suggest

that rice *Tassel seed* gene orthologs are confined to tissues undergoing cell death during the course of normal development. Therefore, a strong connection may exist between cell death and JA signaling, and perhaps this relationship has been conserved during the evolution of maize and rice.

Understanding the unique evolution of each maize SD gene and their corresponding rice ortholog can shed some light on why maize evolved a SD pathway to produce unisexual spikelets whereas rice maintained a cosexual orientation. Elucidating the differences in SD gene expression between maize and rice, at both a developmental and tissue-specific level, will assist in designing strategies to transfer maize-like unisexuality to rice, allowing for the generation of more food for a planet struggling with a growing human population. It remains to be seen whether this outcome can be achieved, either through conventional breeding or through the advancements of current agricultural biotechnology; research in maize SD genes, and their orthologous counterparts in other cereals, will only encourage the development of new and innovative solutions to combat food insecurity in the coming decades.

APPENDICES

Gene	Tissue	Average Δ Ct	Standard Error
<i>rTs1</i>	Root	10.11	0.16
	Shoot	10.48	0.12
	Leaf Base	5.61	0.33
	Leaf Middle	6.56	0.21
	Leaf Apex	7.33	0.09
<i>rTs2</i>	Root	10.12	0.17
	Shoot	9.14	0.11
	Leaf Base	9.47	0.44
	Leaf Middle	9.95	0.34
	Leaf Apex	10.74	0.17
<i>rSk1</i>	Root	12.08	0.78
	Shoot	10.46	0.50
	Leaf Base	10.67	0.78
	Leaf Middle	11.29	0.61
	Leaf Apex	12.74	0.52

Table 9: Δ Ct values generated from multiplexed taqman qRT-PCR experiments involved in this study.

Average Δ Ct and standard errors include all biological and technical replicates for each gene and tissue type.

Singleplex		Multiplex		% difference
Gene	Average Ct	Gene	Average Ct	
eEF-1a-VIC	19.15	eEF-1a-VIC	19.61	-2.40
rTs1-FAM	27.27	rTs1-FAM	26.32	3.40
eEF-1a-VIC	17.38	eEF-1a-VIC	17.68	-1.70
rTs2-FAM	25.25	rTs2-FAM	22.44	11.1
eEF-1a-VIC	17.38	eEF-1a-VIC	17.00	2.20
rSk1-FAM	27.41	rSk1-FAM	24.16	11.9

Table 10: Mean Ct values from Taqman efficiency tests for *rTs1*, *rTs2*, and *rSk1* primer and probe pairs.

Each primer and probe pair was run as a singleplexed and multiplexed reaction in parallel. Reactions were run in triplicate and mean Ct values were acquired. Each reaction used 500 ng of cDNA as a template, converted from a total RNA sample extracted from wild-type japonica rice leaf tissue.

NCBI protein IDs used in phylogenetic analysis of TS1	NCBI protein IDs used in phylogenetic analysis of TS2	NCBI protein IDs used in phylogenetic analysis of SK1
XP_002966811.1	XP002978559.1	XP002966076.1
XP_002961193.1	XP002970482.1	XP002971410.1
XP_001764781.1	NP001105322.1	XP002966074.1
XP_002283123.1	XP002468013.1	XP002971412.1
XP_002283135.1	NP001049848.1	XP002971416.1
XP_002283147.2	XP003558117.1	NP001147693.1
XP_002285574.1	NP001060718.1	NP001151310.1
NP_001233812.1	XP002323034.1	NP001048163.1
NP_001234259.1	XP002308240.1	XP002452530.1
XP_003537948.1	XP002533789.1	XP002452527.1
XP_003539375.1	XP002265892.1	NP001048164.1
XP_003537949.1	XP002325666.1	NP173655.2
XP_002297796.1	XP003552289.1	XP002893223.1
XP_002299250.1	XP003534665.1	NP173653.1
XP_002513441.1	XP003522143.1	NP001031078.1
XP_002323952.1	XP003522142.1	XP002893221.1
XP_002513443.1	XP003623493.1	NP173652.1
XP_003556820.1	NP190736.1	XP002893219.1
XP_003555620.1	XP002877816.1	NP973885.1
XP_003519005.1	XP002277487.2	NP173656.1
XP_003543820.1	XP003521608.1	XP002893224.1
XP_003555640.1	XP003554568.1	XP002891115.1
XP_003601322.1	XP002272942.2	XP003544213.1
XP_002263854.1	XP002515215.1	NP001048165.1
XP_002314229.1	NP001118737.1	XP003570608.1
XP_002520644.1	NP189570.3	XP002437371.1
XP_002513228.1	XP003559196.1	XP002452336.1
XP_003574749.1	XP002328109.1	XP003575223.1
XP_002445903.1	XP002327628.1	XP003579871.1
NP_001062199.1	XP002523859.1	XP003635298.1
NP_001062200.1	XP002328111.1	XP002268637.1
XP_003571069.1	XP002272206.1	XP003598165.1

Table 11: List of protein sequences obtained from BLASTp searches of maize TS1, TS2, and SK1 proteins against the entire NCBI RefSeq database. These were used to construct cladograms using Bayesian inference analysis. Query Q7XV13.2 from the NCBI SWISS-PROT database was included in the TS1 group (see text).

NCBI protein IDs used in phylogenetic analysis of TS1	NCBI protein IDs used in phylogenetic analysis of TS2	NCBI protein IDs used in phylogenetic analysis of SK1
XP_003571227.1	XP002272280.2	XP003531544.1
NP_001046180.1	NP001239862.1	XP002525850.1
NP_001105981.1	XP003546632.1	XP003547073.1
NP_566875.1	XP003538100.1	NP001150098.1
XP_002875714.1	XP003540813.1	XP002454037.1
NP_001105979.1	XP003540812.1	NP001131902.2
XP_002446554.1	XP002272549.1	XP002443889.1
NP_001105978.1	XP002272838.1	XP003571510.1
Q7XV13.2	XP003632648.1	NP001176085.1
XP_003579848.1	XP002281462.1	NP001051322.1
NP_001105977.1	XP002323405.1	XP002466429.1
XP_002468402.1	XP002309406.1	XP002466428.1
NP_001049158.1	XP003554452.1	XP002466427.1
XP_003561993.1	XP002523206.1	NP001051321.2
XP_002890224.1	XP002275768.1	XP003559264.1
NP_564021.1	XP002308449.1	XP002466426.1
NP_177396.1	XP002281352.1	NP001051324.1
XP_002887437.1	XP002281320.2	XP003559257.1
XP_002331196.1	XP002281290.1	NP001051326.1
XP_002304125.1	XP002275639.2	XP002526116.1
XP_002273258.2	XP002275723.2	XP002306070.1
XP_003528556.1	XP002309404.1	XP003554912.1
XP_003547757.1	XP002308448.1	XP002306038.1
XP_003627308.1	XP002323404.1	XP002324098.1
XP_003627309.1	XP002309402.1	XP002526107.1
XP_003554800.1	XP002323403.1	XP002276804.1
XP_003521841.1	XP002322804.1	XP002279299.1
XP_002519072.1	XP002530171.1	XP002272457.1
XP_002519070.1	XP002526911.1	XP002276825.1
XP_003528455.1	XP002530170.1	XP003551755.1
XP_003541736.1	XP002526345.1	XP003551756.1
XP_003531186.1	XP002513332.1	XP003543952.1
XP_003637315.1	XP002513344.1	XP003516840.1

Table 11 continued.

NCBI protein IDs used in phylogenetic analysis of TS1	NCBI protein IDs used in phylogenetic analysis of TS2	NCBI protein IDs used in phylogenetic analysis of SK1
XP_002311724.1	XP002309405.1	XP003543870.1
XP_002314548.1	XP002530176.1	XP003517996.1
XP_002514963.1	XP002275746.2	XP003554914.1
XP_002265505.1	XP003553613.1	XP003516841.1
NP_176923.1	XP003554454.1	XP003554841.1
XP_002887130.1	XP003554453.1	XP003531850.1
XP_002981424.1	XP002466226.1	XP002284331.1
XP_002969954.1	NP001148513.1	XP002281326.2
XP_002964909.1	XP003563948.1	XP002281187.1
XP_002986490.1	NP001051666.1	XP002281171.2
XP_002986491.1	XP003538228.1	XP002284355.1
XP_002964910.1	XP003539230.1	XP002284350.1
XP_002964771.1	XP002513092.1	XP002281210.2
XP_002961703.1	XP002527336.1	XP002281128.1
XP_002994506.1	XP002297758.1	XP002281117.1
XP_002966904.1	XP002265724.1	XP002314683.1
XP_002961107.1	XP003598766.1	NP186859.1
XP_002284535.2	XP002894375.1	XP002884302.1
XP_002280651.1	XP002321040.1	NP001151223.1
NP_001234856.1	XP002321039.1	NP001131410.1
NP_001234098.1	XP002321038.1	XP002448206.1
XP_002891934.1	XP002520695.1	NP001053356.1
NP_175900.1	XP003521529.1	XP003581444.1
XP_003521704.1	XP003521528.1	XP003572739.1
XP_002512386.1	XP002512852.1	XP002454220.1
NP_188879.2	XP002321085.1	NP001169852.1
NP_001237323.1	NP189311.2	XP002460066.1
XP_003528382.1	XP002875338.1	XP002266304.1
NP_001237685.1	XP002275647.1	XP002521042.1
NP_001236153.1	XP003624486.1	XP002862169.1
XP_003597558.1	NP566798.1	NP188864.1
XP_003531596.1	NP001060559.1	XP002883349.1
XP_003531599.1	NP001060558.2	XP003618097.1
XP_003531597.1	XP002461166.1	XP003544918.1
XP_003627173.1	XP003576028.1	
XP_003627174.1		

Table 11 continued.

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