Investigations of self-incompatibility (SI) in perennial ryegrass (*Lolium perenne* L.)

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Abstract

Perennial ryegrass (*Lolium perenne* L.) is one of the most economically and environmentally important grass species for the temperate zone. It maintains effective self-incompatibility (SI), which promotes outbreeding as well as limits the efficient production of inbred lines and hybrids. SI in *L. perenne* is controlled by the *S* and *Z* loci, mapping to linkage groups 1 and 2, respectively. None of the gene products has been identified so far. Comparative mapping has identified regions on rice chromosomes 5 (R5) and 4 with synteny to regions of *L. perenne* genome containing the *S* and *Z* loci, respectively. Markers were developed from the syntenic rice genomic region to refine the *S* and *Z* maps. The closest flanking markers had a map distance of 2 cM from *S* and 0.2 cM from *Z*. SI cDNA libraries were developed from *in-vitro* pollinated stigma subtracted with unpollinated stigma to identify SI components and SI response related genes. Through a BLAST search, candidates identified from the SI libraries that were orthologous to sequences on the *S* and *Z* flanking regions on rice R4 and R5 were the prime candidate SI genes. Altogether ten SI candidate genes were identified with incompatible response associated differentially expression pattern: a rapid increase in expression within two minutes after pollen-stigma contact and reaching a maximum between 2-10 minutes, implying their roles in the SI response. Attempts were carried out to determine the linkage relationships between the identified candidates and the *S* or *Z* loci. Large fine scale mapping populations were developed individually for the *S* and *Z* loci to generate high resolution maps of *S* and *Z* towards map-based cloning. Tightly linked markers were identified mapping at a distance of 1.4 cM from *S* and 0.9 cM from *Z*. The studies performed in this project have implications on both the underlying genetic control and the associated biochemical responses involved in *L. perenne* SI. The closely linked markers for *S* or *Z* could be applied in future marker assisted selection breeding programmes and map-based cloning.

Dedication

This thesis is dedicated to my dearest parents, who give me endless love and support throughout my life. It is dedicated to my grandmother and grandparents, whose care and encouragement I am always indebted. It is also dedicated to my departed grandfather, whose love and trust is my forever inspiration.

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Contents

Table of contents

List of figures

List of tables

CHAPTER 1

INTRODUCTION

1.1 General introduction

Perennial ryegrass (*Lolium perenne* L., 2n=*2x=14*; DNA content = 2034 Mbp: Bennett *et al*., 1976) is one of the most economically and environmentally important grass species, native to Europe, temperate Asia, and North Africa and widely distributed throughout the world (Hannaway *et al*., 1999). It is cultivated as a forage crop for ruminant animals for dairy and meat, and wool production and it is also widely used for turf (Wilkins and Humphreys, 2003; Humphreys *et al*., 2006). The value of ryegrass keeps increasing for its conventional agriculture use as well as for the development of environmentally friendly biofuels and raw material, and application in leisure activities (King *et al*., 2008). Perennial ryegrass is a member of the Poaceae family and is, therefore, related to many important and more extensively studied cereals, such as rice, wheat, barley, maize and oats. Perennial ryegrass is an outcrossing species where self-incompatibility (SI) is governed by the complementary action of two gametophytically expressed and highly polyallelic genes, *S* and *Z* (Cornish *et al*., 1979). The effectiveness of SI limits to a certain level the efficient production of inbred lines and hybrids in plant breeding. As a consequence, the breeding of turf and forage ryegrasses involves generating synthetic varieties from polycrossing selected multiple mother plants. The production of synthetics would make it possible to exploit heterosis effects. On the other hand, in the process of selecting important traits, homozygosity of regions of the genome and homogeneity of these regions across the breeding population would be developed. If the selected traits are linked to *S* and *Z*, seed yield of the breeding population could be seriously compromised. Up to date, none of the gene products of *S* and *Z* has been identified in any grass species. All Poaceae species so far studied possess a common SI system (reviewed by Baumann *et al*., 2000). Studies on perennial ryegrass would contribute to a better understanding of the SI systems in other species in terms of both function and evolution.

From a practical standpoint, having gene specific markers for *S* and *Z* could lead to ways of (1) developing new breeding systems to better exploit the huge heterotic potential of the species and (2) maximising seed yield potential through ensuring maximum allelic diversity in breeding populations.

1.2 Introduction to self-incompatibility

SI has been known in flowering plants for over a century since Darwin's description in 1876 (Darwin, 1876). SI is considered to be one of the most important strategies used by flowering plants to circumvent the tendency towards self-fertilization and is defined as "the inability of a fertile hermaphrodite seed plant to produce zygotes after self-pollination" (de Nettancourt, 1977). SI enables the pistil to differentiate self and non-self pollen of the same species, so that the self (incompatible) pollen is selectively inhibited during pollination, at a species dependent specific stage. Since plants can not move about and choose a mating partner, the effectiveness of SI mechanisms is of crucial importance for promoting outcrossing, maintaining heterozygosity, and ensuring the evolutionary success of flowering plants.

Heteromorphic self-incompatibility has been found in 24 families and in more than 167 genera (Ganders, 1979). The flowers produced by different species are morphologically distinct with regard to style length and anther level. This topological barrier together with some possible derivative biochemical barriers prevents mating action (Barrett and Cruzan, 1994). Up to date, research has been performed in *Gaertnera* (Pailler and Thompson, 1997) *Erythroxylum* (Pailler *et al*., 1998), Rubiaceae species (Faivre, 2002), *Fagopyrum* (Matsui *et al*., 2003, 2004), *Pentanisia* (Massinga *et al*., 2005) and *Primula* (McCubbin *et al*., 2006). Proteins potentially involved in SI reactions have been detected in *Turnera* (Athanasiou and Shore, 1997) and buckwheat (*Fagopyrum esculentum*) (Miljuš-Đukić *et al*., 2004), but their

functions are unknown. A single diallelic *S*-locus, containing at least seven genes, has been identified to co-regulate floral characteristics and biochemical incompatibility (Kurian and Richards, 1997). Genes acting downstream of the *S*-locus genes have recently been identified in *Primula*, but the molecular genetics of heteromorphic SI are still uncharacterized (McCubbin *et al*., 2006).

Homomorphic self-incompatibility is more widespread and occurs in over half of the flowering plant families (de Nettancourt, 2001). Classic genetic studies in the early 20th century revealed two major classes of homomorphic SI systems, gametophytic and sporophytic (de Nettancourt, 1977). Homomorphic refers to the group of plants in which flowers produced by all individuals of a self-incompatible species have the same morphological character. In many cases, SI is controlled by a single genetic locus, the *S*-locus, with a large number of alleles. Two or more multi-allelic loci controlled SI systems are also existing. It has been revealed in the well studied single-locus SI systems that at least two genes, one expressed in pistil and the other in pollen or pollen tube, form a complex at the *S*-locus, therefore the term of '*S* haplotype' has been introduced to determine the SI specificity in the single-locus SI systems (Nasrallah, 2005). In gametophytic SI (GSI), the SI phenotype of pollen is determined by its own (haploid) *S* haplotype. The pollen is rejected when the *S*-haplotype of the haploid pollen matches either of the two *S*-haplotypes of the diploid pistil. In sporophytic SI (SSI), the SI phenotype of pollen is determined by the *S*-haplotype of its diploid parent. The pollen is recognized as self and rejected if either of the two *S*-haplotypes of its parent matches one of the two *S*-haplotypes of the pistil. The diploid expression of SSI allows dominance-recessive interactions to occur individually between *S*-alleles in pollen and stigma, which complicates the SSI system as described later in section 1.4.1 (Figure 1.1).

In both systems, the style becomes incompatible at a late bud stage, 1-4 days before pollen maturity at anthesis. In vitro pollination of the immature style with pollen from a mature flower of the same plant can lead to some seed set. Break down of self-incompatibility can be achieved under a variety of natural stress conditions and other manipulations such as heat and CO_2 treatment of the style, lectin pretreatment of the stigma surface, and physical disruption of the stigmatic barrier (de Nettancourt, 1977). This facts lead to the hypothesis that self-fertilization is inhibited on stylar/stigmatic side. The relationship between pollen type, stigma type, site of pollen rejection and the SI system involved had been reviewed in a large number of SI species by Heslop-Harrison and Shivanna (1977). The pollen inhibition usually takes place on or immediately below the stigma in species with a dry stigma and within the style, or in rare circumstances, the ovary in species with a wet stigma. Details of the physiology and molecular genetics of homomorphic SI systems are described in the following sections.

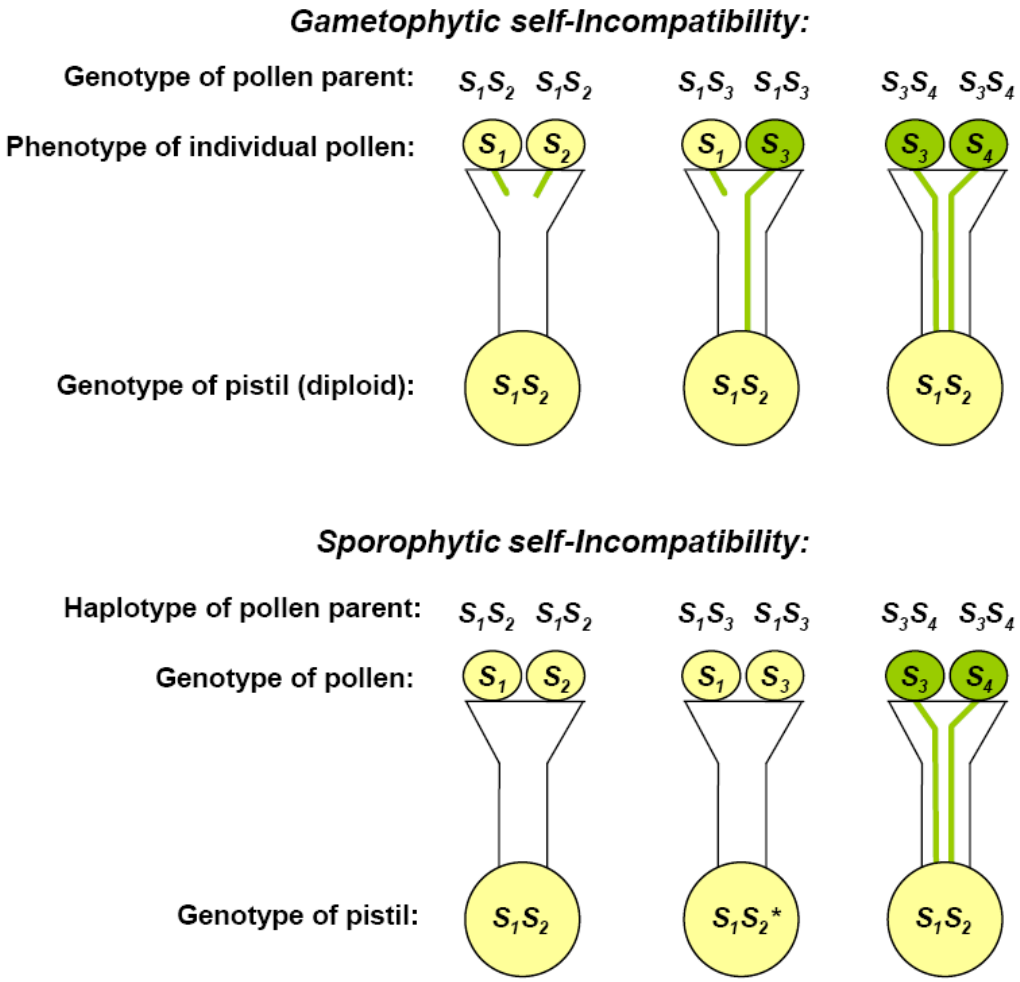


Figure 1.1 Genetic control of two major homomorphic self-incompatibility systems controlled by a multi-allelic *S*-locus where pollen and pistil are independently expressed. In the gametophytic system, inhibition of the pollen occurs when the haplotype of the individual pollen grain matches with either haplotype in the diploid pistil. Thus, both S_1 and S_2 pollen are inhibited in a S_1S_2 style; only S_3 pollen produced by S_1S_3 plant will grow successfully in a S_1S_2 style. Where there is no match of alleles, for example S_3S_4 pollen on a S_1S_2 style, all pollen is compatible and pollen tubes will grow. In the sporophytic system, when a haplotype in the pollen parent is matched with either haplotype of the pistil (e.g., S_1S_2 or S_1S_3 pollen on S_1S_2 pistil), pollen is arrested at the stigma surface. Where there is no match, pollen tubes grow to the embryo sac. Because it is the genotype of the diploid pollen parent that matters, dominance interactions are possible between *S*-haplotypes. In the case of S_1S_3 pollen and S_1S_2 pistil (indicated by *), if S_3 is dominant to S_1 in the pollen or S_2 is dominant to S_1 in the pistil, the pollen from S_1S_3 parent will be compatible. Incompatible pollen is indicated with the same yellow color as pistil. Compatible pollen and pollen tube are shown in green color.

1.3 Physiological features of SI

The process of pollination initiates when pollen grains from anthers, the male reproductive organs, are deposited on the stigma of pistils, the female reproductive organs. On the stigma, pollen absorbs water and forms a pollen tube which invades the pistil. The pollen tube, carrying all the cellular contents including sperms, first grows between the walls of the stigmatic cells, then travels through an extracellular matrix (ECM) in the transmitting tissue of the style and finally arrives at the ovary, where it targets an ovule that contains an egg (Palanivelu and Preuss, 2000). The tube then bursts and delivers the sperm, followed by fertilization. Interactions between stigma and pollen have been studied to understand self-incompatibility responses, using centrifugation (Luu *et al*., 1997), detergent assays and spring displacement experiments (Zinkl *et al*., 1999). Self-incompatible pollen is rejected at some point in the pollination process, depending on the species. This may happen at hydration, germination, during growth through the style, in the ovule, or even post-fertilization, implicating that there are a variety of mechanisms involved in different SI systems.

SSI has been identified in phylogenetically divergent families of the Brassicaceae, Asteraceae, Convolvulaceae, Polemoniaceae and Malvaceae, which suggests multiple origins of SSI (Hiscock and Tabah, 2003). Most studies are concentrated on species of the Brassicaceae. Hydration of incompatible pollen in *Brassica* is preceded by a latent period of between 30-90 min, during which time signals passing from the pollen coat to the stigma are responsible for the activation of the SI system. Differences in Ca^{2+} signalling were observed between SC and SI repspones in *B. napus*. However, the lower intensity of Ca^{2+} peaks at the early stage of incompatible pollination suggests a more critical role of Ca^{2+} signalling in SC other than SI response (Dearnaley *et al.*, 1998). A stigmatic response is stimulated to interrupt hydration and arrest grain development. The SI response is focused at the pollen-stigma interface as self

grains are not inhibited metabolically, but are physiologically isolated from the subjacent stigmatic papilla, which ensures that a single papilla can simultaneously permit development of a compatible pollen grain and reject an incompatible pollen grain (Dickinson, 1995). Kroh (1966) has reported that incompatible pollen grains can be "resurrected" by transferring them to a stigma where they are compatible. This suggests that the rejection of incompatible pollen is reversible. However, further evidence of the mechanism is needed to explain this physiological observation.

GSI systems have been described in more than 60 families, including the Solanaceae, Liliaceae, Rosaceae, Papaveraceae and Poaceae (Charlesworth *et al*., 2005; McClure and Franklin-Tong, 2006; Baumann *et al*., 2000). In the Solanaceae, both the compatible pollen and incompatible pollen germinate and a pollen tube grows through the transmitting tract of the style. There, a polysaccharide, callose, is deposited in the walls and forms plugs at regular intervals. However, the growth of the incompatible pollen tube is arrested by the time it has reached about one-third of the way through the style. The callose deposits become irregular; the tube wall becomes thickened and the tip becomes abnormal. The callose deposits swell and burst within the style in most cases. In *Papaver rhoeas*, by comparison, rejection of pollen takes place on the stigmatic surface. *P. rhoeas* also has a dry stigmatic surface compared to the wet, lipid-rich exudate found on the surface of the Solanaceae (Elleman *et al*., 1992). Inhibition of incompatible pollen in *P. rhoeas* is rapid, taking place on a time scale of minutes, compared with the relatively slow inhibition in the Solanaceae. Three possible phases have been proposed for the *Papaver* SI response. First, a very rapid but reversible inhibition of tip growth takes place, followed by a 'commitment' phase, during which processes are triggered that lead to the irreversible degradative processes detected in the 'late'

phase (Wheeler *et al*., 2001). These fundamental differences implicate that gametophytic SI systems may have evolved independently several times.

1.4 Molecular genetics of SI

1.4.1 Sporophytic self-incompatibility

The molecular basis of SSI has been most extensively studied in the Brassicaceae, particularly in crop *Brassica* species. Recently, studies are also being carried out on *Ipomoea trifida* (Convolvulaceae) (Kowyama *et al*., 2000) and *Senecio squalidus* (Asteraceae) (Hiscock, 2000; Hiscock *et al*., 2003). Evidences have shown that orthologues of *Brassica* stigmatic SI determinants in these two families are not involved in the SSI responses, suggesting novel SSI mechanisms different from that of the Brassicaceae (reviewed by Hiscock and Tabah, 2003).

In *Brassica*, both the female and male determinants of SI have been identified (Nasrallah, 2002). The female determinant is the *S*-locus receptor protein kinase (*SRK*), a single-pass transmembrane serine/threonine kinase, expressed in the stigma epidermis (Stein *et al*., 1991). The hyper-variability regions within its receptor domain were predicted to be responsible for the *S*-specificity (Hiscock and Tabah, 2003). Another stigma *S*-linked gene, the *S*-locus glycoprotein (*SLG*), similar to the extracellular domain of SRK with the same allele, has been reported to enhance the ability of stigma to reject incompatible pollen (Takasaki *et al*., 2000). In the self-incompatible *Arabidopsis lyrata*, the *S*-locus appears to lack *SLG*, implying that *SRK* alone determines *S*-specificity in the stigma and *SLG* acts to enhance the strength of the SI reaction (Schierup *et al*., 2001).The male determinant is the *S*-locus cysteine-rich protein (*SCR*) gene (Schopfer *et al*., 1999), also designated as *S*-locus pollen protein 11 (*SP11*) (Takayama *et al*., 2000). It encodes small secreted hydrophilic and positively charged proteins

of 50 to 59 amino acids, expressed mainly in the anther tapetum which breaks down to form the pollen coating, where the mature SCR/SP11 protein accumulates. *SCR/SP11* and *SRK* are tightly linked and recombination between them is suppressed, which would otherwise lead to the breakdown of the SI function (Casselman *et al*., 2000). However, the order, orientation and distance between them and other *S*-locus genes is different (Boyes *et al*., 1997; Cui *et al*., 1999; Takayama *et al*., 2000; Fukai *et al*., 2003). Comparative analysis of the *S* locus of *A. lyrata* and *Brassica* indicates that the *S* locus of Brassicaceae is a dynamic locus undergoing repeated rearrangements, deletions and insertions (Kusaba *et al*., 2001). Insertion of retrotransposons has recently been reported as the reason for the differences of the *S*-locus lengths between *Brassica oleracea* and *B. rapa* (Fujimoto *et al*., 2006).

The expression of *SRK* in the plasma membrane of stigma epidermal cells and of *SCR/SP11* in the pollen coat is tightly regulated and peaks just before anthesis when flowers are self-incompatible matured (Schopfer *et al.*, 1999; Takasaki *et al.*, 2000). The interaction between *SRK* and *SCR/SP11* activates the SI response in an allelic-specific manner, when the *S*-receptor domain of SRK and SCR/SP11 pollen ligand are encoded by the same *S* haplotype (Kachroo *et al*., 2001; Takayama *et al*., 2001). A model for the mechanism of SSI is illustrated in Figure 1.2. After pollination, the SCR/SP11 protein is delivered to the surface of a stigma epidermal cell then transferred to the plasma membrane within the region of pollen contact. The interaction of SRK and SCR/SP11 induces transphosphorylation on serine and threonine residues in the kinase domains of presumably dimerized SRKs (Takayama *et al.,* 2001). Two thioredoxin-H-like proteins (THL1 and THL2) associate with SRKs and act as inhibitors in the absence of SCR/SP11 to prevent auto-phosphorylation and maintain the inactivate state of SRKs (Cabrillac *et al.,* 2001). Activation of SRK accompanied with SLG triggers a signal cascade that results in the arrest of pollen tube development. The downstream

signalling reactions are under identification. *M* locus protein kinase (MLPK), a membrane anchored cytoplasmic serine/threonine protein kinase, involves in the SI signalling (Murase *et al.*, 2004) through direct interactions with SRK (Kakita *et al.*, 2007). A stigma-specific Armadillo repeat-containing protein ARC1 has been identified to interact with the kinase domain of SRK in a phosphorylation-dependent manner (Stone *et al*., 1999). A recently identified U-box motif in ARC1 suggests a role for ubiquitination in the SI response (Azevedo *et al*., 2001). The direct interaction of ARC1 and SRK implicates the role of MLPK as a coreceptor within the SRK receptor complex. Characterization of the *SCR/SP11* high-affinity binding site revealed that the integral and membrane-anchored forms of SRK exhibited high-affinity binding to SCR/SP11, but the soluble form of SRK (eSRK) exhibited no high-affinity binding (Shimosato *et al*., 2007). Furthermore, the artificially dimerized form of eSRK exhibited high-affinity binding, suggesting that the membrane anchorage is necessary for SRK to obtain the high-affinity dimeric form (Shimosato *et al*., 2007). The mechanism of pollen inhibition remains to be characterized. Recently, Ca^{2+} signalling related actin reorganization and likely depolymerization has been found in papilla cells of *B*. *rapa* after self-pollination (Iwano *et al*., 2007). The same research identified that the dynamics of actin cytoskeleton leads to structure changes of vacuoles in the papilla cell, which in turn regulates hydration and germination of pollen.

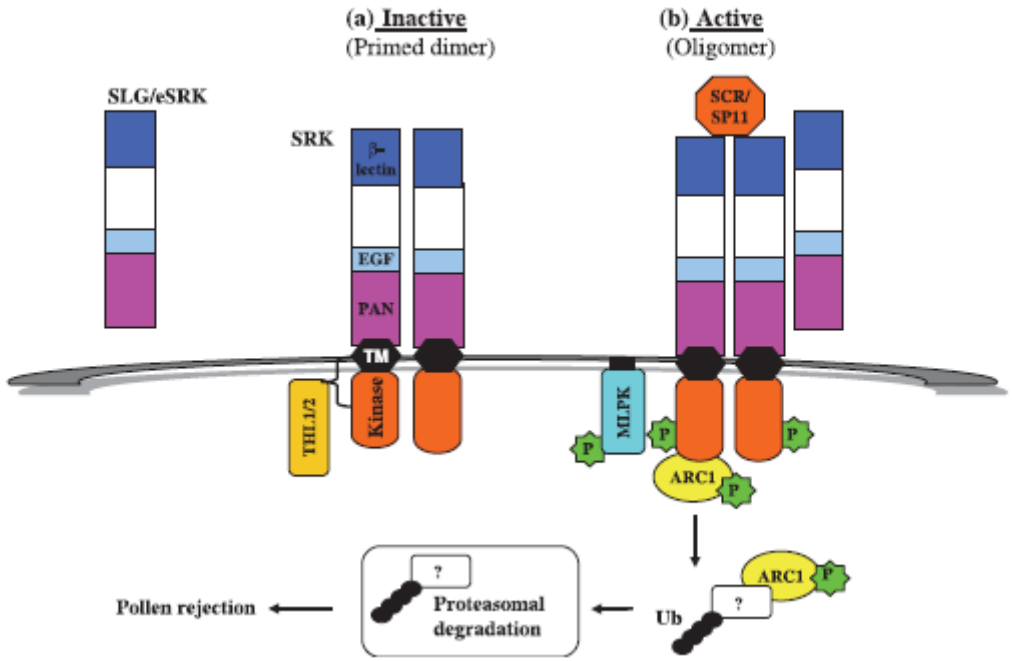


Figure 1.2 Model of SI response in *Brassica*. (a) Compatible reaction. In the absence of incompatible pollen, SRK is inhibited by THL1 and THL2. This inhibition is released upon the binding of the haplotype-specific pollen ligand SCR/SP11. SLG and eSRK represent low affinity binding sites for SCR/SP11. (b) Incompatible reaction. SCR/SP11 ligand binds to and activates the haplotype-specific SRK. SLG can enhance the strength of the SI response but is not indispensable in the SRK complex. Activated SRK induces transphosphorylation of serine and threonines residues in their kinase domains, and some of these phosphorylation sites might involve in the actions of downstream signaling proteins such as ARC1 and MLPK (Figure taken from Sanabria *et al.*, 2008).

A particular characteristic in SSI are dominance-recessive interactions between *S* haplotypes as a result of diploid sporophytic determination. The interactions can occur independently for pollen and stigma, leading to complicated compatibility/incompatibility pattern and differences in reciprocal pollination. Recent molecular studies on dominance have grouped haplotypes of *SRK*s, *SLG*s and *SCR*/*SP11*s into two classes, class I or class II (Shiba *et al.* 2002). Class I *SCR/SP11*s are generally dominant or co-dominant, expressed both sporophytically in tapetum and gametophytically in microspores and pollen, whereas class II haplotypes are only expressed in tapetum and recessive to class I *SCR/SP11* haplotypes (Kusaba *et al.*, 2002; Shiba *et al.*, 2002). Studies of class I and class II *SCR/SP11*

heterozygotes showed suppressed expression of class II *SCR/SP11* haplotypes but no effect on class I *SCR/SP11* expression, revealing the RNA level regulation of dominance interaction in pollen (Kusaba *et al*., 2002; Shiba *et al*., 2002). Most recently it has been reported that DNA methylation is involved in the recessive *SCR/SP11* allele gene silencing and thus determines the dominance reactions (Shiba *et al*., 2006). In the stigma, however, dominance interactions are not as common as in pollen and not regulated by differential expression of *SRK* alleles, but during interaction of *SRK* with *SCR/SP11* or downstream signaling factors such as *ARC1* (Hatakeyama *et al*., 2001).

1.4.2 Gametophytic self-incompatibility

In most families, GSI has been found to be controlled by a single locus, but more complex systems exist. In grasses, SI is thought to be controlled by two loci, named *S* and *Z* (Hayman, 1956). Four loci control is found in sugarbeet, *Beta vulgaris* (Larsen, 1977), *Ranunculus acris* (Osterbye, 1975) and *Lilium* (Lundqvist 1991). In the case of the single *S*-locus GSI, two mechanistically different systems have been investigated extensively at the molecular level. One is the S-RNase system, originally found in members of the Solanaceae and later reported in the Rosaceae and Plantaginaceae (formerly known as Scrophulariaceae) (Lee *et al*., 1994; Murfett *et al*., 1994; Xue *et al.*, 1996; Stephenson *et al*., 2000; Cheng *et al*., 2006). Evolution studies have revealed a single origin for the S-RNase GSI system before the separation of the Asteridae and Rosidae (Igic and Kohn, 2001; Steinbachs and Holsinger, 2002; Vieira *et al*., 2008a). The other single locus GSI system is found in *P. rhoeas* (Foote *et al.*, 1994).

1.4.2.1 S-RNase mechanism

In the S-RNase-based SI system, incompatible pollen germinates and grows normally on the stigma, and the pollen tube is arrested only by the time it has reached about one-third of the way through the style. Analysis of stylar proteins from *Nicotiana alata* led to the cloning and sequencing of the first *S*-gene (Anderson *et al*., 1986) and this allowed the identification and cloning of a large number of alleles of this gene from members of the Solanaceae, Rosaceae and Plantaginaceae families (Huang *et al*., 1994; L; Xue *et al.*, 1996; Sassa *et al*., 1997). Sequence comparison revealed that the proteins encoded by the pistil *S*-gene exhibit homology to the catalytic domain of two fungal RNases, Rh from *Rhizopus niveus* and T_2 from *Aspergillus oryzae* (McClure *et al*., 1989). The evidence that the S proteins degraded rRNA in incompatible pollen confirmed their ribonuclease activity, and they were since referred to as S-RNases (Gray *et al*., 1991). Subsequent transgenic studies, using both loss- and gain-of-function approaches, established that the S-RNases were functional pistil *S*-locus components and their catalytic activity was crucial for the rejection of incompatible pollen (Lee *et al*., 1994; Murfett *et al*., 1994). Studies performed in *Pyrus pyrifolia* detected a possible relationship between self-S-RNase, actin depolymerization and DNA degradation, implicating a S-RNase triggered programmed cell death (PCD) in incompatible pollen tubes (Wang *et al*., 2008a). It has been revealed that S-RNases are highly polymorphic with two hypervariable regions (HVa and HVb) concomitant five highly conserved regions (C1 to C5) (Ioerger *et al*., 1991). However, where the *S*-specificity resides is still unknown.

For the pollen S protein, two candidate genes have been reported. The first is *PhSBP1* (*Petunia hybrida* S-RNase binding protein 1). It was isolated from *Petunia hybrida* using S-RNase as bait in a yeast two-hybrid system (Sims and Ordanic, 2001). However, it does not exhibit a *S*-haplotype-specific interaction with S-RNase, therefore cannot be the pollen S

determinant. The second is an F-box gene *SLF* (*S*-locus F-box; also referred to as *SFB*), first identified by sequencing the genomic region around the *S-RNase* gene from *Antirrhinum hispanicum* (Plantaginaceae) (Lai *et al*., 2002) and was recently obtained from several *Prunus* (Rosaceae) species (Entani *et al*., 2003; Ushijima *et al*., 2003; Yamane *et al*., 2003). SLF is considered to be the strongest pollen *S* candidate gene for its *S*-locus location, *S*-haplotype-specific polymorphism and pollen-specific expression. Later studies in *Petunia inflata* were performed by transforming S_2S_3 plants with $PiSLF_2$ gene, and only S_3-pollen lost SI but not S_2-pollen, demonstrating the pollen *S* identity of *SLF* gene (Sijacic *et al*., 2004). F-box proteins are known to function by directing their specific substrates into an ubiquitin-mediated protein degradation pathway (Craig and Tyers, 1999). SLF might function through a high affinity to non-self-S RNases for breakdown and a low affinity to self-S RNases for the intact ribonuclease activity in the SI response, as have been reported in *Petunia inflate* (Hua and Kao, 2006; Hua *et al*., 2008). Competitive interaction between pollen *S* alleles, that polyploid plants pollen carrying two different pollen *S* alleles fail to function in SI, have been reported in the Solanaceae (Sijacic *et al*., 2004; Tsukamoto *et al*., 2005), but not in *Prunus* (Hauck *et al*., 2006). Biochemical studies in *Petunia inflata* revealed the strong interaction between non-self PiSLF and S-RNase (Hua and Kao, 2006). Recently, three functional domains have been identified for PiSLF, named FD1, FD2 and FD3, and their S-RNase-binding properties investigated by *in-vitro* binding assays (Hua *et al*., 2007). It is then proposed that FD2 of PiSLF is the binding domain with strong interactions with a domain common to all S-RNases, and that FD1 and FD3 together determine the *S*-specificity of PiSLF with a negative effect on the strong interaction between self S-RNase and FD2 of PiSLF (Hua *et al*., 2008).

There is evidence that S-RNase alone is not sufficient and other stigma factors not linked to *S*-locus are required for SI response (Bernatzky *et al*., 1995; Tsukamoto *et al*., 2003). Group 1 factors are required for S-RNase expression (McClure *et al*., 2000) and Group 2 factors such as HT-B, 120K and the 4936-factor (McClure *et al*., 2000; O'Brien *et al*., 2002; Hancock *et al*., 2005) are involved in the cytotoxic activity of S-RNase (Goldraij *et al*., 2006).

Two molecular models have been proposed so far for the S-RNase based inhibition of pollen in an *S*-haplotype specific manner (Figure 1.3). The first model involves S-RNase degradation (Entani *et al*., 2003). SLF belongs to F-box family proteins which can bind Skp1 and cullin-like proteins to form an E3 ubiquitin ligase complex, leading to degradation of ubiquitylated substrates by the 26S proteasome. Through interaction of S-RNase and SLF, in a compatible pollination (e.g., S_1-RNase/SLF_2), S-RNase is ubiquitylated and then degraded by the 26S proteasome, and in an incompatible pollination (e.g., S_1-RNase/SLF_1), S-RNase in certain ways evades degradation and the cytotoxic activity of S-RNase then degrades the pollen rRNA, leading to the arrest of pollen tube growth. The proposed biochemical mechanism of *Petunia inflata* SI (Hua *et al*., 2008) involves protein degradation as well. The formation of a stable S_1-RNase/$PiSLF_2$ complex upon compatible pollination would lead to subsequent ubiquitination of S-RNase, while the unstable state of the S_1-RNase/$PiSLF_1$ complex formed in the case of incompatible pollination would free the S-RNase from degradation. However, the degradation model does not propose the functions for the known factors (e.g., HT-B, 120K) and the evidence from immunolocalization experiments that no large-scale S-RNase degradation in compatible pollen tubes (Goldraij *et al*., 2006) leads to the prediction of the alternative model. In this model, S-RNase, HT-B and 120K are transported into a pollen vacuole. In a compatible pollination, non-self S-RNase and SLF interaction facilitates HT-B degradation and S-RNase remains compartmentalized, resulting in compatibility. While in

self interaction of S-RNase and SLF, the release of S-RNase from the vacuole reinforces its cytotoxic activity, leading to pollen rejection (McClure and Franklin-Tong, 2006).

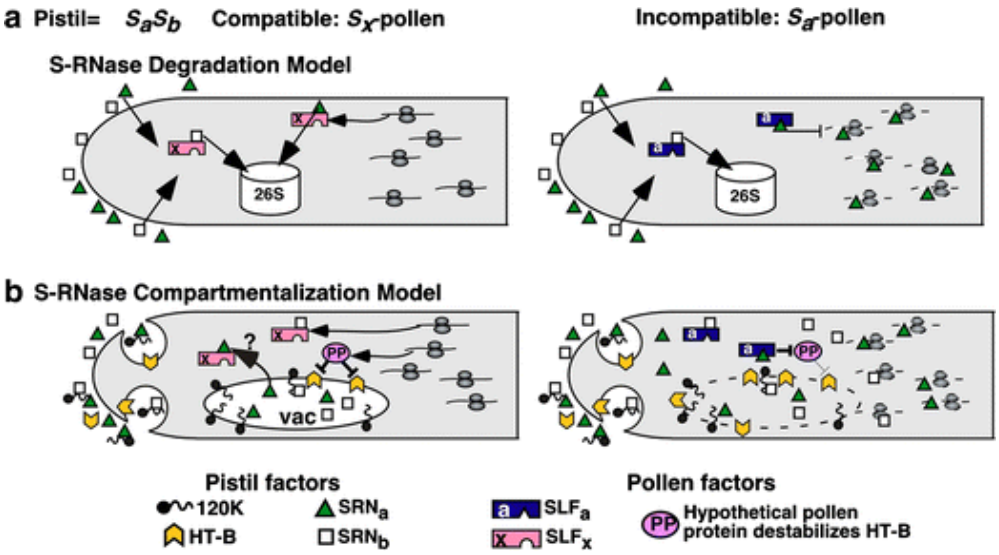


Figure 1.3 Models for S-RNase-based SI. On a *SaSb* pistil, compatible pollination with S_x pollen is shown on the left and incompatible pollination with *Sa* pollen is shown on the right. Symbols for all factors are shown below. (a) S-RNase degradation model: S-RNase enters the pollen tube cytoplasm from the extracellular matrix (ECM) (*arrows*). A compatible non-self S-RNase/SLF interaction (*left*) results in ubiquitylation and degradation by the 26S proteasome and pollen tube therefore grows normally. An incompatible self S-RNase/SLF interaction (*right*) does not result in S-RNase degradation; cytotoxicity results in RNA degradation and pollen tube growth is inhibited. (b) S-RNase compartmentalization model: S-RNase, 120K, and HT-B are taken up by endocytosis and are enclosed in a pollen vacuole. In a compatible interaction (*left*), S-RNase remains compartmentalized without cytotoxicity. A hypothetical pollen factor (PP) induces HT-B degradation in compatible pollen tubes. It is not known how S-RNase gains access to SLF (*arrow*, *question mark*). In an incompatible interaction (*right*), the action of PP is inhibited, the pollen vacuole breaks, S-RNase is released into the cytoplasm, resulting in RNA degradation and pollen tube growth inhibition (Figure taken from McClure and Franklin-Tong, 2006).

Molecular studies have suggested different recognition mechanism of *Prunus* S-RNase-based SI from that of Solanaceae and Plantaginaceae SI (Sonneveld *et al*., 2005). Differences between pollen *S* in *Prunus* (*SFB*) and Solanaceae (*SLF*) have recently been reported (Hauck *et al*., 2006; Sassa *et al*., 2007) and several similar F-box genes, named *SFBB* (*S* locus F-box brothers) have been identified as pollen *S* genes of apple (*Malus domestica*) and Japanese pear

(*Pyrus pyrifolia*) (Cheng *et al*., 2006; Sassa *et al*., 2007), implying a mechanism diversity of S-RNase based GSI. In *Prunus*, several pollen-expressed F-box genes with low allelic sequence polymorphism (*SLFL*s) other than *SFB* have been identified in the *S* locus and its flanking regions (Entani *et al*., 2003; Ushijima *et al*., 2003). These genes (*SLFL1*, *SLFL2*, and *SLFL3*) are more closely related to *SFBB* genes of the Maloideae than to *Prunus SFBs* (Matsumoto *et al*., 2008). Studies on *SLFLs* of *Prunus avium* did not identify direct involvement of *SLFL1* in the GSI reaction or pollen-tube growth while the functions of *SLFL2* and *SLFL3* are undetermined (Matsumoto *et al*., 2008). The divergency of pollen *S* genes in the S-RNase-based SI system leads to concerns on the evolutionary relationship between SLF/SFB and the corresponding S-RNase (Newbigin *et al*., 2008). S-RNases in the Rosaceae, Solanaceae, and Plantaginaceae have a single evolutionary origin (Igic and Kohn, 2001; Steinbachs and Holsinger, 2002; Vieira *et al*., 2008a), but *SLF/SFB* genes have a closer intrafamily relationships with *SLFL/SFBB* genes than with the *SLF/SFB* genes of other taxa (reviewed in Newbigin *et al*., 2008), implicating a much younger origin of the pollen *S* genes than their corresponding S-RNases. Functional characterization and structure analysis of *S* locus of these different species will shed light on the variation and evolution of this GSI system.

1.4.2.2 Self-incompatibility in *Papaver*

SI in *Papaver rhoeas* is also gametophytically controlled by a single, multi-allelic locus (Lawrence *et al*., 1978). However, the stigmatic *S*-gene and the mechanisms involved in pollen inhibition differ dramatically with that found in the Solanaceae, where the action of cytotoxic RNases is involved. In *P. rhoeas*, the SI response does not involve the degradation

of rRNA (Franklin-Tong *et al*., 1991) and is mediated by a complex signaling cascade, which relates to the physiological differences found between their SI responses (see section 1.3).

The stigmatic S proteins of *Papaver* are small extracellular signaling molecules, interacting with the pollen component which is believed to be a plasma membrane receptor. One putative pollen receptor is an S protein binding protein (SBP) (Hearn *et al*., 1996). It binds specifically to stigmatic S proteins but in a haplotype-indifferent manner, suggesting its accessory receptor role rather than pollen S receptor itself. Most recently, the *Papaver* pollen S gene, *PrpS* (*Papaver rhoeas* pollen S), has been identified to be a single copy gene linked to the stigma S determinant (Wheeler *et al.*, 2009) . Based on the knowledge of components involved in the SI reaction, a model for the pollen tube inhibition in *Papaver* has been proposed (Franklin-Tong and Franklin, 2003; McClure and Franklin-Tong, 2006; Franklin-Tong, 2007; Bosch *et al.*, 2008). Inhibition of the incompatible pollen is mediated by the activation of a Ca^{2+}-dependent signalling cascade through interaction of a pistil S-protein and a transmembrane receptor in pollen. A rapid increase of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) is induced by the SI response in incompatible pollen (Franklin-Tong *et al*., 1993, 1995, 1997), leading to the loss of high apical $[Ca^{2+}]_i$ which is a key characteristic of growing pollen tubes. The increase of $[Ca^{2+}]_i$ initiates the intracellular signalling network, inducing the following downstream reactions: phosphorylation of soluble inorganic pyrophosphatases (sPPases) (de Graaf *et al*., 2006) which is required for pollen tube tip extension, activation of a mitogen-activated protein kinase (MAPK) p56 (Rudd *et al*., 2003) which might induce PCD (Li *et al*., 2007b), rapid depolymerization of the pollen actin cytoskeleton (Geitmann *et al.*, 2000; Snowman *et al.*, 2002) through severing activity of actin-binding proteins (ABPs) (Huang *et al*,. 2004), and programmed cell death involving cytochrome c (cyt c) leakage induced caspase-like-activity (Thomas and Franklin-Tong, 2004; Bosch and Franklin-Tong, 2007)

(Figure 1.4). The incompatible pollen is in this way inhibited and does not renaissance. It was recently shown that the pollen microtubule cytoskeleton is an early target of the SI signaling network in *Papaver* (Poulter *et al*., 2008).

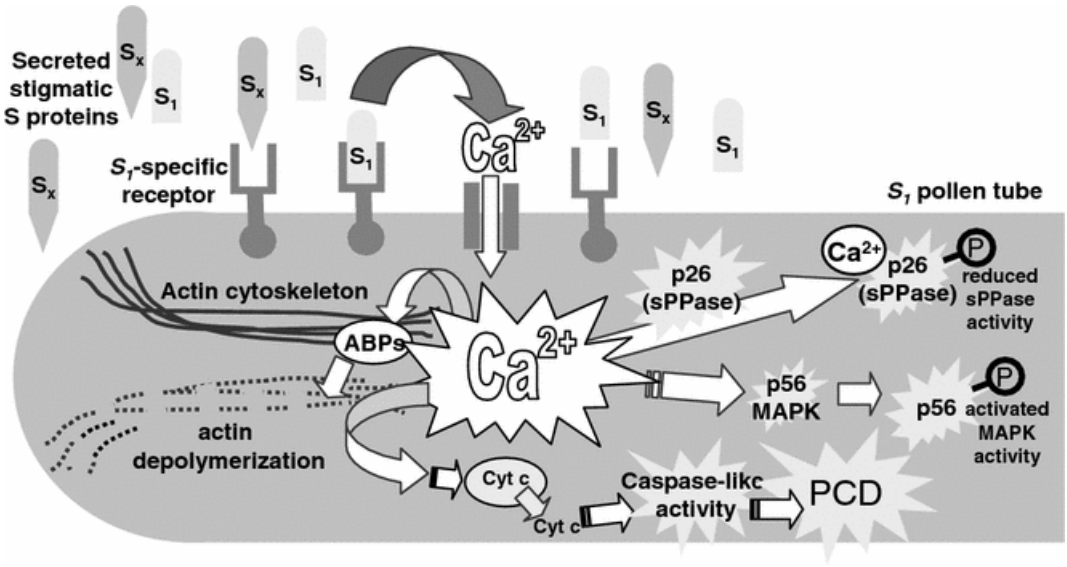


Figure 1.4 Model for pollen tube inhibition in the *Papaver rhoeas* GSI system. The stigmatic S proteins are small secreted protein ligands and interact with the pollen S receptor in a haplotype-specific manner (e.g. S_1 protein with an S_1 pollen receptor) to stimulate an incompatible reaction. This triggers an intracellular Ca^{2+} signaling cascade involving immediate increase of cytosolic free Ca^{2+} and loss of high apical $[Ca^{2+}]_i$ within the pollen. Two targets are rapidly modified: phosphorylation of sPPase and depolymerization of F-actin, implying the rapid arrest of tip growth. p56-MAPK is activated and may be involved in PCD. PCD is triggered, comprising a caspase-like activity, cyt c leakage and DNA fragmentation. This ensures that incompatible pollen does not resume growth (Figure taken from McClure and Franklin-Tong, 2006).

1.5 Self-incompatibility in grasses

In the grass family Poaceae, self-incompatibility has been known for over a century and is present in at least 16 genera (Yang *et al*., 2008). Studies in *Secale cereale* by Lundqvist (1954) and *Phalaris coerulescens* by Hayman (1956) showed that SI in grasses is controlled gametophytically by two multiallelic and independent loci, *S* and *Z*. The incompatibility phenotype of the pollen grain is determined by its haploid genome and depends upon the

combination of *S* and *Z* alleles in the pollen grain. A pollen grain is incompatible when both its *S* and *Z* alleles are matched in the pistil. Until now, the *S*-*Z* system has been identified in many other species (Baumann *et al*., 2000). The relationships between the grass *S*-*Z* two-locus SI system and the well studied single *S*-locus SI systems have been reviewed in Yang et al. (2008).

1.5.1 Physiological features

Many studies of the grass self-incompatibility response have been carried out in the early 1980s, based on the physiological aspects of pollen-stigma interaction. Though under gametophytic control, there are some anomalous SI features in grasses. First, different from most families with gametophytic systems where the pollen is binucleate (one vegetative cell and one generative cell) at the time of dispersal, the grass pollen is trinucleate (a vegetative cell and two gametes). Second, grass pollen is short-lived with a high respiratory rate compared with the pollen of most gametophytic families. Third, on the pistil side, whereas most genera with gametophytic SI systems have stigmas with surface fluid, the grass stigma is of the dry type. These three characteristics are in resemblance with features of sporophytic SI. Furthermore, the pollen tube inhibition occurs very fast near the stigma surface, which is found in most families with a sporophytic SI system as well as in *Papaver* which has a GSI like grasses.

Knox and Heslop-Harrison (1971) reported the release of intine-held antigens by the pollen grains of *Phalaris tuberosa* on the stigma surface within five to ten minutes after pollen-stigma contact, during which time pollen tube growth was controlled in incompatible pollination. The fact that the antigens remained spread on the stigma surface in compatible pollination implied the possible role of the antigens as compatible recognition materials

(Knox and Heslop-Harrison, 1971). The grass family has unusually fast pollen germination speed and tube growth rate. Pollen rehydration by uptake of water from the stigma is essential to germination and the feathery form of the stigma reduces the capacity of the pollen-tube transmitting tracts. It was then hypothesized that incompatibility occurs rapidly at the stigmatic surface before effective penetration. Otherwise, the transmitting tracts would be saturated and the compatible pollen tube growth would then be blocked (Heslop-Harrison, 1979b). Evidence has shown that in incompatibility reactions the water flow to pollen grains is restricted at the receptive part of the stigma papilla, leading to the inhibition of pollen germination (Heslop-Harrison, 1979a). Through observation of stained pollen tubes, incompatible responses were observed near the stigma surface within two minutes in *Gaudinia fragilis* and *Secale cereale* after the recognition event of pollen and stigma (Shivanna *et al*., 1982) and in rye, within 90 seconds after the contact of incompatible pollen (Heslop-Harrison, 1982). The inhibited pollen tubes are short, distorted and occluded with callose deposits although the length of inhibited tubes is variable. In *Hordeum bulbosum,* however, the rejection is much slower, after the tube tip penetrates the stigma cuticle (Heslop-Harrison and Heslop-Harrison, 1980) and in some individuals of *Alopecurus partensis*, inhibited pollen tubes even reached the transmitting tracts (Shivanna *et al*., 1982). After penetration, the water required for the pollen tube growth was transferred from the hydrated grain but not the stigma tissues and the released enzymes, such as pectinase and cutinase, by the emerging tube tip were responsible for the dissolving of the cuticle of stigma papillae (Heslop-Harrison and Heslop-Harrison, 1981).

Heslop-Harrison (1982) has proposed that the grass incompatibility response may contain at least three elements: the self-recognition step governed by the *S* and *Z* loci, the rejection response, and an additional control that determines the rate at which the growth of the tube is

arrested. Later, Wehling et al. (1994) reported pollen protein phosphorylation and involvement of Ca^{2+}-induced signal transduction in rye SI. Significant phosphorylation activity was reported in self-pollinated pollen grains and decreased activity was found associated with the loss of SI. Disruption of the self incompatibility response was observed after treating the incompatible stigmas with different protein kinase inhibitors and Ca^{2+} antagonists (Wehling *et al*., 1994). A model for the SI reaction in rye has been proposed, suggesting that the pollen S- and Z- determinants are possibly pollen grain plasma membrane located protein kinases with extracellular receptor domains. The stigma S- and Z- determinants act as signal molecules, by interacting with "self" pollen partners, induce a Ca^{2+} mediated signal cascade, finally leading to pollen tube inhibition (Wehling *et al*., 1994).This model is consistent with the rapid response of SI in grasses and shows similarity with *Papaver* and sporophytic SI systems. Up to date, the detailed components of the SI responses in grasses have yet to be characterized.

1.5.2 Genetics of self-incompatibility in grasses

Although self-incompatible and self-compatible species are found in the same genus, the self-incompatibility frequency is higher in perennial species than in annual species. The two-locus incompatibility system in grasses is more complex as the two loci are complementary in action; therefore it has features distinct from those of single locus systems. These include the differences in the degree of compatibility and in reciprocal crosses between two plants. The degree of compatibility, referred to the percentage of compatible pollen, can be 0%, 50%, 75% or 100% depending on the genotypes (Figure 1.5). If a plant with the genotype $S_1S_2Z_1Z_3$ crosses with $S_1S_1Z_1Z_2$ as the pollen donor, 50% of the pollen grains will be compatible, while in the reciprocal cross, 75% of the pollen will be compatible (Figure 1.6).

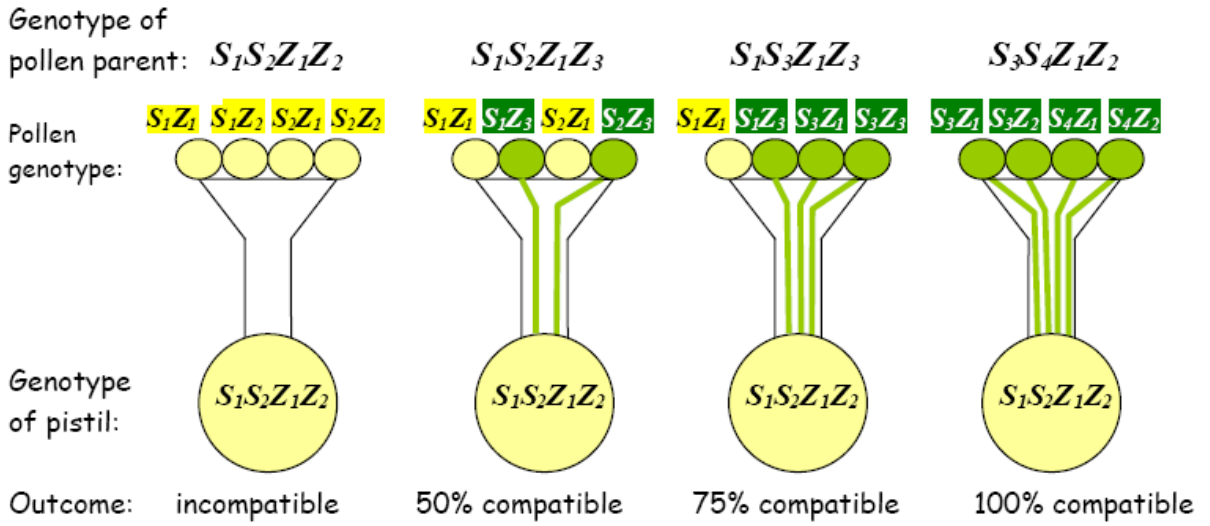


Figure 1.5 Genetic control of GSI by two multiple-allelic loci *S* and *Z*. When both *S* and *Z* alleles in pollen are matched in the pistil, incompatibility occurs, pollen growth is inhibited. Otherwise pollens are compatible. Degree of compatibility can be 0%, 50%, 75% and 100% compatible, depending on the genotypes of pollen and stigma.

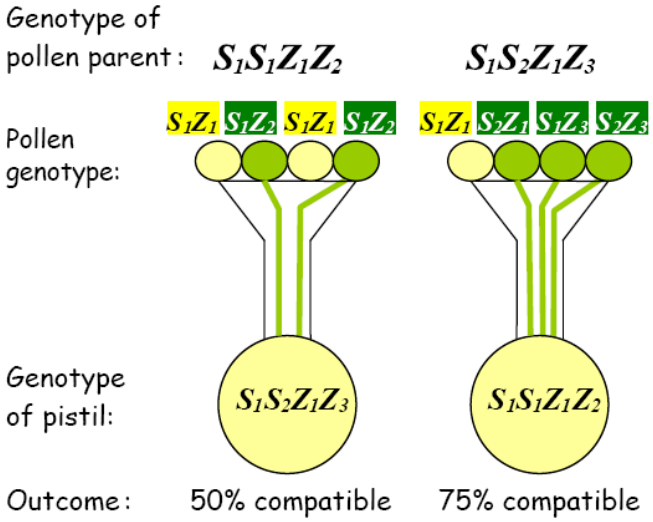


Figure 1.6 Reciprocal cross between plants of the genotype $S_1S_2Z_1Z_3$ and $S_1S_1Z_1Z_2$ produces different degree of compatible pollen. When $S_1S_1Z_1Z_2$ is pollen donor, crossing with $S_1S_2Z_1Z_3$ pistil, 50% of pollen is compatible; while when $S_1S_2Z_1Z_3$ is pollen donor, crossing with $S_1S_1Z_1Z_2$ pistil, 75% of pollen is compatible.

Furthermore, studies on the breakdown of SI have revealed the existence of self-compatible pollen mutants and the involvement of an additional locus. A self-compatibility (*SC*) locus independent of the *S* and *Z* incompatibility loci has been reported in *L. perenne* (Thorogood

and Hayward, 1991). At least three self-compatible mutants, at the *S* and *Z* loci and at least a third locus (*T*), have also been reported in *P. coerulescens* (Hayman and Richter, 1992). In *S. cereale*, three incompatibility loci, *S*, *Z* and *S5* (analogous with *T* in *Phalaris*) have been identified (Voylokov *et al*., 1998).

Li et al. (1994) reported a putative *S* gene clone, named *Bm*2, identified from *P. coerulescens*. But the expression of the *Bm*2 gene was barely detectable in other SI grasses species such as *S. cereale*, *H. bulbosum* and *L. perenne* (Li *et al*., 1997). Later studies revealed that *Bm*2 represents a thioredoxin-like gene linked to the *S*-locus, about 1 cM away (Baumann *et al*., 2000). Hackauf and Wehling (2005) identified a putative ubiquitin-specific protease (UBP) gene showing a pistil specific expression pattern and cosegregation with the *Z*-locus in *S. cereale*. It is still undetermined if the UBP gene is a component of the *Z*-locus or a linked gene with suppressed recombination around the *Z*-locus.

To date none of the gene products of *S* and *Z* have been isolated, but identification of markers or genes linked to *S* or *Z* infers the genetic locations of self-incompatibility loci. The *S*-locus was now known to be located on chromosome 1R in rye by linkage to an isozyme phosphoglycoisomerase *PGI-2* and a leaf peroxidase *Prx-7,* with recombination frequencies of 16.7% and 15.8%, respectively (Gertz and Wricke, 1989). The *Z*-locus was located on chromosome 2R in rye and linked with beta-glucosidase and esterases 4/11 with recombination frequencies of 16.7% and 17.5–20%, respectively (Fuong *et al*., 1993). The *T*-locus was located on chromosome 5R in rye and linked to the Esterase 5-7 complex with recombination at a level of 28.8–36.0% (Fuong *et al*., 1993). Recent mapping analysis in *Secale* (Voylokov *et al*., 1998; Hackauf and Wehling, 2005), *Phalaris* (Bian *et al*., 2004) and *Lolium* (Thorogood *et al*., 2002) have confirmed syntenic chromosomal locations of *S* and *Z* on linkage groups (LG) 1 and LG2, respectively, in accordance with the Triticeae consensus

map (Armstead *et al*., 2002; Jones *et al*., 2002b; Sim *et al*., 2005) and also identified closely linked molecular markers, which may facilitate comparative mapping and map-based cloning.

1.5.3 Self-incompatibility in ryegrass

Perennial ryegrass (*L. perenne*) is an outcrossing, wind-pollinated species. It exhibits the *S*-*Z* two-locus GSI system. The *S* locus has been mapped on LG1 and the *Z* locus has been mapped on LG2 (Thorogood *et al*., 2002). The putative co-localization of the *S* and *Z* loci with two seed yield quantitative trait loci (QTL) of *L. perenne* has recently been reported (Studer *et al*., 2008b). The *S*-gene is known to be linked to *PGI-2* (Gertz and Wricke, 1989). There was also evidence of linkage between either the *S*- or *Z*-locus and the isozyme glutamate oxalacetatetransaminase, *GOT/3* (Thorogood and Hayward, 1992). Distorted segregation of the *GOT/3* locus in some selfs of self-compatible *Lolium* plants (derived from self-fertile *Lolium temulentum*) occurred (Thorogood and Hayward, 1992). This distortion was then thought to be due to linkage to *S* or *Z*. As *S* is linked to *PGI/2* which is in turn unlinked to *GOT/3*, it was assumed that it was the *Z* locus that was responsible for the distorted ratios. However, distortion also occurs on linkage group 3 where *GOT/3* is located (Jones *et al*., 2002b) and it was determined by Thorogood *et al*. (2002) that this was not in fact due to linkage with one of the incompatibility loci which were found to be on LG1 (*S*) and LG2 (*Z*). Instead a pleiotropic effect of *S* or a locus closely linked to *S* explains the distortion on LG3. Additionally, a self-fertility locus has been mapped on LG5 of *L. perenne*, in a position which is likely to be orthologous to the *Secale S5* and *Phalaris T* loci (Thorogood *et al*., 2005). Recently, transcribed derived fragments (TDFs) involved in the SI in *L. perenne* have been identified using cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis (Van Daele *et al*., 2008b). Some TDFs were homologous to genes

involved in other SI systems, such as protein kinases, actins, a GTP-binging protein and ubiquitin-related proteins (Van Daele *et al*., 2008b). Genomic locations of these SI-related genes have been further determined using derived markers and a large number of them have been mapped to LG1 and LG2 (Van Daele *et al*., 2008a), but their functions in the SI response are still uncharacterized.

With the available comprehensive information for model plant species such as rice and *Arabidopsis*, it is now possible and has proven to be useful to use comparative genetics in map-based cloning. Comparative mapping work has demonstrated that regions of the *Lolium* genome show a potentially useful degree of conservation with the rice and the Triticeae genomes (Sim *et al*., 2005; Armstead *et al*., 2002; Jones *et al*., 2002b). These genetic linkage maps can be used to aid map-based cloning of the SI genes. Current evidence suggests that plants from the same phylogenetic family will possess a common self-incompatibility system and studies on one given species of the Poaceae would also contribute to a better understanding of the SI systems in other grass species in terms of both function and evolution. Genetic improvement of grasses by conventional grass breeding programs, based on selecting observable phenotypes from different genetic varieties, is slow due to self-incompatibility and their perennial nature. Advances in genomics and molecular genetic technologies, such as marker assisted selection (MAS), QTL mapping and functional genetics, can enhance conventional grass improvement. Through elucidating the molecular basis and the underlying control genes of self-incompatibility in the grasses, an effective breeding device for plant breeders can be expected and a better understanding of the diversity of SI systems and mechanisms can be attained.

The objectives of the thesis were (1) to fine map the *S* and *Z* loci in *L. perenne* via comparative genetics, using expressed sequence tags from related grass species as well as rice genomic sequence and mapping information; (2) to establish SI cDNA libraries for identifying genes involved in the *L. perenne* SI response and within the stigmatic candidate genes for the *S* and *Z* loci using a comparative genetics approach; (4) to verify expression pattern of identified candidate genes during the incompatibility response; (5) to determine genetic linkage relationships between candidate genes and the *S* or *Z* locus; and (6) to develop fine mapping populations in order to identify tightly linked markers for the *S* and *Z* loci.

CHAPTER 2

MATERIALS AND METHODS

2.1. Comparative genetics approach for marker development and candidate SI gene identification

2.1.1 Plant material

The *Lolium perenne* mapping family p150/112, the core mapping family for the International Lolium Genome Initiative (ILGI) was described in detail by Jones et al. (2002b). It was developed based on a cross between a multiply heterozygous *L. perenne* parent of complex descent as pollinator and a double-haploid as female parent (Jones *et al*., 2002b). One hundred and twenty-five individuals from this family, genotyped for *S* and *Z*, were included for the specific molecular marker generation for the *S* and *Z* loci (Thorogood *et al*., 2002).

2.1.2 Marker development based on comparative genetics

Previous comparative mapping work (Sim *et al*., 2005; Armstead *et al*., 2002; Jones *et al*., 2002b) had identified that the regions of the *L. perenne* genome to which the *S* and *Z* loci mapped, showed a degree of conserved synteny with rice chromosomes 5 and 4, respectively. Consequently, primers suitable for the amplification of *Lolium* genomic DNA in regions physically close to the *S* and *Z* loci, based on the comparative relationship with rice, were developed. This was achieved by identifying Osa1 (the MSU Rice genome annotation) rice loci in these regions (http://rice.plantbiology.msu.edu/) and by carrying out multiple sequence alignments of these rice genes with expressed sequence homologues from barley, wheat and maize, in order to identify conserved nucleotide sequences. Primers were designed to be anchored in exons and to span intronic regions, to maximise the chances of identifying polymorphisms and to generate polymerase chain reaction (PCR) products of a suitable size for analysis on agarose gels (c.200-1500bp). Primers were named after the corresponding

Osa1 rice locus to facilitate cross-species comparisons. The software package MACAW version 2.0.5 (Schuler *et al*., 1991) was used for multiple sequence alignment and visualisation. Sequence data were obtained through www.gramene.org or from the Osa1 Rice Database at http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/. Sequences selected to perform alignment for primer design were listed in Appendix Table A. Details of primer sequences used were listed in Table 3.2.1.

2.1.3 Genetic mapping

Segregation data used in previous genetic mapping analyses of the ILGI family was available (Armstead *et al*., 2002; Jones *et al*., 2002b, Thorogood *et al*., 2002). Segregation data from the newly developed *S* and *Z* linked markers was combined with the available data sets and recombination frequencies and map distances were calculated using Joinmap 3.0 (Van Ooijen and Voorrips, 2001) with the Kosambi mapping function.

2.1.4 Identification of possible gene duplications in the S and Z regions of the rice genome

The initial mapping work identified two markers on *L. perenne* LG2 developed from rice which flanked the *Z* locus (04g54940 and 04g55570, section 3.2.1). In order to explore the possibility that the two locus *S* and *Z* system had evolved as the result of a gene duplication, using the Osa1 pseudomolecules database, all the predicted rice coding sequences between the flanking markers on rice C4 were searched using the Basic Local Alignment Search Tool (BLAST) (http://rice.plantbiology.msu.edu/blast.shtml) against all the predicted rice sequences from rice C5 and high probability alignments were identified.

2.1.5 Molecular marker analysis

DNAs from the ILGI family were extracted by the CTAB method (Doyle and Doyle, 1987) and amplified in 10 µl volume PCR reactions with 0.05 µl Taq DNA Polymerase (5 $U \cdot \mu l^{-1}$) (Roche), containing 1 µl of 10 x buffer, 1 µl of dNTPs (2 mM each), 0.05 µl of each forward and reverse primer (100 µM), 2.5 µl of DNA template (10 ng-50 ng), and 5.35 µl ddH_2O. The reactions were carried out as touchdown PCR using the following conditions: a one minute initial denaturing step at 94°C, followed by 10 cycles each containing a 30 seconds denaturing step at 94°C, a 60 seconds annealing step at 60°C (with the temperature reduced by 1°C each cycle) and a 60 seconds extension step at 72°C, followed by 30 cycles of a 30 seconds denaturing step at 94°C, a 60 seconds annealing step at 50°C and a 60 seconds extension step at 72°C.

The PCR products were analysed by gel electrophoresis at 250V through 1%-1.5% agarose gel in 0.5 x TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.0), with 100 bp DNA ladder (Promega) as a molecular weight marker. After staining with ethidium bromide (0.5µg/ml), the gel was photographed under UV light.

2.1.6 Lolium BAC library screening and BAC sequencing

2.1.6.1 PCR-based screening

The *Lolium* BAC library containing 5 genome equivalents was PCR screened as described by Farrar et al. (2007). DNA pools of microtitre plate-grown BAC clones were generated to enable a PCR-based screening of the library. 10 µl PCR reactions were performed with 0.05 µl Taq DNA Polymerase (5 $U \cdot \mu l^{-1}$) (Roche), containing 1 µl of 10 x buffer, 1 µl of dNTPs (2 mM each), 0.05 µl of each forward and reverse primer (100 µM), 1 µl of BAC DNA (10 ng-

50 ng), and 6.85 µl ddH_2O. Thermal cycling was performed as touchdown PCR beginning with 1 minute at 94°C, followed by 10 cycles of 30 seconds at 94°C, 1 minute at 60°C (with the temperature reduced by 1°C per cycle), 1 minute at 72°C, followed by 30 cycles of 30 seconds at 94°C, 1 minute at 50°C, 1 minute at 72°C. The PCR products were analysed by electrophoresis through a 1% agarose gel in 0.5 x TBE buffer, with 100 bp DNA Ladder (Promega) as marker. A PCR screen of 192 DNA pools generated three "hits" per positive BAC clone within the library and the DNA pool numbers represented an 'address' which identified the microtitre plate containing the positive BAC clone.

2.1.6.2 BAC clones rows x columns minipreps

A microtitre plate containing 200 µl per well of LB medium (1.0% Tryptone, 0.5% Yeast Extract, 1.0% NaCl, pH 7.0) was inoculated with a BAC library microtitre plate identified in a PCR screen and grown at 37°C overnight. 75 µl of the culture from each well were pooled in a 1.5 ml tube by rows and columns separately, i.e. 8 row pools (A-H by row) x 12 column pools (1-12 by column) and DNA extracted from each of the 20 pools in miniprep format. The culture was transferred to 1.5 or 2 ml microcentrifuge tubes followed with centrifugation at 10,000 g for 10 minutes. The supernatant was removed and the excess liquid blotted on paper towels. 300 µl of P1 (resuspension buffer, Table 2.1.1) was added to the pellet, mixed by vortexing. 300 µl of P2 (lysis buffer, Table 2.1.1) was added to each tube and mixed by inversion 5 times, leaving it to at room temperature for 5 minutes. 300 µl of P3 (neutralisation buffer, Table 2.1.1) was then added. The mixture was incubated on ice for 10 minutes, and then centrifuged at maximum speed for 10 minutes at 4°C. The supernatant was transferred to a new tube and centrifuged again as above. The supernatant was transferred to a new tube and 650 µl of Isopropanol (Sigma) was added. After incubation on ice for 5 minutes, the tubes

were centrifuged at maximum speed at 4°C for 15 minutes. The supernatant was discarded and the pellet was washed with 500 µl of 70% EtOH. The tubes were then centrifuged at maximum speed for 5 minutes. The supernatant was removed carefully and the pellet was dried by leaving the tube open for an hour at room temperature. 10 µl of EB (elution buffer, Table 2.1.1) was then added to resuspend the pellet. 1 µl of each of the 20 minipreps for one plate was used for a 10 µl PCR as before. Products were analysed on 1% agarose gels and amplification of the right sized product in the row and column dimensions identified the position of the positive clone in the 96-well plate. The selected PCR-positive BACs were scaled up for DNA isolation, followed by sequencing.

2.1.6.3 BAC clone midipreps

Clone midipreps were carried out using QIAGEN plasmid Midi kits (QIAGEN). The candidate colony was inoculated into 100 ml LB medium containing chloramphenicol (50 $\mu g \cdot ml^{-1}$) in an autoclaved flask. The culture was incubated overnight at 37°C with vigorous shaking. The culture was divided between two oakridge tubes, with about 35 ml culture in each tube. The tubes were centrifuged at 5,000 g for 10 minutes. If desired, the remains of the culture were divided between tubes and centrifuged again. The supernatant was discarded and excess liquid was blotted on paper towels. Each pellet was then resuspended in 10 ml of P1 by vortexing. 10 ml of P2 was added to each tube and mixed gently but thoroughly by inverting 4-6 times. After leaving the tubes stand at room temperature for 5 minutes, 10 ml of chilled P3 was added, mixed immediately but gently by inverting 4-6 times and incubated on ice for 15 minutes. The tubes were then centrifuged at 10,000 g for 30 minutes at 4°C. The supernatant was transferred to a new oakridge tube and centrifuged again at 10,000 g for 15 minutes at 4°C. QIAGEN-tip 100 was equilibrated with 4 ml of Buffer QBT (equilibration

buffer, Table 2.1.1) and the column was allowed to empty by gravity flow. The above supernatant from the same culture was combined, applied to a QIAGEN-tip and allowed to enter the resin by gravity flow. The tips were then washed twice with 10 ml of Buffer QC (washing buffer, Table 2.1.1). 1 ml of Buffer QF (elution buffer, Table 2.1.1) warmed to 65°C was added for 5 times and the elution was collected in a new clear oakridge tube. After precipitation DNA with 3.5 ml of room temperature isopropanol, the tubes were centrifuged at 10,000 g for 30 minutes at 4°C. The supernatant was removed carefully and 2 ml of room temperature 70% EtOH was added to wash the DNA pellet. The tubes were centrifuged at 10,000 g for 10 minutes. The supernatant was removed carefully, and the pellet was dried overnight by reverse the tube on paper towels. The DNA pellet was then resuspended in 300 μl of EB.

Table 2.1.1 List of composition of QIAGEN buffers

Buffer	**Composition**	
Buffer P1 (resuspension buffer)	Tris·Cl (pH 8.0)	50 mM
	EDTA	10 mM
	RNase A	100 μg ml^{-1}
Buffer P2 (lysis buffer)	NaOH	200 mM
	SDS	1% (w/v)
Buffer P3 (neutralization buffer)	potassium acetate (pH 5.5)	3.0 M
Buffer QBT (equilibration buffer)	NaCl (pH 7.0)	750 mM
	MOPS	50 mM
	isopropanol	15% (v/v)
	Triton® X-100	0.15% (v/v)
Buffer QC (wash buffer)	NaCl (pH 7.0)	1.0 M
	MOPS	50 mM
	isopropanol	15% (v/v)
Buffer QF (elution buffer)	NaCl (pH 8.5)	1.25 M
	Tris·Cl	50 mM
	isopropanol	15% (v/v)
EB (elution buffer)	Tris·Cl (pH 8.5)	10 mM

2.1.7 BAC and PCR products sequencing

Automated fluorescent sequencing was carried out on an ABI 3100 with BigDye Terminator version 3.1 sequencing kit (Applied Biosystems) and results were viewed using the software Chromas (Version 2.31). The BAC DNA was sequenced directly (without subcloning) and PCR products were sequenced after purification with MinElute PCR purification kit (QIAGEN) following the manufacturer's protocol.

2.2 Construction of subtracted SI cDNA libraries

2.2.1 In-vitro pollination method

Plants to be used as pollinators were ready for pollen collection when their inflorescences were about to anthese or had been anthesing for a few days. Plants were still able to produce pollen two or more weeks after the first inflorescences had started anthesis. The flowering stems of a plant were enclosed in a cellophane bag. The plants anthesed during midday. Mature ovaries with stigmas attached were collected from florets of spikelets that were close to anthesis. Spikelets were removed from the plants and ovaries were harvested at some distance from flowering plants to avoid contamination with drifting pollen. Ovaries were placed on 50 mm diameter petri-dishes plated with agar (2.5% Agar, 25% sucrose and 25 ppm Boric acid) which provided a medium on which the ovaries could be supported allowing the feathery stigmas ready for pollen interaction (Lundqvist, 1961). Five to eight ovaries from each chosen plant were placed on a single petri-dish. Pollen, which had been collected in cellophane bags, was shaken onto the surface of the agar, ensuring that only free-flowing,

non-clumped pollen was used. Anthers in the bag were removed prior to pollination to ensure smooth pollen flow onto the plates.

2.2.2 Plant material

The *L. perenne* plants, genotyped for *S* and *Z*, were from the core mapping family ILGI, described in detail by Jones et al. (2002b) and Thorogood et al. (2002). Plants representing all the possible incompatibility responses (Figure 1.5, Yang *et al*., 2008), i.e., with genotypes $S_1S_2Z_1Z_2$, $S_1S_2Z_1Z_3$, $S_1S_3Z_1Z_3$ and $S_1S_3Z_1Z_2$ were used for *in-vitro* pollination and construction of SI suppression subtractive hybridization (SSH) libraries. Two kinds of stigma material were used for subtraction: the unpollinated stigma as the driver and the *in-vitro* pollinated stigma of the same genotype as the tester. Leaf and root material from the different genotypes were also collected. Pollen was collected for the construction of a pollen specific control library. All collected plant materials were placed immediately in liquid nitrogen and then stored at -80°C before RNA extraction.

2.2.3 Total RNA extraction

2.2.3.1 Isolation of total RNA

Total RNA from mature and immature unpollinated stigmas, pollinated stigmas, leaves and roots of each genotype used was extracted using Tri Reagent (Sigma). Stigmas were removed from agar plates, placed in a Lysing Matrix D tube (Q-Biogene) and immediately stored in liquid nitrogen. The tubes were then placed on ice. 1ml of Tri Reagent (Sigma) was added and tubes were immediately placed in a bead Mill 300 (Retsch) for 90 seconds at a frequency of 30 Hz. Other tissues were homogenised with mortar and pestle and 1 ml of Tri Reagent was added to the homogenate. Tubes were then allowed to incubate for ten minutes at room

temperature. 160 µl of ice cold chloroform (Sigma) were added to each tube and mixed by inversion and allowed stand at room temperature for three minutes. Tubes were then centrifuged at 12,000 g for 15 minutes at 4ºC. The supernatant was recovered and the chloroform extraction was repeated. The supernatant was recovered to a new 1.5 ml Eppendorf tube and 0.5 µl (≈10 µg) of Glycogen (Roche) was added along with 500 µl of Isopropanol (Sigma). Tubes were inverted to mix the contents and allowed to incubate at room temperature for ten minutes before being centrifuged at 12,000 g for ten minutes at 4ºC. The supernatant was discarded, paying attention not to disturb the pellet, and 1 ml of 75% EtOH was added and vortexed for five seconds. The tubes were then centrifuged at 12,000 g for five minutes at 4ºC. The ethanol was pipetted off and the last drops were allowed to evaporate by leaving the tube open for five minutes. The pellet was resuspended in 20 µl of nuclease free water (Ambion).

2.2.3.2 DNase 1 treatment of total RNA

Each sample of total RNA was digested with DNase I to remove any contaminating DNA that may have been present. This was carried out using the DNA-*free* kit (Ambion). 2 µl of DNase I buffer was added to the sample along with 1 µl of DNase I and mixed by gentle pipetting up and down. Samples were then incubated for 20 minutes at 37ºC in a heating block. 2 µl of inactivation reagent was then added and mixed by pipetting up and down. Tubes were incubated at room temperature for three minutes before being centrifuged at 10,000 g for 90 seconds at 4ºC. The supernatant containing RNA was recovered to a new Eppendorf tube for storage at -80ºC. The Inactivation Reagent supplied with the kit removes DNase I and divalent cations from the reaction mixture and therefore eliminates the need for a chloroform extraction.

2.2.4 PCR cDNA synthesis

The cDNAs were generated from the total stigma RNA by the Super SMART cDNA Synthesis Kit (BD Biosciences Clontech). The SMART cDNA synthesis technology is able to preferentially enrich for full-length cDNAs and can be subsequently used for PCR-selected subtraction. The first-strand synthesis is performed with a modified oligo(dT) primer (the 3'SMART CDS Primer II A). When reverse transcriptase (RT) reaches the 5' end of the mRNA, a few additional deoxycytidines are added to the 3' end of the cDNA as a result of the RT's terminal transferase activity. The SMART Oligonucleotide with an 3' end oligo(G) sequence creates an extended template by base-pairing with the deoxycytidine overhang. RT then continues transcription till the end of the oligonucleotide, producing full-length single-strand (ss) cDNA with complete 5' end of the mRNA as well as complementary sequences of the SMART oligonuleotide. The primer containing a stretch of identical sequence with 3' SMART CDS Primer II A and SMART oligonucleotide primes the cDNA amplification. In cases of prematurely terminated cDNAs or contaminating genomic DNA which have no SMART anchor sequence, there will be no exponentially cDNA amplification. The SMART oligonuleotide and 3' SMART CDS Primer II A both have an *Rsa* I site to remove the identical sequences from the amplified cDNAs, generating blunt-ended double-stranded cDNAs for following adaptor ligation and subtraction. The synthesis procedure was carried out according to the manufacturer's protocol.

2.2.5 cDNA Subtraction by suppression PCR

Suppression PCR was carried out according to the method described by Rebrikov et al. (2004). Nucleotide sequences were summarized in Table 2.2.4 and Table 2.2.5. Reagents were listed in Table 2.2.6.

2.2.5.1 Adaptor ligation

SI cDNA libraries are being developed to identify stigmatic SI components and genes involved in *Lolium* SI response as well as non-SI related pollen-stigma interactions. Therefore cDNA samples of unpollinated stigma will be used as drivers and cDNA samples of *in-vitro* pollinated stigma will be used as testers. The subtracted libraries will be enriched with differentially expressed genes induced by the incompatibility response. One µl of each purified and *Rsa* I-digested cDNA preparation was diluted with 5 µl of sterile H_2O. For each ligation reaction, 3 µl of sterile H_2O, 2 µl of 5X Ligation Buffer and 1 µl of T4 DNA Ligase (400 U μl^{-1}) were used**.** A ligation Master Mix was prepared for all ligations plus one additional reaction. The reagents were combined in a 1.5 ml microcentrifuge tube. For each tester cDNA mixture, the following reagents were combined in a 0.2 ml microcentrifuge tube in the order shown (Table 2.2.1). The mixture was pipetted up and down to mix thoroughly.

Table 2.2.1 Components of tester cDNA mixtures.

Component	**Tube No.**	
	1	**2**
	Tester 1-1	**Tester 1-2**
Diluted tester cDNA	2 µl	2 µl
Adaptor 1 (10 µM)	2 µl	-
Adaptor 2R (10 µM)	-	2 µl
Master Mix	6 µl	6 µl
Final volume	10 µl	10 µl

2 µl of Tester 1-1 and 2 µl of Tester 1-2 were mixed in a fresh microcentrifuge tube. This was the unsubtracted tester control 1-'c'. The tubes were centrifuged briefly and incubated at 16ºC overnight. 1 µl of EDTA/Glycogen Mix (0.2 M EDTA; 1 mg ml^{-1} glycogen) was added to stop the ligation reaction. The tubes were heated at 72°C for five minutes to inactivate the ligase and briefly centrifuged. The experimental Adaptor-Ligated Tester cDNAs and unsubtracted tester controls were now complete. 1 µl of each unsubtracted tester control (1-'c') was removed and diluted into 1ml of H_2O. These samples were used for PCR amplification. The samples were stored at -20°C.

2.2.5.2 Ligation efficiency test

The ligation efficiency was measured to ensure that at least 25% of the cDNAs had adaptors attached. The PCR reaction was designed by using an adaptor specific primer (P1) and a gene specific primer to amplify fragments that span the adapter/cDNA junctions of Testers 1-1 and 1-2. The intensity of this amplicon was compared to the amplicon amplified using two gene specific primers. Theoretically those two amplicons should be of equal intensity. Gene specific primers (Table 2.2.5) were designed for Elongation factor 1-α (*Elf1-α*) from a conserved region of a multiple alignment of the following sequences: *Arabidopsis* (NM_100667.2), maize (AY109326.1), barley (Z50789.1) and rice (AF030517.1). 1 µl of each ligated cDNA (e.g., the Tester 1-1 and 1-2) was diluted into 200 µl of H_2O. The following reagents were combined in four separate tubes (Table 2.2.2):

Table 2.2.2 List of sample and primer combinations for ligation efficiency test.

	Tube (µl)			
Component	**1**	**2**	**3**	**4**
Tester 1-1 (ligated to Ad1)	1	1	-	-
Tester 1-2 (ligated to Ad2R)	-	-	1	1
Gene-specific 3'primer (10 µM)	1	1	1	1
Gene-specific 5'primer (10 µM)	-	1	-	1
PCR primer P1 (10 µM)	1	-	1	-
Total volume	3	3	3	3

A master mix was prepared for all the reaction tubes plus one additional tube. For each reaction, the reagents were combined in the following order and mixed well by vortexing and were briefly centrifuged: 18.5 µl of sterile H_2O, 2.5 µl of 10 X PCR reaction buffer, 0.5 µl of dNTP mix (10 mM) and 0.5 µl of 50 X Advantage cDNA polymerase mix to a final volume of 22 µl. 22 µl of this master mix were added into each of the four reaction tubes, mixed well by vortexing and were briefly centrifuged. The reaction mix was incubated at 75°C for five minutes in a thermal cycle to extend the adaptors and immediately followed by 35 cycles of 30 seconds at 94°C; 30 seconds at 65°C and 2.5 minutes at 68°C. 5 µl from each reaction were analyzed on a 2% agarose/EtBr (0.5µg/ml) gel in 0.5X TBE buffer alongside a 100 bp DNA ladder (Metabion).

2.2.5.3 First Hybridization

For each of the tester samples, the following reagents were combined in the following order (Table 2.2.3):

Table 2.2.3 Components of first hybridization reactions.

Component	**Hybridization sample 1**	**Hybridization sample 2**
Rsa I-digested Driver cDNA	1.5 µl	1.5 µl
Ad1-ligated Tester 1-1	1.5 µl	-
Ad2R-ligated Tester 1-2	-	1.5 µl
4X Hybridization Buffer	1.0 µl	1.0 µl
Final volume	4.0 µl	4.0 µl

The samples were overlaid with one drop of mineral oil and centrifuged briefly. After incubation in a thermal cycler at 98ºC for 1.5 minutes, the samples were incubated at 68ºC for seven to twelve hours. The second hybridization followed immediately.

2.2.5.4 Second Hybridization

1 µl of driver cDNA, 1 µl of 4X hybridization buffer and 2 µl of sterile H_2O were combined in a sterile tube. 1 µl of this mixture was placed in a 0.5 ml microcentrifuge tube and overlaid with one drop of mineral oil. The tube was incubated in a thermal cycler at 98ºC for 1.5 minutes. The tube now containing the freshly denatured driver was removed from the thermal cycler. To simultaneously mix the driver with hybridization samples 1 and 2, the following procedure was used: A micropipette was set at 15 µl. The pipette tip was gently touched to the mineral oil/sample interface of the tube containing hybridization sample 2. The entire sample was carefully drawn partially into the pipette tip. A small amount of air was drawn into the tip to create a slight air space below the droplet of sample. The pipetting step was repeated with the tube containing the freshly denatured driver. Both samples were contained in the pipette tip now, separated by a small air pocket. The entire mixture was transferred to the tube containing hybridization sample 1 and mixed by pipetting up and down. The hybridization reaction was incubated at 68ºC overnight. 200 µl of dilution buffer was then added and mixed well by pipetting. The reaction was heated at 68ºC for seven minutes in a thermal cycler. The tubes were stored at -20ºC.

2.2.5.5 PCR amplification

1 µl of cDNA (i.e., each subtracted sample from the secondary hybridization and the corresponding diluted unsubtracted tester control) was transferred into an appropriately labelled tube. A master mix for all of the primary PCR tubes plus one additional tube were prepared. For each reaction the components were combined in the following order: 19.5 µl of sterile H_2O, 2.5 µl of 10X PCR reaction buffer, 0.5 µl of dNTP mix (10 mM), 1.0 µl of PCR primer P1 (10 µM) and 0.5 µl of 50X Advantage cDNA polymerase mix to a total volume of 24 µl. The tube was mixed well by vortexing, and briefly centrifuged. 24 µl of master mix were added into each prepared reaction tube. The reaction mix was incubated at 75ºC for five minutes in a thermal cycler to extend the adaptor then immediately followed with 30 cycles of 30 seconds at 94ºC; 30 seconds at 66ºC and 1.5 minutes at 72ºC. 8 µl from each tube were analyzed on a 2.0% agarose/EtBr gel run in 0.5X TBE buffer. 3 µl from each primary PCR mixture were diluted in 27 µl of H_2O. 1 µl of each diluted primary PCR product mixture was then transferred into an appropriately labelled tube. A master mix was prepared for the secondary PCR reactions plus one additional reaction by combining reagents in the following order: 18.5 µl of sterile H_2O, 2.5 µl of 10X PCR reaction buffer, 1.0 µl of nested PCR primer NP1 (10 µM), 1.0 µl of nested PCR primer NP2R (10 µM), 0.5 µl of dNTP mix (10 mM) and 0.5 µl of 50X Advantage cDNA polymerase mix to a final volume of 24 µl. The tube was mixed well by vortexing, and briefly centrifuged. 24 µl of master mix were added into each prepared reaction and immediately run for 15 cycles of 30 seconds at 94ºC; 30 seconds at 68ºC and 1.5 minutes at 72ºC. 8 µl from each reaction were analyzed on a 2.0% agarose/EtBr gel run in 0.5X TBE buffer alongside a 100 bp DNA ladder (Metabion). The reaction products were stored at -20ºC.

2.2.5.6 Subtraction efficiency test

The efficiency of the subtraction was compared by measuring the abundance of a housekeeping gene in the tester cDNA before and after subtraction. *Elf 1-α* and *L. perenne* actin gene (AY014278) were used as the housekeeping genes and primers were designed (Table 2.2.5). The subtracted and unsubtracted secondary PCR products were diluted ten fold in H_2O. The following reagents were combined in 0.2 ml microcentrifuge tubes in the order: 1.0 µl of diluted subtracted cDNA or diluted unsubtracted tester control 1-'c', 1.2 µl of housekeeping gene 5'primer (10 µM), 1.2 µl of housekeeping gene 3'primer (10 µM), 22.4 µl of sterile H_2O, 3.0 µl of 10X PCR reaction buffer, 0.6 µl of dNTP mix (10 mM) and 0.6 µl of Taq Polymerase to a final volume of 30 µl. The tubes were mixed by vortexing and briefly centrifuged. The following thermal cycling program was used for 18 cycles: 30 seconds at 94ºC; 30 seconds at 60ºC and two minutes at 68ºC. 5 µl from each reaction were transferred in a clean tube, and stored on ice. The rest of the reaction was returned to the thermal cycler for three additional cycles. The transfer step was repeated three times (i.e., 5 µl were removed after 24, 27, and 30 cycles). The 5 µl samples (i.e., the aliquots that were removed from each reaction after 18, 21, 24, 27 and 30 cycles) were analyzed on a 2.0% agarose/EtBr gel in 0.5X TBE buffer alongside a 100 bp DNA ladder (Metabion).

Table 2.2.4 List of nucleotide sequences for cDNA subtraction by suppression PCR.

Name	**Sequences and sequence orientations**
Adaptor1	5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3' 3'-GGCCCGTCCA-5'
Adaptor2R	5'-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3' 3'-GCCGGCTCCA-5'
PCR Primer1	5'-CTAATACGACTCACTATAGGGC-3'
NP1	5'-TCGAGCGGCCGCCCGGGCAGGT-3'
NP2R	5'-AGCGTGGTCGCGGCCGAGGT-3'

Table 2.2.5 List of primer sequences of housekeeping genes provided by Dr Stephen Byrne.

Gene	5'- forward primer -3'	5'- reverse primer -3'
Elf 1-α	5'-GGTATCTCCAAGGATGGCCAG-3'	5'-CACCACTGAGCACAATGTTAC-3'
Actin	5'-CCTTCGTCTTGACCTTGCTGG-3'	5'-GGCATTCTCCAGCTCCTGTTC-3'

Table 2.2.6 List of reagents used for cDNA subtraction.

Reagent	components	
5X ligation buffer	Tris-HCl (pH 7.8)	250 mM
	$MgCl_2$	50 mM
	DTT	10 mM
	BSA	0.25 mg ml^{-1}
	ATP	3 mM
4X Hybridization buffer	NaCl	4 M
	HEPES (pH 8.3)	200 mM
	Cetyltrimethyl ammonium bromide (CTAB)	4 mM
Dilution buffer (pH 8.3)	HEPES (pH 6.6)	20 mM
	NaCl	20 mM
	EDTA (pH 8.0)	0.2 mM

2.2.6 Cloning of subtracted cDNAs

2.2.6.1 Purification of amplified cDNAs

Secondary PCR products (section 2.2.4.5) were purified before cloning with the GenElute^{TM} PCR Clean-Up Kit (Sigma) in order to remove other components in the PCR reactions, such as excess primers, primer-dimers, nucleotides, DNA polymerase and salts. Five volumes of Binding Solution were added to one volume of the secondary PCR products and mixed. This mixture was then transferred to a GenElute Miniprep binding column placed in a 2 ml collection tube. The assembled miniprep column was pre-treated with 500 µl of the 'Column Preparation Solution'. The sample was centrifuged at maximum speed for 1 minute and the eluate was discarded. 500 µl of diluted 'Wash Solution' was applied to the column bounded with cDNA and centrifuged for 1 minute at maximum speed. The eluate was discarded and the column was centrifuged for an additional two minutes. The miniprep column was then transferred to a fresh 2 ml Eppendorf tube, 20 µl of sterile ddH_2O was added and incubated

for 1 minute at room temperature. The column was then centrifuged for 1 minute at maximum speed to elute the cDNA which was used in the subsequent cloning.

2.2.6.2 Cloning into pGEM®-T Easy Vector and transformation

For cloning of PCR fragments the pGEM®-T Easy Vector system (Promega) was applied. Successful cloning of an insert is identified by blue/white color screening due to the interruption of the coding sequence of β-galactosidase in the vector. The pGEM®-T Easy Vector and Control Insert DNA tubes were briefly centrifuged and contents were collected at the bottom of the tubes. Ligation reactions were set up as described below (Table 2.2.7).

Table 2.2.7 Components of ligation reactions for cloning.

	Standard Reaction	**Positive Control**	**Background Control**
2X Rapid Ligation Buffer	5 µl	5 µl	5 µl
pGEM®-T Easy Vector (50 ng)	1 µl	1 µl	1 µl
subtracted cDNA (purified)	3 µl	-	-
Control Insert DNA	-	2 µl	-
T4 DNA Ligase (3 Weiss units μl^{-1})	1 µl	1 µl	1 µl
deionized water	-	1 µl	3 µl

The reactions were mixed by pipetting and incubated overnight at 4°C. Two LB/ampicillin/IPTG/X-Gal plates (1.5% agar, 100 µl ml^{-1} ampicillin, 0.5 mM IPTG, 80 µl ml^{-1} X-Gal in LB medium) were prepared for each ligation reaction. The plates were equilibrated to room temperature prior to plating. The tubes containing the ligation reactions were centrifuged and contents were collected at the bottom of the tube. Tube(s) of frozen JM109 High Efficiency Competent Cells (Promega) were removed from storage and placed in an ice bath until the cells had just thawed (approximately five minutes). The cells were mixed by gently flicking the tube. 50 µl of cells were carefully transferred into each ligation tube. The

tubes were gently flicked to mix and placed on ice for 20 minutes. The cells were then heat-shocked for 45-50 seconds in a water bath at exactly 42°C without shaking. The tubes were immediately returned to ice for two minutes. 950 µl room temperature SOC medium (2% Tryptone, 0.5% Yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM Mg^{2+} stock and 20 mM Glucose) were added to the tubes. The tubes were then incubated for 1.5 hours at 37°C with shaking at about 150 rpm. The cells were then pelleted by centrifugation at 1,000 *g* for ten minutes, resuspended in 200 µl of SOC medium, and 100 µl plated on each of two LB/ampicillin/IPTG/X-Gal plates. The plates were incubated overnight (16 to 24 hours) at 37°C. White colonies, generally containing inserts, were then inoculated into individual wells of 96-well plates containing LB medium supplemented with 100 µg ml^{-1} ampicillin, and incubated overnight at 37˚C with shaking. The clones were stored in 15% sterile glycerol (VWR International) at -80˚C.

2.2.7 Differential screening of clones

Differential screening was performed to remove clones that did not contain a differentially expressed transcript. Inserts of clones were amplified and identified as being differentially expressed depending on their hybridization patterns after hybridization with forward and reverse subtracted cDNA probes.

2.2.7.1 cDNA insert analysis

Insert cDNAs of selected clones from subtracted cDNA libraries were amplified using a colony PCR technique in order to verify the presence of a cDNA insert and for the following differential screening analysis. The 20 µl PCR was carried out with Taq DNA Polymerase,

containing 1 U Taq polymerase (New England Biolabs), 1x ThermoPol buffer, 50 µM dNTPs, 300 nM each forward and reverse pUC/M13 primer (the binding site is contained in pGEM®-T Easy Vector), and 2.5 µl of bacterial culture. The reactions were carried with two minutes at 94˚C, followed by 22 cycles of 30 seconds at 94˚C and three minutes at 68˚C.

2.2.7.2 Preparation of cDNA dot blots

Amplified cDNAs were dot-plotted in duplicate onto positively charged nylon transfer membranes (Schleicher & Schuell) by a 96-well replicator (Sigma-Aldrich). Each 5 µl of amplified cDNA product and 0.5 µl of 1 M NaOH were combined in a 96-well plate. The plate was incubated for five minutes at 37°C or heated 5 minutes in a boiling H_2O bath and was put on ice. 5 µl of 2 M ammonium acetate (pH 7) were added. The plate was briefly centrifuged to mix contents. The membrane was wetted thoroughly in deionized water, then soaked in 1 M ammonium acetate (pH 7.0) prior to use. 2 µl of each mixture was transferred using a micropipettor to a nylon membrane on top of two sheets of dry filter paper. The membrane was briefly blotted to remove excess liquid before spotting sample. Two identical blots were prepared for hybridization with subtracted pollinated and unpollinated stigma cDNA probes. The prepared sample was cross-linked to the membrane using a UV linking device (UVIlink, UVItec) at 254 nm. Total exposure should be approximately 120 mJ for damp membranes.

2.2.7.3 Probe labelling and differential screening

Forward and reverse subtracted probes were generated from secondary PCR products prepared as described in section 2.2.5.5. Secondary PCR products were digested with *Rsa*I to

remove adaptors that would result in non-insert specific hybridization. Digested products were purified as described in section 2.2.6.1 and labelled. Gene Images AlkPhos Direct Labelling and Detection System (GE Healthcare) was used for screening of differentially expressed cDNAs according to the manufacturer's protocol. After probe labelling, hybridization and washing, the signal was detected with ECF^{TM} substrate, scanned on a Typhoon9410 (GE Healthcare) at excitation spectrum 430 nm and emission at 560 nm. Differential gene expression was analysed using ImageQuant software version 5.2 (GE Healthcare) using default settings.

2.2.8 DNA sequence analysis

Clones in each library exhibiting a stronger hybridization signal with pollinated stigma probe (forward) compared to the unpollinated stigma probe (reverse) were sequenced by a commercial sequencing company (AGOWA). Sequences were vector trimmed (Vector NTI software, Invitrogen). Sequences of clones were compared to rice genomic sequences through BLAST (http://rice.plantbiology.msu.edu/blast.shtml). Regions for *S* and *Z* loci as identified in previous comparative mapping work (Sim *et al*., 2005; Armstead *et al*., 2002; Jones *et al*., 2002b) were preferentially screened. The screening was based on the assumption of synteny between rice and *L*. *perenne* genomic regions.

The Gene Ontology (GO) terms assigned to each putative homologue were also collected. The GO ontologies are built from a set of structured, controlled vocabularies for describing the roles of genes and gene products across organisms (The Gene Ontology Consortium, 2000). Three independent categories of GO are constructed: (1) molecular function (MF), (2) biological process (BP) and (3) cellular component (CC). MF describes the biochemical activity a gene product has or carries as a potential. BP describes the biological objective a

gene or gene product involved in, which is accomplished by one or more ordered assemblies of molecular functions. CC refers to the location in the cell where a gene product functions. Within each of these ontologies there is a more specific layer of terms, which are further subdivided into low-level "child" terms. The ontologies are structured in the form of directed acyclic graphs where each child term may have one or multiple higher level "parents" (The Gene Ontology Consortium, 2001). GO terms can be used to get an overview of functional categories that are enriched in a genome or cDNA collection from a gene expression analysis. This can be achieved using "GO slims" which contain a list of high-level terms of the three ontologies to which all other low-level child terms can be mapped up to (Gene Ontology Consortium, 2004). GO slim set was specifically generated according to the analysis by taking the first level of terms in the GO database of the three ontologies. The program "GO Terms Classification Counter" (http://www.animalgenome.org/bioinfo/tools/countgo/) was then used to classify all the GO terms up to the parent terms in the GO slim file for the rice homologies identified from the transcripts in the SSH cDNA libraries.

2.2.9 Reverse transcriptase PCR

RNA was isolated from various tissues as described in section 2.2.3.1. Pollinated stigmas were harvested at 20 minutes and one hour after *in-vitro* pollination. First-strand cDNA was synthesized in 20 µl reactions using 1 µg of total RNA from each sample. cDNA synthesis was performed using an Oligo$(dT)_{23}$ primer (Sigma-Aldrich) and SuperScript III Reverse Transcriptase (Invitrogen) according to manufacturer's protocols. The products were diluted tenfold with dH_2O and 1 µl was used as template for subsequent PCR. Primer pairs were designed with Primer Express 2.0 from vector trimmed clone sequences. PCR was carried out in 20 µl reactions containing 1 U Taq polymerase (New England Biolabs), 1 x ThermoPol

buffer, 50 µM dNTPs, 500 nM each forward and reverse primer with thermal cycling of 2 minutes at 94˚C, 30 cycles of 30 seconds at 94˚C, 30 seconds at 60˚C and 40 seconds at 72˚C, followed by 10minutes at 72˚C. PCR products were analyzed on a 1.5% agarose/EtBr gel in 0.5 x TBE buffer alongside a 100 bp DNA ladder (Metabion).

2.2.10 Full length cDNA amplification by rapid amplification of cDNA ends (RACE)

Full length cDNAs of selected candidates were generated with the SMART RACE cDNA Amplification Kit (BD Biosciences Clontech). The kit provides a method for performing both 5'- and 3'-rapid amplification of cDNA ends (RACE). Following the first-strand cDNA synthesis, both 5' and 3'- RACE PCR reactions were performed according to the manufacturer's protocol. The RACE products were then cloned into pGEM T-Easy vector as described in section 2.2.6.2. Eight to ten different independent clones of each RACE product were sequenced by AGOWA (Germany) in order to obtain the maximum length of sequence at the 5' end. The full length cDNA sequences were generated by combining 5' and 3' RACE products sequences through their overlapping region. Sequences were searched for their function domains using InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/).

2.3 Real-time PCR analysis of candidate SI genes identified in the SI cDNA libraries

2.3.1 Plant materials and cDNA preparation

Self-pollinated stigmas were collected in a pollination time course of 0 min, 2 min, 5 min, 10 min, 20 min and 30 min after *in-vitro* self-pollination. The methods of *in-vitro* pollination and total RNA extraction were described in section 2.2.1 and 2.2.3.1, respectively. About 10 ng of total RNA from collected stigma samples were reverse transcribed to first strand cDNA using

oligo(dT) as the primer and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol.

2.3.2 Primer design

Primers for the ten identified candidates (see Chapter 4) were designed with their SSH sequencing results (vector trimmed) using Primer Express 2.0 software (Applied Biosystems). A total of four reference genes were tested in this real-time study to identify the most suitable genes for data normalization. The four reference genes, which have been applied in various studies, were *actin*, *Elf1-α*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *tubulin*. The source of their sequences and where they were reported as reference genes were listed in Table 2.3.1. Primer Express 2.0 was used to design their primers. For real-time PCR analysis using SYBR Green dye, the key parameters of primer design were low primer-dimer formation and length of amplicon between 100 bp to 200 bp. The sequences of primers for each candidate and reference gene are listed in Table 2.3.2.

Table 2.3.1 Source of reference gene sequences and applications.

Gene	**Species**	**Accession**	**Gene application reference**
Actin	*L. perenne*	AY014279	Petersen *et al*., 2004; Nicot *et al*., 2005
Elf1-α	*Hordeum vulgare*	Z50789	Nicot *et al*., 2005; Jain *et al*., 2006
GAPDH	*L. perenne*	EF463063	Kim *et al*., 2003; Petersen *et al*., 2004
Tubulin	*L. perenne*	AY742902	Kim *et al*., 2003; Suprunova *et al*., 2004

Table 2.3.2 Sequences of primers for each reference gene and SI related candidate genes.

Gene	5'…forward primer…3'	5'…reverse primer…3'
Actin	AGCGGGAAATTGTCAGAGACA	GAGCTGCTCCTAGCAGTTTCCA
Elf1-α	CCTGGTCACCGTGACTTCATC	GCGTGTCTGGCCATCCTT
GAPDH	CGTCTGCGGTGTCAATGC	CGAGTGGGTGGTGGTCATG
Tubulin	ACAGCAAATGTGGGACTCCAA	TCTGCTCATCAACTTCCTTTGTG
Can3	TCAAGGTGAAGAAGCACGAC	CAACAACATAGCAGGATGGC
Can4	GGCTGCACGTCTTCACTCTA	ACAACACGACTCCAAAGCTG
Can10	CCAGGTGTGGTTGCTTCAGA	GTGGAATGGAGTATGTTCTTCATCA
Can18	TCGCAGGCGTTAACATTTCA	CGCTTCTTCGTGCTCTGAGA
Can94	AACCCTTACGCCAGGTTAACG	CCTCCCCCAGAGGAATGTTC
Can130	CCATGAGCTAGGGTGGATCAG	ACGGCGAGAAGCCATACTAACT
Can135	CGGGCAGGTACATCAATCAGT	TGGCAGTTCATTTCGAACTCCTA
Can136	AGCTGCGCAAGGACATTCTC	TCGGCGTCTTTGGACACATA
Can139	TGGGCGAAGCTAAGTTGATGA	GTGGCCAGACAAGCTCACATT
Can151	GCCGTCCCCATGGTATTTC	CTCCACCAGCCCCACAAG

2.3.3 Primer optimization

A mixture of stigma cDNAs (synthesised as described in section 2.3.1) was used as the template for primer optimization using the gradient PCR programme function of the Px2 thermocycler (Thermo Electron Corporation). Each 20 µl PCR contained 1 µl of cDNA, 1 U of Taq polymerase (New England Biolabs), 1 x ThermoPol buffer, 200 µM of dNTPs and 200 nM of each forward and reverse primer. A master mix of 12 reactions plus one additional for each primer pair was prepared. It was then aliquotted into a row of a 96 well PCR plate. The following gradient PCR programme was performed: 2 minutes at 94˚C, 35 cycles of 30 seconds at 94˚C, 30 seconds at 55-70˚C and 40 seconds at 72˚C. The 55-70˚C gradient was broken up into the following 12 annealing temperatures: 55.1˚C, 55.5˚C, 56.3˚C, 57.7˚C, 59.4˚C, 61.4˚C, 63.3˚C, 65.3˚C, 67.6˚C, 69.0˚C, 69.7˚C and 70.2°C. PCR products were analyzed by gel electrophoresis on a 2% agarose/TBE gel alongside a 100bp DNA ladder (Metabion). The optimal annealing temperature of each primer pair was designated by the amplification of specific PCR product with no primer-dimer.

2.3.4 Quantitative Real-time PCR analysis

2.3.4.1 Real-time PCR assay

Real-time PCR was performed using the SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) on an ABI 7000 (Applied Biosystems) according to the manufacturer's instructions. Each 25 µl PCR consisted of 1 µl cDNA, 12.5 µl SYBR Green JumpStart Taq ReadyMix, 0.25 µl Internal Reference Dye and 500 nM of each forward and reverse primer. For each gene analysed, a no template control (NTC) was included by substituting the cDNA template with deionised H_2O. All reactions were carried out in triplicate with thermal cycling of 2 minutes at 94˚C, 45 cycles of 20 seconds at 94˚C, 30 seconds at X˚C and 40 seconds at 72˚C. The annealing temperature X for each primer pair was determined by primer optimisation (section 2.3.3). To determine the specificity of the PCR, the melting curve analysis of amplified products was performed at the end of the cycling using the dissociation curve method on the ABI 7000. Results of real-time PCR (threshold cycle Ct) were analyzed by qBase version 1.3.5 following its workflow (Hellemans and Vandesompele, 2006).

2.3.4.2 Determination of PCR amplification efficiency

The PCR amplification efficiency is required for the calculation of relative quantities. For each primer pair, a serial dilution series of cDNA was set up to determine the PCR efficiency: 1:5, 1:10, 1:20, 1:50 and 1:100. All reactions and thermal cycling set up were the same as described in section 2.3.4.1. The calculation of amplification efficiencies was carried out in qBase by assigning the cDNA of the first dilution point (1:5) as 100 ng, and subsequent dilution series as 50 ng, 25 ng, 10 ng and 6.67 ng.

qBase used Ct values and their relative quantities to generate a standard curve with linear regression. The slope of the curve and the standard deviation of the residuals (S_e) were calculated by the following formulas:

$$slope = \frac{\sum_{q=1}^{h}\left(Q_q - \overline{Q}\right)\left(Ct_q - \overline{Ct}\right)}{\sum_{q=1}^{h}\left(Q_q - \overline{Q}\right)^2}$$

$$Se = \sqrt{\frac{\sum_{q=1}^{h}\left(Ct_{q,measured} - Ct_{q,predicted}\right)^2}{h-2}}$$

('h' refers to the number of standard curve points (with known quantity Q))

The base of amplification efficiency (E) and its standard deviation (SD(E)) were thereafter calculated using the formulas:

$$E = 10^{\left(\frac{-1}{slope}\right)}$$

$$SD(E) = \frac{E \cdot \ln(10) \cdot Se}{slope^2}$$

2.3.4.3 Evaluation of normalization with reference genes

The use of multiple reference genes are recommended to produce more reliable data. Identification of the most stable reference genes in qBase was done by calculation of the coefficient of variation (CV) of normalized reference gene relative quantities and the reference gene expression stability parameter (M), which is defined as the average pairwise variation of a particular gene with all other reference genes (Vandesompele *et al*., 2002b). The most appropriate reference genes for normalisation were selected by stepwise exclusion of

reference genes with high M value. The programme "geNorm" was developed for performing this evaluation and the principles are described by Vandesompele et al. (2002b).

2.3.4.4 Calculation of relative quantities

Relative quantification was carried out using a modified ΔΔCt method of Pfaffl (2001), adjusting for differences in PCR efficiency and for normalization with multiple reference genes. Normalised relative quantity was calculated with the formula $\frac{E_{goi}^{\Delta Ct,goi}}{\sqrt[f]{\prod_{0}^{f} E_{ref_0}^{\Delta Ct,ref_0}}}$ by qBase version 1.3.5 (Hellemans and Vandesompele, 2006). The calculation steps are detailed in Hellemans et al. (2007). Results of relative quantities were exported in excel format for further visualisation of results using SigmaPlot 9.0.

2.4 Development of fine mapping populations for identification of tightly linked markers for the *S* and *Z* loci

2.4.1 Plant material

The ILGI population (p150/112) was constructed at the Institute of Biological, Environmental and Rural Sciences (IBERS; formerly Institute of Grassland and Environmental Research), Aberystwyth. 96 of the ILGI individuals, phenotyped and genotyped for the *S* and *Z* loci, were selected for determining map distances between candidate genes or simple sequence repeat (SSR) markers and the *S* or *Z* locus. The fine mapping populations for *S* and *Z* locus were developed as described in section 6.1.4 so that only a single heterozygous *S* or *Z* genotype was obtained in the respective mapping population. 1406 progeny individuals were

germinated for the *S*-population and 1276 individuals were germinated for the *Z*-population. The selfs or contaminants in the progenies were screened as described in section 2.4.3 and excluded from the linkage analysis.

2.4.2 Genomic DNA preparation

Genomic DNA from 96 ILGI plants were provided by IBERS, with a concentration of approximately 15 ng μl^{-1}. For plants of the *S*- and *Z*-population, genomic DNA was extracted either by the CTAB method (Doyle and Doyle, 1987) or with the MagAttract 96 DNA plant core kit (QIAGEN).

2.4.2.1 CTAB method

For each plant, three leaf blades of approximately 70 mm in length were placed in a 2 ml Eppendorf tube. All tubes were placed in a freeze dryer with caps open for two days. A single sterile glass ball was then placed in each tube and milled on a bead Mill 300 (Retsch) for 1.5 minutes at the maximum frequency. 1 ml of CTAB extraction buffer (Table 2.4.1), preheated to 65°C, was added along with 9 µl of β–mercaptoethanol to each tube and incubated for 10 minutes at 65°C with vortexing every 2-3 minutes. 500 µl of chloroform:isoamyl alcohol was then added and the tube inverted to mix. Samples were then centrifuged at 13000 rpm for 10 minutes. The aqueous layer was transferred to a new 1.5 ml Eppendorf tube and 700 µl of pre-chilled isopropanol was added. After vortexing, tubes were placed at -80°C for 15 minutes to allow the DNA to precipitate followed by centrifugation at 13000 rpm for 15 minutes at 4°C. The supernatant was discarded and the pellet was washed in 70% ethanol then centrifuged at 130000 rpm for 5 minutes at 4°C. The pellet was re-suspended in 250 µl of sterile ddH_2O. 2

µl of RNase A (10 mg ml^{-1}) was added to each sample and the mixtures were incubated at 37°C for 30minutes. 500 µl of chloroform:isoamyl alcohol was added and after mixing, the tubes were centrifuged at 13000 rpm for 10 minutes. The aqueous layer was transferred to a 1.5 ml Eppendorf tube and 500 µl of isopropanol was added. The mixtures were incubated at -80°C for 15 minutes followed by centrifugation at 13000 rpm for 20 minutes at 4°C. The supernatant was discarded and the pellet was washed in 70% ethanol then centrifuged at 130000 rpm for 5 minuets. The pellet was re-suspended in 100 µl of sterile ddH_2O. The isolated genomic DNA was quantified on a Safire plate reader system (Tecan) using absorbance measurements at 260 vs 280 nm. The DNAs were then diluted to a concentration of approximately 25 ng μl^{-1} and arranged into 96 well plates.

Table 2.4.1 Composition of CTAB extraction buffer.

Component	**Amount**
CTAB	1.5% w/v
NaCl	1.4 M
EDTA (pH 8.0)	20 µM
Tris-HCl (pH 8.0)	100 µM

2.4.2.2 Isolation of genomic DNA using the MagAttract 96 DNA plant kit with a robotic system

Three leaf blades per plant of approximately 70 mm in length were placed in a well of a 96 well deep well collection plate. The collection plates were placed in a freeze dryer without a lid for two days. A single sterile glass ball was then placed in each well of the deep well plate and milled on a bead Mill 300 (Retsch) for 10-15 minutes at the maximum frequency until the leaf material was ground to a fine powder. 600 µl of RLT buffer (QIAGEN) was added into each well and the plates were left on a shaker for 5-10 minutes to mix samples. The plates were then centrifuged at 1500 rpm for five minutes followed by the DNA extraction

procedure with a Microlab Star Robot (Hamilton) according to the manufacture's protocol. 65 µl of RB buffer (QIAGEN) was added to each well of a 96-well flat-bottom microplate (QIAGEN). 20 µl of resuspended MagAttract Suspension A (QIAGEN) was then added to each well of the 96-well flat-bottom microplate containing RB buffer. 200 µl of each supernatant from the plant lysates in the deep well collection plate was transferred to the 96-well flat-bottom microplate containing MagAttract Suspension A and RB buffer. The samples were mixed thoroughly by pipetting up and down several times then incubated for two minutes at room temperature. The mixing step was repeated and the samples were incubated for another two minutes at room temperature. The flat-bottom microplate was placed onto the magnet bed for 20 seconds to separate the MagAttract particles. The supernatant was then discarded. 200 µl of RPW buffer (QIAGEN) was added to the pelleted MagAttract particles, resuspending thoroughly by pipetting up and down. The flat-bottom microplate was then placed onto the magnet bed for 20 seconds to separate the MagAttract particles. The supernatant was discarded. 200 µl of ethanol was added to resuspend the MagAttract particles by pipetting up and down to mix thoroughly. The flat-bottom microplate was then placed onto the magnet bed for 20 seconds to separate the MagAttract particles and the supernatant was discarded. After repeating the ethanol washing step, the MagAttract particles were left at room temperature for 5–10 minutes to dry. 100 µl of AE buffer (QIAGEN) was added to each well to resuspend the MagAttract particles, followed by vortexing and then incubated for 5 minutes at room temperature. The flat-bottom microplate was then placed onto the magnet bed for one minute to separate the MagAttract particles. The supernatant was transferred to a clean 96-well round-bottom microplate and was ready to use. The general yield of DNA extracted with MagAttract 96 DNA plant kit (QIAGEN) was between 5-50 ng μl^{-1}.

The 96-well plates, containing genomic DNA samples from the *S*- and *Z*-population, were

used in the subsequent PCRs.

2.4.3 SSR markers analysis

SSR markers that have been mapped to *Lolium* LG1 and LG2 were chosen from publicly available sources (Table 2.4.3) as well as licensed markers from IBERS, Aberystwyth, UK and ViaLactia Biosciences, Auckland, New Zealand (Table 2.4.4). The SSR forward primers were fluorescently 5'-labelled with one of four fluorochrome moietes – 6-carboxyfluorescein (FAM) (Metabion), VIC®, NED™ or PET™ (Applied Biosystems). Each SSR marker was first screened in the parents (p235/40/13 and p232/88/10) of the *S*- and *Z*-population and polymorphic SSRs were used for further linkage analysis in the *S*- and *Z*-populations, respectively. As described in section 6.1.4., recombinants were revealed as homozygous for the SSR marker locus and the recombination frequency between the marker and the *S* or *Z* locus was converted to their linkage distance (in cM). SSR marker Rv1112 (Gill *et al*., 2006) was used to screen for self progeny or contaminants in the *S*- and *Z*-population. The genotype of one parent p235/40/13 for Rv1112 was homozygous (e.g. 'aa'), while the other parent p232/88/10 was genotyped as another homozygous for Rv1112 (e.g. 'bb'). Therefore, a cross progeny was genotyped as heterozygous (e.g. 'ab') for Rv1112 and the selfs progeny or contaminants were identified as homozygous for Rv1112 ('aa' or 'bb').

Genomic DNAs were amplified through 10 µl PCR with 0.06 µl Taq DNA Polymerase (5 U μl^{-1}) (New England Biolabs), containing 1 µl of 10 x PCR buffer, 0.2 µl of dNTPs (10 mM each), 0.25 µl of each forward and reverse primer (10 µM), 1 µl of genomic DNA template (25 ng), and 7.24 µl of ddH_2O. The reaction conditions varied for each primer source (Table 2.4.2) with available information of the specific annealing temperature (AT) for each SSR marker. PCR products were incubated at 65ºC for 30 minutes to avoid poly A peaks. PCR

products labelled with one of each of the four fluorescence dyes were pooled as follows: 0.5 µl of VIC, 1 µl of FAM, 2 µl of PET and 2 µl of NED. Pooling was carried out with different amounts of products according to the fluorescence dye to normalise differences in dye strength. 0.5 µl of the pooled products and 9.5 µl of formamide/sizing standard mix (25 µl of sizing standard; 950 µl of formamide) were mixed and placed on a 96-well plate (Applied Biosystems). The mixed products were separated using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and sized using the GeneScan^{TM}500 LIZ$^{®}$ size standard (Applied Biosystems). Allele scoring was performed using GeneMapper v3.7 (Applied Biosystems).

SSRs tightly linked to the *S* or *Z* loci were further analysed on the ILGI population to compare the map distances on different populations.

Table 2.4.2 PCR conditions for SSR markers grouped by source of primers.

Sources	Tm	Time	Cycles	Reference
CRC, Australia	94ºC AT 72ºC	1 min 30 secs 1 min	10 cycles (touch down -1ºC per cycle)	Jones *et al*., 2001
	94ºC AT 72ºC	1 min 30 secs 30 secs	30 cycles	
Kubik et al.	94ºC	1 min		Kubik *et al*., 2001
	94ºC AT 72ºC	1 min 1 min 2 mins	30 cycles	
	72ºC	10 mins		
The Noble Foundation, USA	95ºC	5 mins		Warnke *et al*., 2004
	95ºC AT 72ºC	1 min 1 mins 1 min	35 cycles	
	72ºC	10 mins		
Lauvergeat et al.	94ºC	3 mins		Lauvergeat *et al*., 2005
	94ºC AT 72ºC	30 secs 30 secs 30 secs	10 cycles (touch down -1ºC per cycle)	
	94ºC AT 72ºC	30 secs 30 secs 30 secs	30 cycles	
	72ºC	5 mins		
ViaLactia, New Zealand	95ºC	10 mins		Gill *et al*., 2006
	94ºC AT 72ºC	1 min 30 secs 1 min	10 cycles (touch down -1ºC per cycle)	
	94ºC AT 72ºC	30 secs 30 secs 10 mins	25 cycles	
	72ºC	10 mins		
IBERS, UK	96ºC	5 mins		King *et al*., 2008
	96ºC AT 72ºC	15 secs 30 secs 30 secs	40 cycles	
	72ºC	4 mins		

Table 2.4.3 Details of tested SSR markers from publicly available sources: marker name, forward and reverse primer sequences, linkage group, annealing temperature and reference.

Name	Sequences (5' – 3')	LG	Tm (ºC)	reference
LpACT15H3	Forward: GACATCCATGCAAAATGTAAG Reverse: TCGTCACTTGCAAATATGAAC	1	60	King *et al*., 2008
LpACA31C9	Forward: CTGAATGCGCAAGCCGCATTC Reverse: CGATTTGCAAAACCGATGGTG	2	60	
LpHCA17C11	Forward: ACCGGGACAAAGGGCTAGTAC Reverse: CGCTCGATCGATCTGATCGTG	2	60	
rv0244	Forward: CAGATTGCAACTCACGAGGA Reverse: ATTTTCCCGTCTGTATTGCG	1	65	Gill *et al*., 2006
rv1112	Forward: TTTGTTAGCCCGTCCTTACCTA Reverse: AACATGTGGAATGGAATGGAAT	5	60	
B1-B6	Forward: GGAGCTGCATCTTTCTTGCT Reverse: GCAAACCCAGACACCCATTA	1	55	Lauvergeat *et al*., 2005
B3-B7	Forward: AGGCGACCAATACGTCTGTC Reverse: ATCTCTGATGGCTTTGTGGC	1	55	
B3-D12	Forward: GGGCATCACTGAGAAGAGGA Reverse: TACAAAGGAAGTCGGGCATC	2	50	
B4-D7	Forward: CGGGAGCTCTCTCTCCTTCT Reverse: TCCAGAACCTTCTCGAGGTC	1	55	
LpSSR021	Forward: AACAAGTCAATGGACAGATT Reverse: TTTGTTTCCCTTTTGG	2	50	Jensen *et al*., 2005b
LpSSR027	Forward: CACCACCTTCTCCAAC Reverse: AACAAGCACTTAGGAACA	1	55	
LpSSR057	Forward: TAGCCTCCAGAAACAAAGTC Reverse: CATAGCAGTACAGCCAGTCA	1	50	
LpSSR076	Forward: CCCATACTTCGAGGCATAAA Reverse: AAATTCCCCCATCAGAGAAC	2	52	
LpSSR085	Forward: GCCAGATCCCTTGTAGAAG Reverse: GCACCATTTAAAACCAAAGA	1	57	
LpSSR112	Forward: GACCCCGAGACAGCCTA Reverse: ACGCATATGGTCTTCAGAA	2	55	

Name	Sequences (5' – 3')	LG	Tm (ºC)	reference
M15185	Forward: GGTCTGGTAGACATGCCTAC Reverse: TACCAGCACAGGCAGGTTC	2	55	Kubik *et al*., 2001
M16B	Forward: TGCTGTGGCTCTTGTGAC Reverse: AGCCGAGGCTCAGCTCGA	1	65	
M4136	Forward: AGAGACCATCACCAAGCC Reverse: TCTGGAAGATTTCCTTG	2	55	
PR3	Forward: GTATAGTACCCATTCCGT Reverse: GCCGCCCTGCCATGCTG	2	60	
PR8	Forward: AGGGTTCGTCTGCATTC Reverse: GCCGTCGCACCCCTG	1	60	
PR24	Forward: TGCTGTGATGCTGAATG Reverse: GTATAGTACCCATTCCGTTGTC	2	55	
PR25	Forward: AGGGTTCGTCTGCATTC Reverse: CCTGCATACATTCATCCA	1	55	
PR39	Forward: CATTCATCCACGTTAGAC Reverse: CTTCCACGACTGCTTC	1	55	
PRE	Forward: CATTCATCCACGTTAGAC Reverse: GTTAGGTTCGTCTGCAT	1	50	

Table 2.4.4 List of tested SSR markers on *Lolium* LG1 and LG2 with license proprietary.

Sources	Name	LG
ViaLactia, New Zealand	rv0117	1
	rv0252F	1
	rv0327F	1
	rv0746F	1
	rv1087F	1
	rv0033	1
	rv0062F	2
	rv0122	2
	rv0188F	2
	rv0226F	2
	rv0696	2
	rv0981F	2
	rv1117F	2
	rv1164F	2
	rv1239F	2
	rv1269F	2
	rv1282F	2
	rv1384	2
The Noble Foundation	NFFa136	2
CRC, Australia	LPSSRK13C10	2
	LPSSRK12E03	2
	LPSSRK12D11	1
	LPSSRK10F08	1
	LPSSRHXX238	1
IBERS, UK	LpACT26F10F	1
	LpACT27C2F	2
	LpACT2G6F	2
	LpACT1B4F	2

2.4.4 Development of mapping markers

Markers were developed to enable mapping of the *Lolium* G10-protein candidate (as identified by a comparative genetics approach (Chapter 3)) and the ten SI candidate genes (identified in SI SSH libraries (Chapter 4) and differentially expressed in real-time PCR analysis (Chapter 5)). Primers were designed to amplify longer sequences than were needed for real-time PCR expression analysis in order to increase the chances of identifying polymorphisms. For the candidates where full length cDNA sequence information is available

(*Can3*, *Can10*, *Can94*, *Can130*, *Can135* and *Can136*, refer to Chapter 4), several sets of primer pairs were designed for each cDNA to cover different regions of the full length sequence. For the remaining four candidate genes, primers were designed on their SSH sequencing results. Primers were designed with Primer3 (V0.4.0) (http://frodo.wi.mit.edu/primer3/input.htm) and analysed as described in section 2.1.5. Primer pairs were first tested on a subset of ILGI plants (8 to 12 random genotypes) and the pair which yielded visible polymorphisms on an agarose gel was selected for each candidate gene and further mapped on the ILGI population. For the candidates where all primer pairs amplified a single band, the primer pair that generated a larger amplicon was selected and amplified on ILGI genotypes of all different genotypes ($S_1S_2Z_1Z_2$, $S_1S_3Z_1Z_2$, $S_1S_3Z_1Z_2$ and $S_1S_2Z_1Z_3$). The amplified products were directly sequenced by a commercial sequencing company (AGOWA). The sequences were aligned with MACAW (v 2.0.5) to identify single nucleotide polymorphisms (SNPs). A SNP is evident as a variation in nucleotides in the sequencing chromatographs between two aligned sequences. SNP(s) that resided in a restriction site of a restriction enzyme were utilised as a cleaved amplified polymorphic sequence (CAPS) marker. The previously identified *Lolium* G10-protein gene CAPS marker (refer to Chapter 3) and the developed CAPS markers for SI candidate genes were tested on a subset of ILGI plants and those yielding polymorphisms were applied to mapping on the whole ILGI population. Sequence tagged site (STS) markers were developed as described in section 2.1.2. Three expressed sequence tagged (EST) markers (LpGK1, LpGK2 and LpCadelp) for the *Z* locus, were developed and provided confidentially by Dr. Bruno Studer/University of Aarhus, Denmark within a collaborative initiative for linkage analysis on the ILGI population. The EST, CAPS and STS markers showing close linkage with the *S* or *Z*

loci were further tested on the *S*- or *Z*-population for analysing recombination frequencies. Sequences of selected primer pairs were listed in Table 2.4.5.

Table 2.4.5 Sequences of primers for mapping SI candidate genes and the *Lolium* G10-protein to *S* or *Z* locus.

Name	**5'…forward primer…3'**	**5'…reverse primer…3'**
Can3	GAGGTACAAACCAGCAGTGA	TATGGGTCATGAACCTCGAG
Can4	GATGGCAAGAAATGTGCGGT	GAGGTACCCAAGAAGTTGGA
Can10	CCTGATCATAGTGGAAGTAG	GTTTCTTGATACTGCTGGAC
Can18	CGTTCGACATCCTCTACTGT	AAGGTTGTAAGCTGGCAGGT
Can94	CGTGGATACAGAATGCCAGC	CGATGTCAACGTCGAAGATG
Can130	CAGCTCCGGCATGTTGAAA	CGAGTGGAGCGAGGAGAAGA
Can135	CGGGCAGGTACATCAATCAGT	TGGAAACCCATGACAGAGTCCT
Can136	GCGCAAGGACATTCTCATGA	GCTGAAGCCACTTTCTGGAC
Can139	AAGAACAGCAGCGCTACTGA	AGAAGCAGGTTGCGAGAAGA
Can151	CTAGTCAAGCACCATAGTGC	ACCTCCTCCGGTATTCT
Lolium G10	GCCTAAGATAAAGACGAGCC	TGCATAACCTTGGTCCAAGC

2.4.5 Linkage analysis

Segregation data of selected SSRs, EST markers and several previously identified STS markers (Chapter 3) on the ILGI population were compared to the segregation pattern identified for *S* and *Z* genotypes to determine recombination frequencies between markers and the respective locus. Multipoint linkage analysis of markers and the *S* or *Z* locus on the ILGI population was performed using the software package JoinMap® V 3.0 (Van Oijen and Voorrips, 2001) with a log of odds (LOD) score of 5.0. The marker order was calculated at a LOD score larger than 1.0 or recombination threshold value (REC) smaller than 0.40 and a jump threshold in goodness-of-fit of 5.0. Recombination frequencies for markers on the *S*- and *Z*-population were calculated with the number of homozygous individuals divided by the number of genotypes in the mapping population. The Kosambi function was applied to calculate genetic distances in centiMorgan (cM) with the following formula (Kosambi, 1944):

$M = 1/4 \ln[(1+2r)/(1-2r)]$ (M: map distance; r: recombination frequency)

Graphical presentations of the genetic linkage maps were produced using MapChart V2.2 software (Voorrips, 2002).

CHAPTER 3

COMPARATIVE MAPPING AND THE IDENTIFICATION OF CANDIDATE GENES FOR THE *S* AND *Z* LOCI

3.1 Introduction

3.1.1 Comparative genetics in the 'Crop Circle'

Comparative analysis performed on grasses have shown that, though grasses are very different in terms of genome size, ploidy level and chromosome numbers, the synteny of genetic markers in chromosomal regions is very well conserved between different grass genomes (Moore *et al*., 1995; Devos and Gale, 1997; Gale and Devos, 1998; Devos and Gale, 2000; Devos, 2005). Based on the conservation of genome segments, consensus maps can be developed across related species. The first grass consensus map or 'Crop Circle' (Moore *et al*., 1995) aligned the genomes of seven different grass species using rice as a reference genome. After subsequent refinements, genome relationships between eleven grass species (rice, wild rice, foxtail millet, sugar cane, sorghum, pearl millet, maize, the Triticeae cereals, oats, rye and meadow fescue) were established (Devos and Gale, 2000; Devos, 2005). To gain further information on the gene orders within the conserved genome segments, sequence-based high resolution analysis has been performed to identify gene colinearity (Tikhonov *et al*., 1999; Keller & Feuillet, 2000; Dubcovsky *et al*., 2001; Ramakrishna *et al*., 2002ab). Different studies have shown that colinearity can often be identified, though disruptions have been discovered as a result of small rearrangements, such as gene insertions/deletions, gene inversions, tandem gene duplications and gene translocations (Ramakrishna *et al*., 2002a; Song *et al*., 2002; Illic *et al*., 2003; Alm *et al*., 2003; Ma *et al*., 2005; Armstead *et al*., 2007b; Bennetzen, 2007).

Comparative restriction fragment length polymorphism (RFLP) maps can be developed using "anchor probes", which have good hybridization ability across different species and are used to evaluate the conservation of linkage segments (Van Deynze, http://greengenes.cit.cornell.edu/anchors/). With the increasing availability of ESTs through

large scale sequencing, comparative mapping can be performed more efficiently. The rice genome has been used as a model for comparative mapping in grasses because of the availability of various genomic resources (www.gramene.org; http://rice.plantbiology.msu.edu/) and the whole genome sequence (Goff *et al*., 2002). Rice sequences can be used as anchor probes or to identify ESTs and STSs from other grasses that can be aligned for comparative mapping.

Comparative genetics is especially important for less characterised crop species where limited genomic information is available. It has been used for the generation of new markers in different grass species for increasing map density and map-based cloning (Kilian *et al*., 1997; Jones *et al*., 2002b; Yu *et al*., 2004; Kuraparthy *et al*., 2008; Armstead *et al*., 2006a), identifying chromosomal regions containing QTLs (Sarma *et al*., 1998; Hu *et al*., 2003; Armstead *et al*., 2004; Caldwell *et al*., 2004; Jensen *et al*., 2005a; Srinivas *et al*., 2008) and identifying genes of interest (Laurie, 1997; Devos and Gale, 1997; Armstead *et al*., 2005). With the development of Gramene (http://www.gramene.org/), the online open data resource, studies based on comparative genetics in grasses are growing rapidly.

3.1.2 Comparative genetics in Lolium

The use of heterologous RFLP markers as anchor probes from wheat, barley, oat and rice to construct a genetic map of perennial ryegrass (*Lolium perenne*) has revealed the comparative relationships between *L. perenne* and the other Poaceae species (Jones *et al*., 2002b; Armstead et al., 2002). As described in the 'Crop Circle' (Devos, 2005), synteny and colinearity of the genome structure of *L. perenne* and *Festuca pratensis* are well conserved compared to the genome structure of the Triticeae (Jones *et al*., 2002b; Alm *et al*., 2003). Recently, syntenic relationships between rice and *Lolium/Festuca* hybrid genomes have being

explored through introgression mapping (King *et al*., 2002; King *et al*., 2007ab) and a good level of synteny has so far been established between *Lolium/Festuca* chromosome 3 and rice chromosome 1 (King *et al*., 2007a). The linkage groups of *L. perenne* were numerically assigned to coincide with the homoeologous groups of the Triticeae species (Jones *et al*., 2002b), and based on the relationships with the rice genome, synteny has been established between the *L. perenne* and rice genomes (Figure 3.1.1) (Jones *et al*., 2002b, Sim *et al*., 2005).

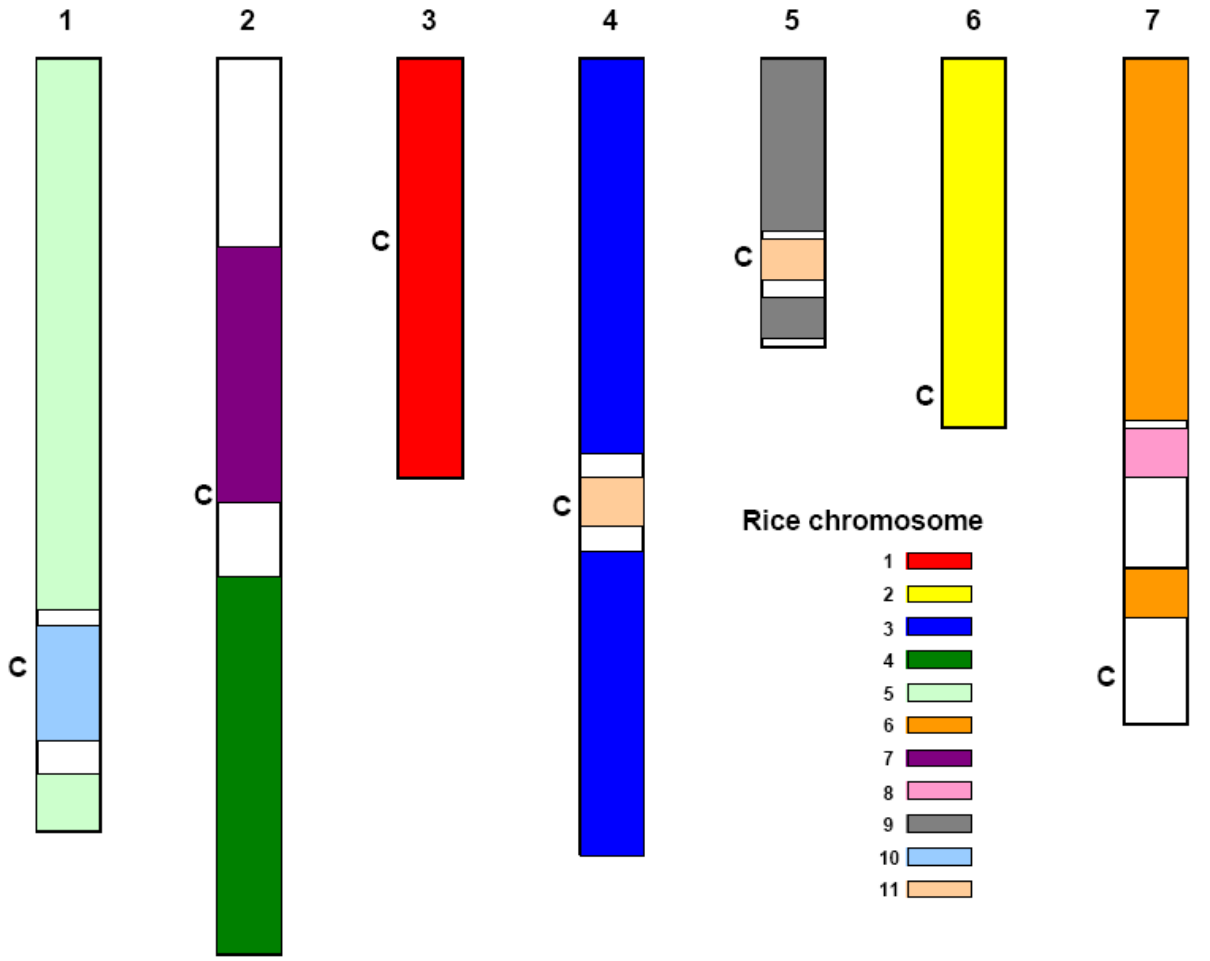


Figure 3.1.1 *Lolium perenne* linkage group (LG) view showing comparative relationship with the rice genome. Colored boxes represent conserved syntenic segments of rice chromosomes. Each color corresponds to one rice chromosome. Conserved synteny has been established between *L. perenne* LG1 with rice chromosome 5; LG2 with rice chromosome 7 and 4; LG3 with rice chromosome 1 and 5; LG4 with rice chromosome 3; LG5 with rice chromosome 9 and 11; LG6 with rice chromosome 2; and LG7 with rice chromosome 6 and 8. Three chromosomal rearrangements have been observed on L. perenne LGs 1, 4 and 5 relative to rice. LG1 has an insertion of a fragment of rice chromosome 10 between two distinct fragments of rice chromosome 5, LG4 the insertion of a fragment of rice chromosome 11 between two distinct fragments of rice chromosome 3, and LG5 the insertion of a fragment of rice chromosome 11 between two distinct fragments of rice chromosome 9. The *L. perenne* centromere location on each linkage group is indicated by 'C'. White boxes indicate that a syntenic relationship could not be found (Jones *et al.*, 2002b; Sim *et al.*, 2005).

With this comparative information on the conserved syntenic relationships and genome rearrangements, it is possible to transfer genetic information from well-characterised grass species, such as rice, to *L. perenne* for the identification of markers, QTLs and candidate genes of interest. Studies have been carried out within recent years and include for example,

(a) the evaluation of a heading-date QTL in *L. perenne* which showed synteny with the rice *Hd3a* heading-date locus (Armstead *et al*., 2004); (b) the identification of orthologous *Hd1* and *CONSTANS (CO)* genes in *L. perenne* from rice and barley genes (Armstead *et al*., 2005); (c) the identification of the QTL for the vernalization response in *L. perenne* in an orthologous location for the wheat *VRN1* (Jensen *et al*., 2005a); (d) comparative mapping of crown rust resistance (CRres) gene(s) in *Lolium/Festuca*, oats and rice (Armstead *et al*., 2006b); (e) isolation of a staygreen gene in *Lolium/Festuca* (Armstead *et al*., 2006a), which is homologous to the *I* locus in pea (as identified originally by Mendel) that determines cotyledon colors (Armstead *et al*., 2007a); and (f) comparative mapping and candidate gene identification of factors affecting flowering and fertility in ryegrass (Armstead et al., 2008ab). Comparative mapping has been applied to identify markers for *S* and *Z* loci that control self-incompatibility in grasses (Baumann *et al*., 2000). The *S* locus on rye (*Secale cereale*) has been located on LG1 on a F_2 population containing 80 individuals with two RFLP markers derived from wheat (Senft and Wricke, 1996; Voylokov *et al*., 1998): *Xiag249* with a map distance of 2.7 cM and *Xpsr544* with a map distance of 4.5 cM from the *S* locus. The *S* locus on *Phalaris coerulescens* was delimited to a region of 0.26 cM on LG1 between two RFLP markers *Xwg811* and *Xpsr168* on a fine mapping population containing 862 progeny (Bian *et al*., 2004). The closest flanking markers for the *Z* locus have been developed from orthologous genomic regions on rice chromosome 4. They are located on rye LG2 with a map distances of 0.5 cM and 1 cM from *Z*, respectively, on a population of 204 progeny (Hackauf and Wehling, 2005). With the development of comparative genetics in *Lolium*, it is possible to use the rice genome as a template for developing tightly linked genetic markers and possibly identifying candidate genes for the *S* and *Z* loci.

3.1.3 ILGI mapping population of Lolium perenne

The ILGI mapping population is an intraspecific one-way pseudo-testcross population, containing 183 progenies (Jones *et al*., 2002ab). It was derived from a cross between a doubled-haploid plant (DH290) as female and a multiple heterozygous F1 pollinator with complex descent (Romanian collection no.Ba 9982 x 3613 (a polycross of early North Italian x 'Melle' or 'S23'). Genetic linkage maps for ILGI population were developed (Bert *et al*., 1999; Jones *et al*., 2001; Armstead *et al*., 2002; Jones *et al*., 2002ab) containing molecular markers of isozymes, RFLP, randomly amplified polymorphic DNAs (RAPDs), AFLP, SSR markers, SNPs and STSs.

Isozymes were the first molecular markers used for genetic mapping in plants (Tanksley and Orton, 1983) and have been used successfully in grass variety and population studies (reviewed in Wang *et al*., 2001). They are codominant markers, require no DNA and are transferable across species. However, the laborious assay procedure, limited numbers and inclination to posttranslational modification are their limitations for use. RFLPs were the first common used molecular DNA based markers for mapping studies (Lander and Botstein, 1989) and have been extensively used for genetic map constructions in plants. RFLPs are codominant markers and reproducible, but the time-consuming procedure and requirement of large amounts of DNA are their limitations. RAPDs (Welsh and McClelland 1990; Williams *et al*., 1990), SSRs or microsatellites (Tautz 1989; Weber and May 1989) and AFLPs (Vos *et al*., 1995) are PCR-based markers. They overcome many of the limitations of RFLPs. However, RAPDs also have the disadvantages of being dominant markers, showing low-specificity and poor reproducibility across laboratories (Jones *et al*., 1997). Their highly heterozygous, codominant, and PCR-based nature has made SSRs the molecular markers of choice for genetic mapping and diversity studies (Mba *et al*., 2001). They are found

throughout both the transcribed and non-transcribed regions of a genome (Varshney *et al*., 2005). The increasing availability of ESTs in plants also provides a potential source for development of SSRs (Eujayl *et al*., 2001). AFLPs generate highly reproducible fragments in a multi-band profile which can reveal extensive polymorphism. Using this method, previous knowledge of nucleotide sequence is not required and the number of fragments detected in a single reaction can be adjusted by selection of specific primer sets. The disadvantage of AFLPs is that they are dominant markers and allelic bands are often difficult to identify (Powell *et al*., 1996b). SNPs are known to be the basis of most differences between alleles (Rafalski 2002). They are the most abundant source of DNA polymorphism and considered the marker of choice nowadays for their allele-specificity.

The ILGI map of *Lolium* developed by Bert et al. (1999) covers a map distance of 930 cM based on 463 AFLP markers. Jones et al. (2002b) reported a map distance of 811 cM containing RFLP, AFLP, isoenzymes and ESTs. This map has been further enhanced by integration of 93 SSR loci (Jones *et al*., 2002a), composing the ILGI genetic map with a total number of 258 loci. The ILGI mapping population has been used in a number of studies in comparative genetics, genomics and QTL analysis, including comparative genome analysis (Armstead *et al*., 2002; Jones *et al*., 2002b), flowering date (Shinozuka *et al*., 2005; Armstead *et al*., 2008a), seed-set (Armstead *et al*., 2008b), winter hardiness-associated traits (Yamada *et al*., 2004), herbage quality traits (Cogan *et al*., 2005) and self-incompatibility and self-fertility (Thorogood *et al*., 2002, 2005).

3.1.4 Aim of the study

The aim of the study described in this chapter is to develop tightly linked STS molecular markers for the *S* and *Z* loci by comparative genetics, using the *L. perenne* ILGI mapping

population, which has been well characterised in terms of *S* and *Z* loci (Thorogood *et al*., 2002). STS markers are sequence-specific primers generated from a known sequence that specify a unique locus within the target genome (Tragoonrung *et al*., 1992). These primers detect variation in allelic, genomic DNA and have an advantage of being able to distinguish between homozygotes and heterozygotes. Sequences used for the generation of STS markers normally include both coding and non-coding genomic DNA, based on pre-existing sequence knowledge. In contrast to AFLP and SSR markers, primer binding sites are more-likely to be conserved across species for STS primers derived from protein coding sequences. Taylor et al. (2001) reported the application in *L. perenne* using STS primers from wheat and barley and showed that STS markers offer a reliable PCR-based system for mapping orthologous loci across distantly related species and hence, aid in the alignment of genetic linkage maps from divergent species. With the availability of the whole genome sequence from rice as well as ESTs and genomic sequences from rice, barley, wheat and some other Triticeae species (http://www.gramene.org/), STS markers can be developed in conserved regions for mapping *Lolium* S and Z loci. The method and results are described in detail in this chapter. The approach to identify a possible SI candidate gene using comparative genetics is also discussed.

3.2 Results and Discussion

3.2.1 Development and mapping of STS markers

Comparative mapping work has demonstrated that regions of the *Lolium* genome show a potentially useful degree of conservation with rice and the Triticeae genomes and this can be used for the development of mapping strategies within *Lolium* species (Sim *et al*., 2005; Armstead *et al*., 2002; Jones *et al*., 2002b).

Based on the comparative sequencing alignments in defined regions of the rice genome, markers were designed and tested on genomic DNA of the ILGI family with the aim of flanking the *S* and *Z* loci regions as described in section 2.1.2. Primer pairs yielding no visible polymorphisms on agarose gels were rejected. The segregation patterns of primer pairs that gave polymorphic amplification products (Figure 3.2.1, Figure 3.2.3) were compared to the segregation patterns previously identified for *S* and *Z* genotypes (Thorogood *et al*., 2002) to calculate the map distances between the marker and the respective locus. Several new STS markers (Figure 3.2.1) around the *S* and *Z* loci were developed, with the closest flanking markers mapping at a distance of 2 cM from *S* and 0.2 cM from *Z*. Their relative genetic distances (cM) on LG1 and LG2 in accordance with the Triticeae consensus map are shown in Figure 3.2.2. The positions of *S* and *Z* loci on the map are included. The sequences of the STS primers developed are given in Table 3.2.1. The comparative maps of *L. perenne* LG1 with rice C5 and *L. perenne* LG2 with rice C4 including the developed STS markers are shown in Figure 3.2.4.

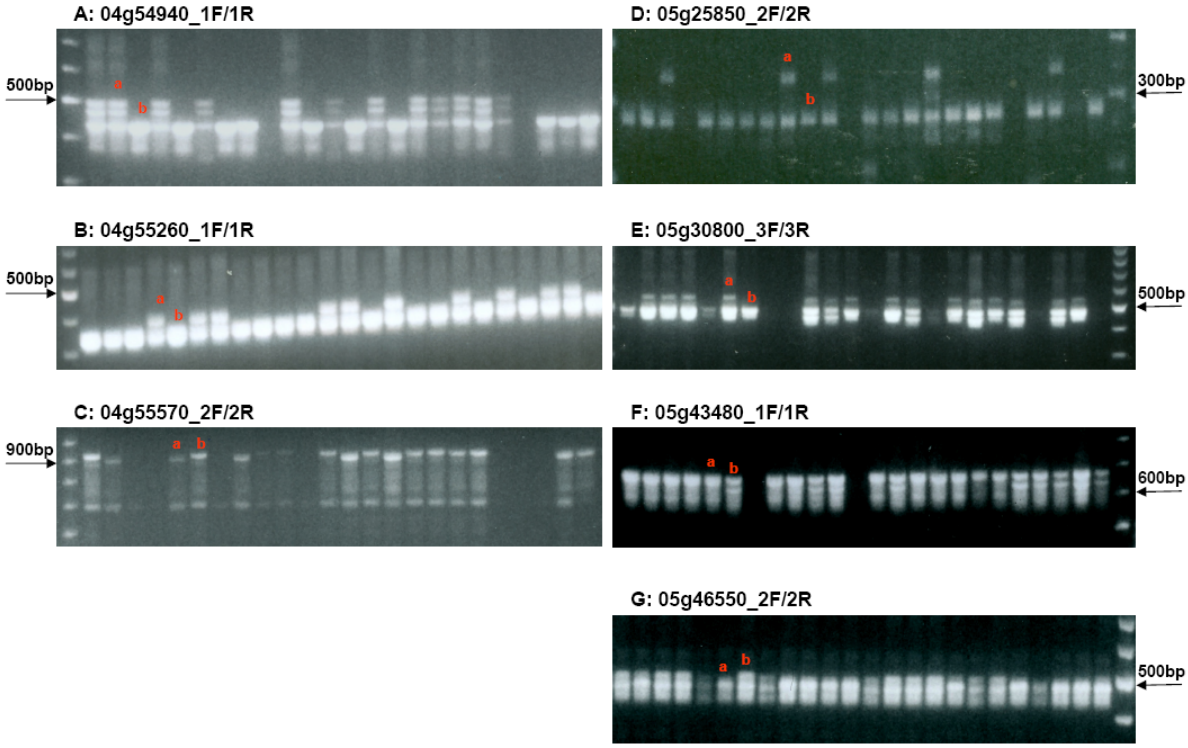


Figure 3.2.1 Primer pairs gave polymorphic amplification products and were mapped to the *S* or *Z* locus. Each gel picture shows amplified products from genomic DNA of 24 ILGI plants using the rice genome derived primers. The two genotypes for scoring segregation patterns of the markers are indicated with "a" and "b". A single band of the 100 bp DNA ladder is indicated on each gel. The three markers A, B and C were derived from gene sequences on rice C4 and were mapped to the *Z* locus (Figure 3.2.2). Markers D, E, F and G were derived from gene sequences on rice C5 and were mapped to the *S* locus (Figure 3.2.2).

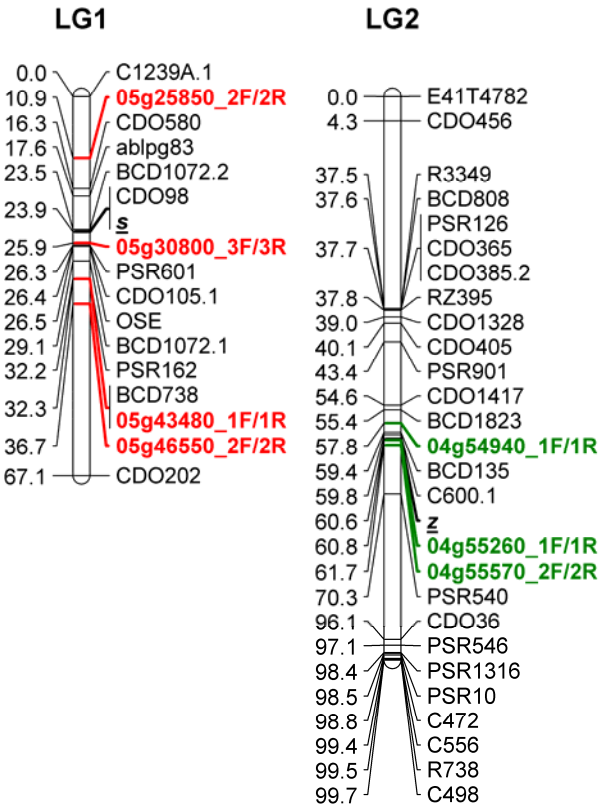


Figure 3.2.2 The genetic maps show the genetic distance (cM) of markers on *L. perenne* linkage groups 1 and 2. *S* and *Z* loci are marked in bold and are underlined. STS markers on LG1 and LG2 are highlighted in red and green colors, respectively. The closest STS marker to the *S* locus is 05g30800_3F/3R and mapped 2 cM away. The closest marker to the *Z* locus is 04g55260_1F/1R and mapped 0.2 cM away. The maps were drawn using MapChart 2.2 (Voorrips 2002).

Table 3.2.1 Primer sequences for STS markers developed in the *L. perenne* ILGI mapping family. Primers were named after the corresponding Osa1 rice locus.

Marker	**5'…forward primer…3'**	**5'…reverse primer…3'**
05g25850_2F/2R	GCTTTGGATAAAGAGGCCAA	TGCTCCCAGACATCAATTCC
05g30800_3F/3R	CCTCAGACCATGATGCTTGG	TCCTCTTGCACTGCCTTGTT
05g43480_1F/1R	CGGCCTGAGGTATTGGAGGG	TGCTTCCGAGCTGAGGTGAG
05g46550_2F/2R	TTGGCCAGATTTGAGAATGA	ACTTCAATGGGTTCTGCTGC
04g54940_1F/1R	ATCCTCAGCTGGGTCAATG	GAATCTTGTAGTTCTGGATCATGTC
04g55260_1F/1R	CAGACTTGGGCATCAGATGA	CTTCTAGGTGACATGTTGG
04g55570_2F/2R	TCAGGTAAACAAGGGCAATC	TTCCCAGGCTTAGGATTGTC

Three primer pairs gave polymorphic amplification products (Figure 3.2.3) which mapped to different *L. perenne* chromosomes than expected from synteny with rice. Two of these,

05g39960 and 05g29860, which were expected to map to LG1 near to *S*, in fact mapped to LG4 close to another marker, BCD1421, which has also been mapped to rice chromosome C5, thus indicating a further region of the rice genome showing a degree of conserved synteny with LG4 (Jones *et al*., 2002b). The third marker, 04g55290, which was designed to amplify the G10 candidate gene for *Z* from *L. perenne* LG2 (see section 3.2.2), actually amplified a marker which mapped to LG3 where the distortion locus with *S* was identified (Thorogood *et al*., 2002). The position of this marker was consistent with the expected position of LOC_Os01g63890 from rice C1, one of the G10 protein orthologues present in the rice genome.

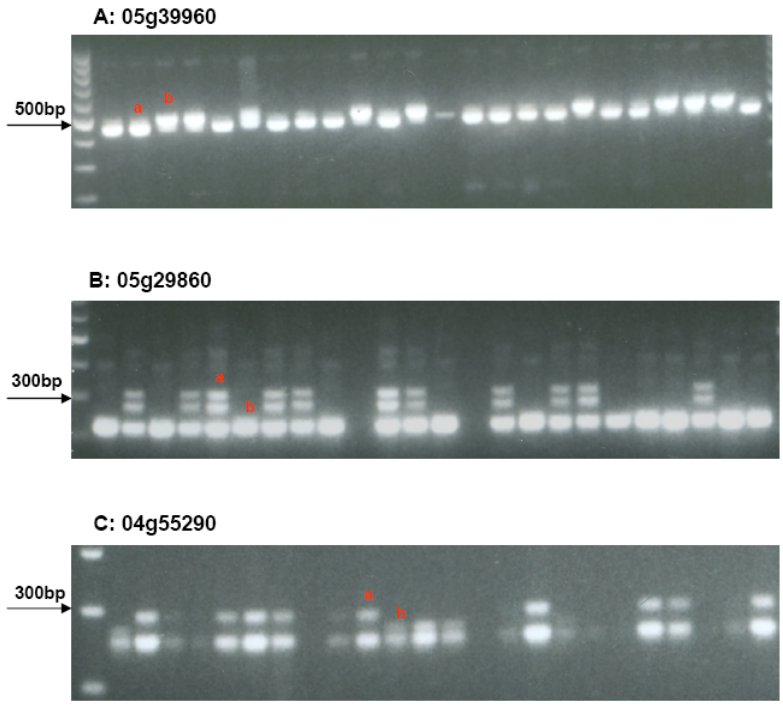


Figure 3.2.3 Primer pairs gave polymorphic amplification products and were mapped to different *L. perenne* chromosomes than that *S* or *Z* is located. Each gel picture shows amplified products from genomic DNA of 24 ILGI plants using the rice genome derived primers. The two genotypes for scoring segregation patterns of the markers are indicated with "a" and "b". A single band of the 100 bp DNA ladder is indicated on each gel. Markers A and B were derived from gene sequences on rice C5 and were mapped to *L. perenne* LG4. Marker C was derived from gene sequence on rice C4 and was mapped on *L. perenne* LG3.

3.2.2 Identification of a candidate SI gene

Through a BLAST search using rice sequences on rice chromosomes C4 in the region defined by the markers flanking *Z* (LOC_Os04g54940_1F/1R to LOC_Os04g55570_2F/2R, see Figure 3.2.2) against all the sequences on C5, two similar genes (G10-proteins) (LOC_Os05g37390 and LOC_Os04g55290 according to Osa1 rice gene models, Table 3.2.2) were identified physically located in regions that would be expected to correspond to the positions of the *S* and *Z* loci on *L. perenne* LG1 and LG2 (Figure 3.2.4). In addition, there were two similar G10-type proteins on rice C1 and C12 (LOC_Os01g63890 and LOC_Os12g05410, Table 3.2.2) (Figure 3.2.5A). Interestingly, the corresponding syntenic regions of *L. perenne* on LG3 and LG5, respectively, also have associations with self-incompatibility. Thorogood et al. (2002) identified that the segregation of loci on LG3 was distorted, apparently, in accordance with the genotype of the *S* locus on LG1. Additionally, Thorogood et al. (2005) also reported that the LG5 contained a major QTL for self-fertility which, in terms of comparative genetics, could be associated with the positions of the *T* locus in rye.

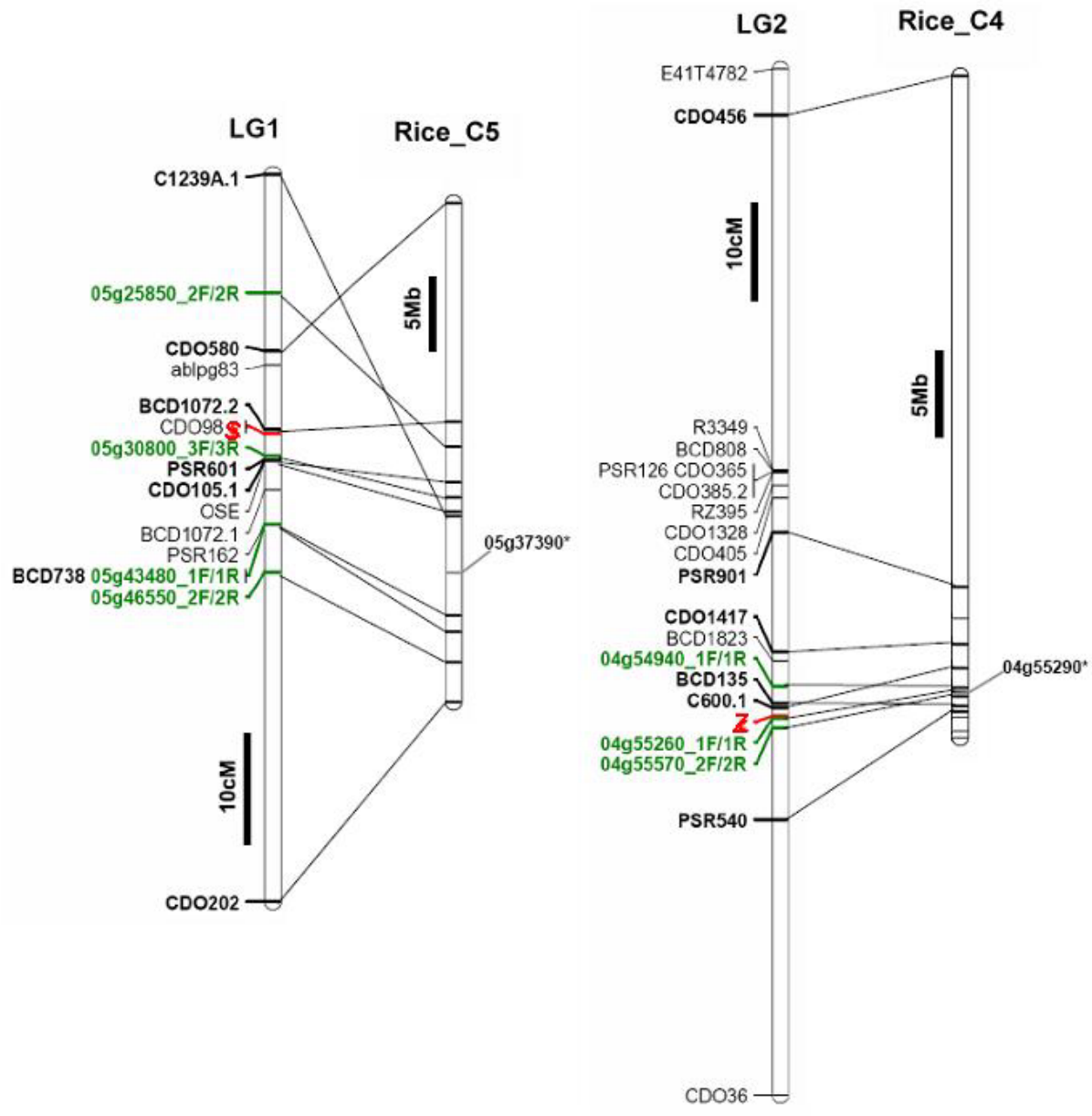


Figure 3.2.4 Comparative maps of *L. perenne* LG1 and LG2 with rice C5 and C4. Markers (bold type) on *L. perenne* LG1 and LG2 were linked to their physical position on the rice C5 and C4 pseudomolecules. STS markers are highlighted with green color. Two G10-proteins, LOC_Os05g37390 and LOC_Os04g55290 (on the right of the rice chromosomes), are physically located in regions that would be expected to correspond to the positions of the *S*- and *Z*-locus (bold italics in red and underlined) on *L. perenne* LG1 and LG2, respectively.

Table 3.2.2 BLAST results from Osa1 showing the G10-protein homologs on rice chromosomes 1, 4, 5 and 12. LOC_Os04g55290 corresponds to the region of the *S* locus on *L. perenne* LG1; LOC_Os05g37390 corresponds to the region of the *Z* locus on *L. perenne* LG2; LOC_Os01g63890 is possibly in the region of the *S* distortion locus on *L. perenne* LG3 and LOC_Os12g05410 is possbily in the region of the self-fertility locus on *L. perenne* LG5. These four sequences show high similarity and have possible associations with self-incompatibility loci in *L. perenne*.

Accession	Description	Hit Score	E value	Identity %
LOC_Os04g55290.1	protein G10 homolog 1, putative, expressed	2190	1.0e-93	100.00
LOC_Os04g55290.2	protein G10 homolog 1, putative, expressed	2190	1.0e-93	100.00
LOC_Os04g55290.3	protein G10 homolog 1, putative, expressed	2190	1.0e-93	100.00
LOC_Os01g63890.1	protein G10 homolog 1, putative, expressed	2154	4.2e-92	99.09
LOC_Os04g55290.4	protein G10 homolog 1, putative, expressed	1910	5.0e-81	98.98
LOC_Os05g37390.1	protein G10 homolog 2, putative, expressed	1677	1.5e-70	86.99
LOC_Os05g37390.2	protein G10 homolog 2, putative, expressed	1677	1.5e-70	86.99
LOC_Os12g05410.1	protein G10 homolog 3, putative, expressed	1308	6.9e-54	77.63
LOC_Os12g05410.2	protein G10 homolog 3, putative, expressed	1308	6.9e-54	77.63

In addition, examination of the rice genomic sequence on rice C1, C4 and C5, identified very similar “expressed or hypothetical” proteins (Table 3.2.3) in the neighbourhood of the G10-proteins. The expressed proteins on C1 and the hypothetical protein on C4 were very similar. The expressed protein on C5 was more divergent (Figure 3.2.5B). No equivalent sequence on rice C12 was found. These “next door” genes did not have any annotations or identifiable motifs, thus their functions remain unknown. However, the apparent conservation of association with the G10-proteins at three of the physical locations in rice indicates that this physical association may be significant for G10-protein functions and that they may also play a role in self-incompatibility reactions.

Lundqvist (1956) assumed that the two-locus SI system in grasses evolved by gene duplication such that the *S* and *Z* genes were similar in structure and function. The finding of highly similar G10-proteins in SI related loci reinforces the possibility that the *S*-*Z* system evolved from a multi-locus duplicated system. It has also been suggested and proven in *Brassica* and Solanaceae that genes encoding the pollen and pistil specificity determinants are tightly linked at the *S*-locus to maintain self-incompatibility (Sijacic *et al*., 2004, Takuno *et al*., 2007). Otherwise, intergenic recombination would cause the breakdown of self-incompatibility by generating different pollen and pistil specificities within a single *S*-haplotype (Sijacic *et al*., 2004). Genetic studies have shown no recombination between the pistil and pollen *S*-allele (de Nettancourt 1977) and the *Brassica* S-locus has been reported to have a recombination suppression mechanism (Boyes *et al*. 1997). In a theoretical gametophytic SI haplotype, the G10-proteins neighbours could represent the pollen/pistil specific counterparts to the G10-proteins, though the degree of conservation is far less for these 'companions' than it is for the G10-proteins themselves. G10-proteins were first identified as being recruited for translation during *Xenopus* oocyte maturation (McGrew *et al*., 1989)**.** They are highly conserved in a wide range of eukaryotic species. As yet, neither the G10-proteins nor the associated similar expressed proteins have defined functions in animal or plant systems. However, the C-terminal of the G10 protein is rich in cysteine, similar to the *Brassica* male determinant SCR (Schopfer *et al*., 1999), which makes the G10-protein gene a promising *Lolium* SI candidate for further investigation, therefore the following attempt was made to identify the *Lolium* G10-protein genes.

Table 3.2.3 BLAST results from Osa1 showing similar "next door" gene proteins on rice chromosomes 1 (LOC_Os01g63880), 4 (LOC_Os04g55280) and 5 (LOC_Os05g37340, LOC_Os05g37400). No equivalent was identified on chromosome 12 or other locations.

Accession	Description	Hit Score	E value	Identity %
LOC_Os04g55280.1	conserved hypothetical protein	7890	0.	100.00
LOC_Os01g63880.1	expressed protein	5312	2.6e-235	94.35
LOC_Os05g37400.1	expressed protein	1004	5.1e-60	66.57
LOC_Os05g37400.2	expressed protein	1004	5.1e-60	66.57

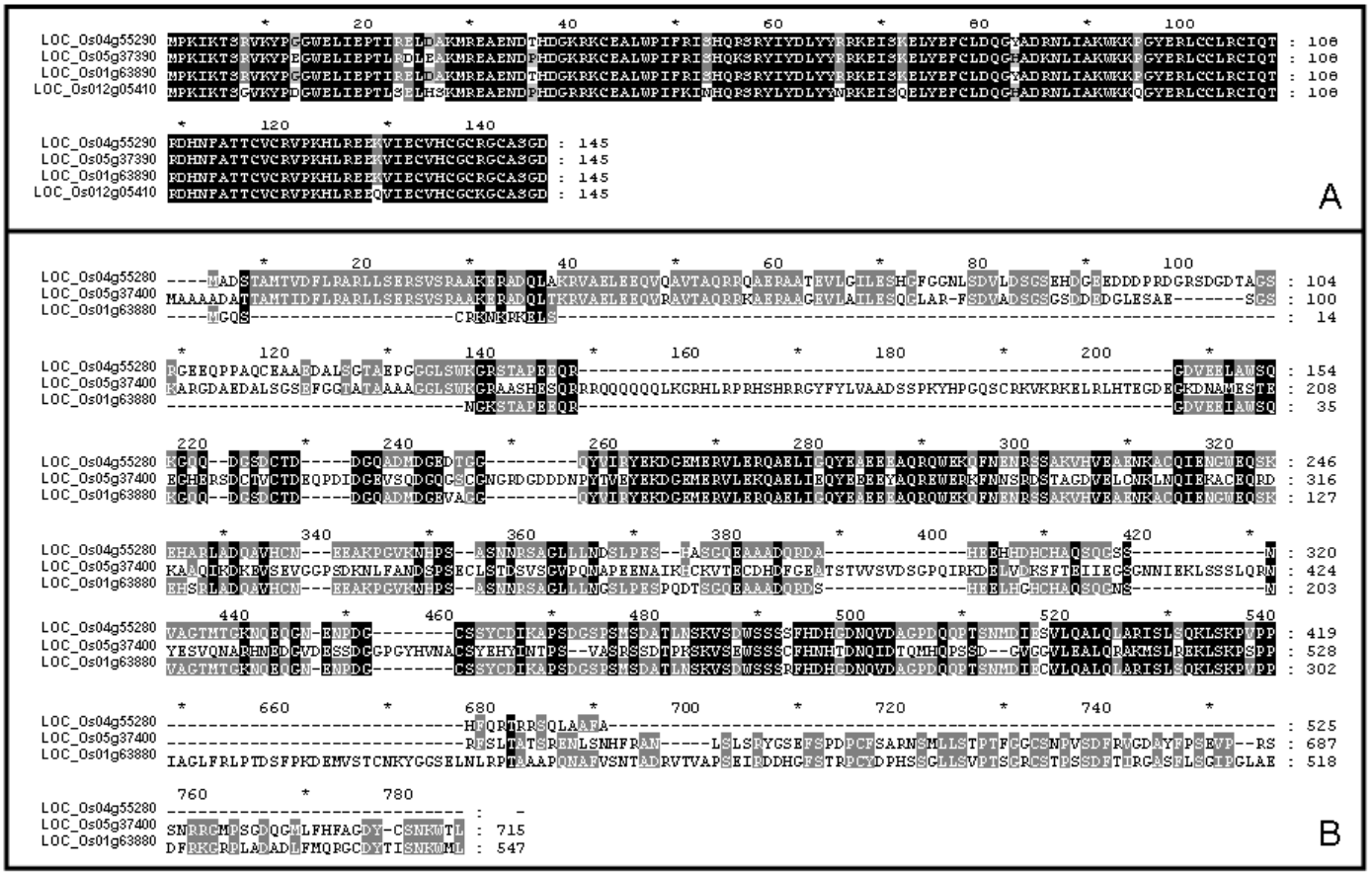


Figure 3.2.5 Potential candidates for involvement in the self-incompatibility response in *L. perenne* were identified through BLAST searches and genetic synteny. Sequence alignments of Osa1 rice gene models for the candidates are shown in A. G10-proteins: the four G10-protein homologues on rice C4 (LOC_Os04g55290), C5 (LOC_Os05g37390), C1 (LOC_Os01g63890) and C12 (LOC_Os12g05410) are very similar; B. 'companion' proteins: the conserved hypothetical protein on C4 (LOC_Os04g55280) and the expressed protein on C1 (LOC_Os01g63880) are similar, while the expressed protein on C5 (LOC_Os05g37400) shows more differences. The alignments were performed using GeneDoc (http://www.psc.edu/biomed/genedoc).

3.2.3 Lolium BAC library screening for SI candidate genes

With the availability of a *Lolium* BAC library in IGER (Farrar *et al*., 2007), primers were designed from the four G10-protein candidate gene homologs in rice. These primers have been used to screen the BAC library to obtain the corresponding *Lolium* gene sequence information. Six clones were identified and have been sequenced. Five of them were highly conserved and the sixth clone showed divergence (Figure 3.2.6). The sequences of the six identified BAC clones and their sequence alignments are shown in the appendix (Appendix A and B). As the *Lolium* BAC library contains five genome equivalents, it is possible that a single gene can be identified in five different clones. However, since our four candidate gene homologs were very similar, it was not possible to discriminate different alleles from different loci.

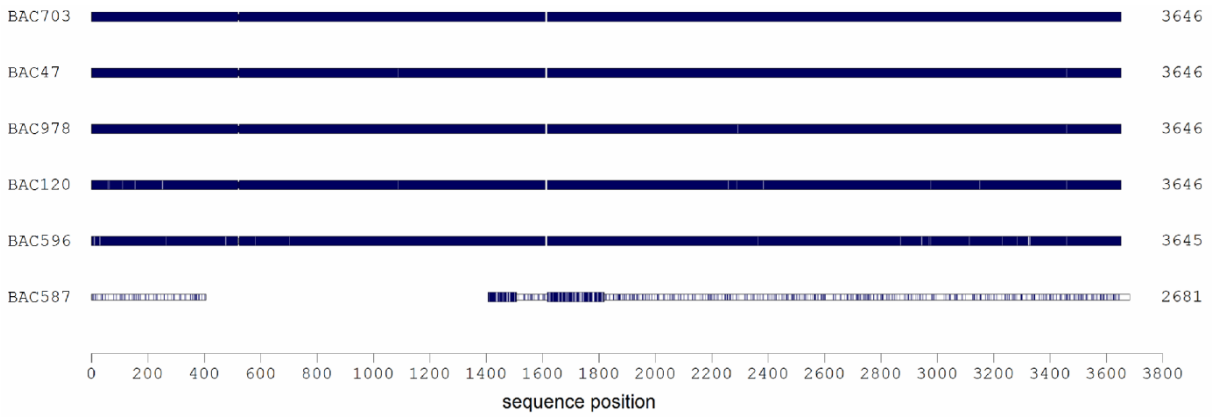


Figure 3.2.6 Schematic representation of sequences from the six identified Lolium BAC clone homologous to the rice G10-proteins. The alignment was performed with MACAW version 2.0.5. The shading represents pair-scores which showed similarities between the sequences. BAC clone #587 was of 75% identity compared to the other five clones in the conversed regions, while the other five BAC clones #703, #47, #978, #120 and #596 were much more conserved with 99% overall identity.

Different *S* and *Z* genotypes from the ILGI family were analysed using primers developed from identified conserved BAC sequences. Though no apparent polymorphism of amplification products were detected on agarose gels, sequencing results of the products

revealed SNPs with regard to the *S* genotypes (Figure 3.2.7). Further mapping of the *Lolium* G10-protein gene on the ILGI population revealed a distance of 20 cM away from the *S* locus. The detailed fine mapping experiment will be described in Chapter 6 of this thesis.

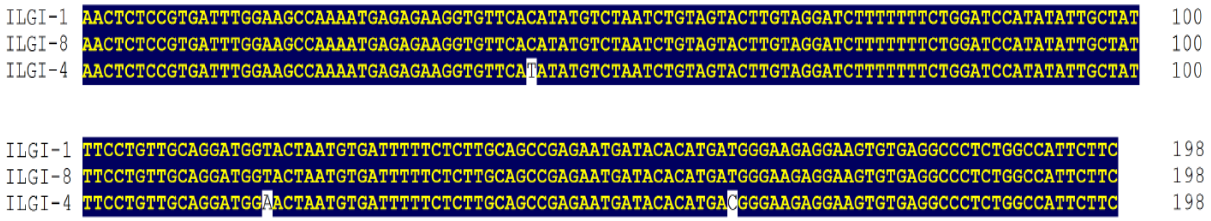


Figure 3.2.7 Alignment of sequences amplified from different *S* genotypes using primers derived from *Lolium* G10-protein conserved BAC sequence. ILGI-1 and ILGI-8 are ILGI plants of the same *S* genotype (S_1S_3), and ILGI-4 has a different *S* genotype of S_1S_2. The shading of pair-score revealed three SNPs. Sequence alignment was performed with MACAW version 2.0.5.

CHAPTER 4

DEVELOPMENT OF SUBTRACTED SI cDNA LIBRARIES AND IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES

4.1 Introduction

Similar to other plant species, in the grass family a cascade response is triggered by the incompatibility reaction after the recognition process between pollen and stigma as determined by *S* and *Z*. This recognition leads to the inhibition of pollen growth in the pistil (Heslop-Harrison, 1982). Gene expression analysis in the pistil during an incompatible pollination can therefore potentially identify genes involved in the SI response and shed light on the signalling cascade.

In the case of S-RNase GSI system, analysis of stylar proteins from *Nicotiana alata* led to the cloning and sequencing of the pistil *S*-gene (Anderson *et al*., 1986) and the same pistil gene was identified in the Solanaceae, Rosaceae and Plantaginaceae with a large number of alleles (Huang *et al*., 1994; L; Xue *et al.*, 1996; Sassa *et al*., 1997). For the *Brassica* SSI system and the *Papaver* GSI system, iso-electric focusing of stigma extracts was used to identify S-linked proteins, leading to the isolation of the *Brassica S*-specific proteins SLGs (Hinata *et al.*, 1982) and the *Papaver* stigma S protein (Foote *et al.*, 1994). Determinants for the grass SI system have not been identified so far. In this chapter, other than the protein approaches performed in the previous SI studies, we applied a genetic approach, differential gene expression analysis, for the purpose to identify the pistil determinants as well as some potential downstream elements involved in the *Lolium* SI response.

By comparing the individual mRNAs from two different samples, genes that are differentially expressed can be identified and their functions determined. A variety of methods have been developed to facilitate the discovery of differentially expressed genes, including cDNA-AFLP (Bachem *et al*., 1996), serial analysis of gene expression (SAGE) (Velculescu *et al*., 1995),

differential display PCR (Liang & Pardee, 1992), representational difference analysis (RDA) (Lisitsyn *et al*., 1993), Real-time Quantitative PCR (Gibson *et al*., 1996), subtractive hybridization (Duguid *et al*., 1988) and hybridization to gene arrays of various formats (Chee *et al*., 1996). The principles of these methods have been reviewed and their applications compared (Kuhn, 2001; Breyne and Zabeau, 2001). This introduction will concentrate on PCR-based methods for qualitative gene expression analysis: cDNA-AFLP and suppression subtractive hybridization (SSH).

4.1.1 cDNA-AFLP technique

cDNA-AFLP is an RNA fingerprinting method derived from AFLP (Bachem *et al*., 1996). It is based on selective PCR amplification of adapter-ligated restriction fragments derived from cDNAs. Three steps are involved: (1) restriction of cDNAs and ligation of an adapter, (2) selective amplification of restriction fragments using primers containing selective nucleotides at the 3' end, and (3) gel analysis of amplified fragments. In most cases, restriction digestion of cDNAs is performed with two restriction enzymes, but use of a single restriction enzyme has also been reported in comparing buds of red and white flowers of the common morning glory (*Ipomoea purpurea*) (Habu *et al*., 1997).

Following the first cDNA-AFLP analysis of differential gene expression during potato tuber development (Bachem *et al*., 1996), cDNA-AFLP has been widely used for the analysis of genes involved in various biological processes, e.g. salt stress related genes in soybean (Umezawa *et al*., 2002); ethylene response in *Arabidopsis* (De Paepe *et al*., 2004); *in vitro* androgenesis induction in maize (Barret *et al*., 2004) and restoration of fertility in sorghum (Pring *et al*., 2006).

cDNA-AFLP allows genome-wide expression analysis without knowledge of sequence information and identifies both known and unknown genes. Because of the stringent hybridization in amplification reactions, the result of cDNA-AFLP is highly reproducible and nearly free of false positives. However, a significant drawback of this technique is the time-consuming series of restriction digestion and PCR amplifications for generating a comprehensive overview of gene expression pattern.

4.1.2 Suppression subtractive hybridization

SSH (Diatchenko *et al*., 1996) is a PCR-based approach to preferentially enrich low abundance differentially expressed genes. It is based on suppression PCR, in which DNA with long and complementary 3' and 5' ends is selectively suppressed during PCR amplification due to the formation of a "panhandle-like" secondary structure (Siebert *et al*., 1995). Combining normalization and subtraction steps, SSH enables the selective amplification, resulting in over 1000 fold enrichment of differentially expressed genes in a single procedure (Diatchenko *et al*., 1996), without physical separation of single-strand (ss) and double-strand (ds) cDNAs (Duguid and Dinauer, 1990). SSH has been shown to be a useful tool for the identification of differentially regulated genes in plants, e.g. genes expressed during flower development in carnation (Ok *et al*., 2003), genes involved in anther abortion in wheat (Chang *et al*., 2006) and salinity stress regulated genes in *Lolium temulentum* (Baldwin and Dombrowski, 2006). Because of the high sensitivity of the SSH method for gene expression analysis, this method will be applied in our study to identify *L. perenne* SI components and SI response related genes.

4.1.2.1 Molecular basis of SSH

Differentially expressed cDNAs are present in the "tester" cDNA but are absent or present at lower levels in "driver" cDNAs (Figure 4.1.1). The tester and driver ds cDNAs are first digested with *Rsa*I, a four-base-pair cutting restriction enzyme that yields blunt ends. The tester cDNA fragments are then divided into two samples (1 and 2) and each is ligated with a different adapter (adapter 1 and adapter 2). The ends of the adapters do not contain a phosphate group, so that only the longer strand of each adapter can be covalently attached to the 5'-ends of the cDNA.

The SSH technique uses two hybridizations. First, an excess of driver is added to each sample of the tester. The samples are then heat-denatured and allowed to anneal, generating type ‘a’, ‘b’, ‘c’ and ‘d’ molecules in each sample (Figure 4.1.1). The concentrations of the high and low abundance ss cDNA tester fractions are normalized, becoming roughly equal, because the re-annealing for homo-hybrid cDNAs is faster for the more abundant molecules due to the second order kinetics of hybridization (James and Higgins, 1985). Meanwhile, the ss cDNAs in the tester fraction are significantly enriched in cDNAs for differentially expressed genes, as cDNAs that are not differentially expressed form heterohybrids with the driver.

In the second hybridization, the two primary hybridization samples are mixed together. Only the remaining normalized and subtracted ss tester cDNAs are able to re-associate and form fractions ‘b’, ‘c’, and new ‘e’ hybrids. The newly formed e hybrids are ds tester molecules with different adapter sequences at their 5'-ends, corresponding to sample 1 and 2. Fresh denatured driver cDNA is added to further enrich the fraction ‘e’ for differentially expressed genes. An extension reaction is performed to fill in the sticky ends of molecules for primer annealing. The fraction ‘e’ containing the differentially expressed tester sequences has heterologous adapters on both ends.

The entire sample is then subjected to PCR to amplify the differentially expressed genes. In all PCR cycles, exponential amplification can only occur with type ‘e’ molecules with a pair of primers which corresponds to the outer part of the adapters 1 and 2, respectively. Type ‘a’ and ‘d’ molecules do not contain primer binding sites, and type ‘c’ molecules can be amplified only at a linear rate. Type ‘b’ molecules contain long inverted repeats on the ends and form a stable “panhandle-like” structure that prevents their exponential amplification. Only type ‘e’ molecules, enriched with differential expressed genes, have different adapter sequence at their ends which allows them to be exponentially amplified using PCR.

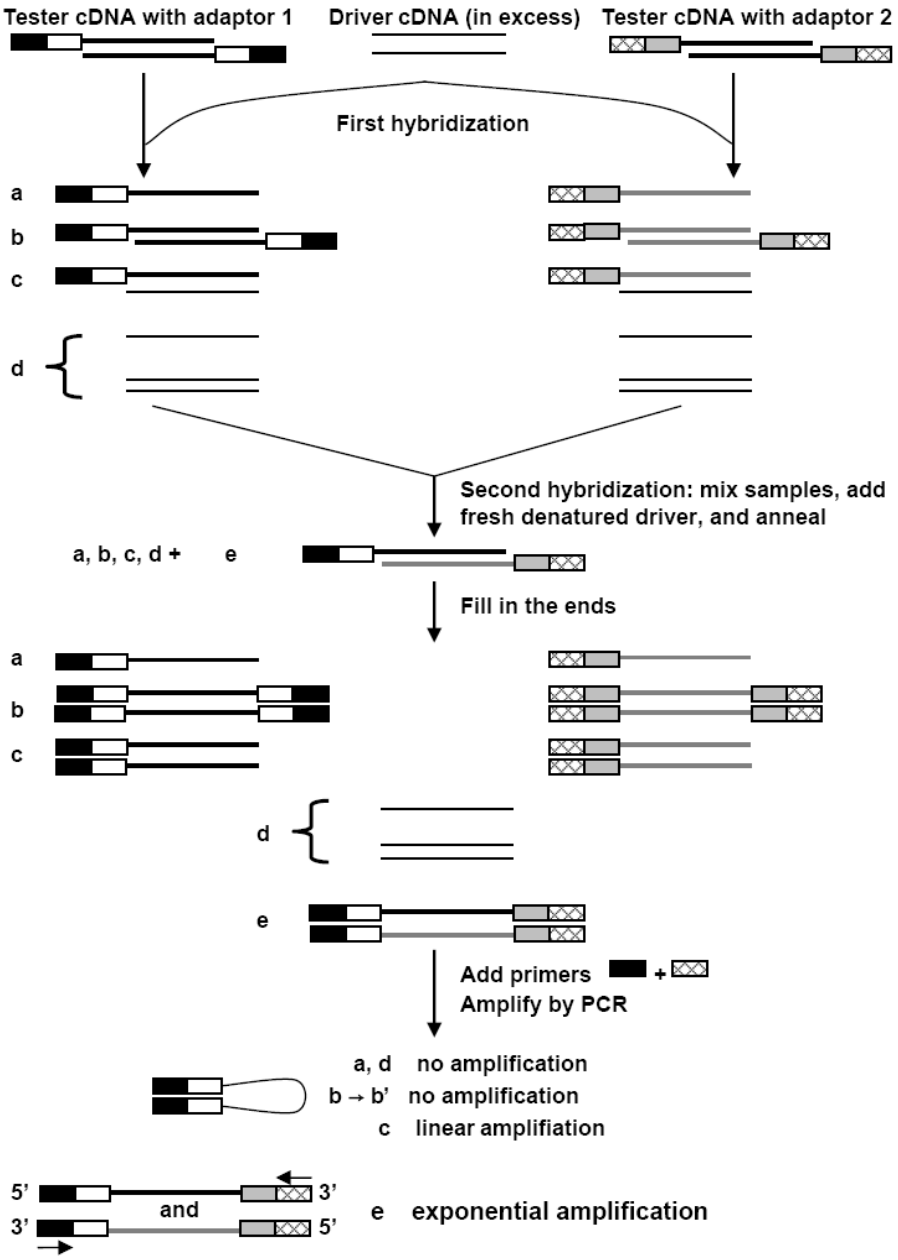


Figure 4.1.1 Outline of the principle of the SSH procedure adopted from Diatchenko et al. (1996). The complete procedure contains two subtractive hybridizations followed by PCR amplification. The first hybridization normalizes the target cDNAs while both hybridizations are designed to enrich target cDNAs. The tester cDNAs are ligated with two different long adapters at their 5' ends. These form a "panhandle-like" structure which suppresses PCR amplification of undesirable cDNAs.

4.1.2.2 Differential screening

SSH greatly enriches differentially expressed genes, however, the subtracted sample will still contain some cDNAs that are common to both the tester and driver samples, generating false positives. This background arises especially when very few differentially expressed genes or less quantitative differences are in the tester and driver. Performing a differential screening therefore can help minimize background and eliminate false positives before carrying out further analysis.

The widely used differential screening approach is to hybridize the subtracted library with ^{32}P-labeled probes synthesized from first-strand tester and driver cDNA. Clones of differentially expressed genes will hybridize only with the tester probe, and not with the driver probe. However, low sensitivity is the disadvantage of this approach as low abundant differentially expressed genes will not be detected (Wang and Brown, 1991).

Here, we used a second differential screening method, bypassing the low sensitivity problem. Forward- and reverse- subtracted cDNAs are made. The forward subtracted cDNA is used for the subtracted library construction. The reverse subtracted cDNA is made with the original tester cDNA as driver and the driver cDNA as tester. The subtracted library is then hybridized with forward- and reverse- subtracted cDNA probes. Clones corresponding to truly differentially expressed genes will hybridize only with the forward-subtracted cDNA probe and clones that hybridize with the reverse-subtracted cDNA probe could be considered as background (Lukyanov *et al*, 1996). Sometime clones hybridize to both forward-subtracted and reverse-subtracted cDNA probes, but with different intensities. When the difference in signal intensity is more than 5- fold, a clone could be a differentially expressed gene. However, when the difference in signal intensity is less than 3- fold, a problem with random

fluctuation of subtraction efficiency might be the explanation. Further analysis of differential gene expression is required to confirm the results of differential screening.

4.1.3 Confirmation of differential screening results

To confirm the results of differential screening and further eliminate false positives, northern blot or reverse transcriptase PCR analysis may be used to analyze differential gene expression. Because northern blot analysis has the drawback of requiring of a large quantity of RNA, in this study the reverse transcriptase PCR method was applied. Candidate clones were sequenced and specific primers were designed. Analysis was then performed with original RNA samples and gene specific primers. Though reverse transcriptase PCR analysis is extremely sensitive, it is merely semi-quantitative, so the differential expression level in samples requires further clarification with quantitative real-time PCR (described in Chapter 5).

4.1.4 Aims

The aim of this experiment was to identify SI related genes in perennial ryegrass by constructing SSH libraries with self-pollinated or cross-pollinated stigmas as tester and unpollinated stigmas as driver. After differential screening, sequence analysis and library comparison, it was hoped that genes involved in pollen-pistil interactions would be identified. Combining this effort with a comparative genetics approach, candidate genes in the predicted *S* and *Z* regions (described in Chapter 3) were the primary SI components or SI response related candidates and were further analyzed.

4.2 Results

4.2.1 Construction of SSH cDNA libraries

4.2.1.1 Development of SI SSH libraries

According to the genotypes of the used ILGI plants, three types of incompatibility outcomes with regard to the *S* and *Z* loci were obtained after *in-vitro* pollination (Table 4.2.1): (1) both *S* and *Z* locus incompatible; (2) single *S* allele incompatible; and (3) single *Z* allele incompatible. Five SSH cDNA libraries were developed (Table 4.2.2). Library 1 was developed from subtracting *in-vitro* self-pollinated mature stigma cDNAs with unpollinated mature stigma cDNAs, hence both *S* and *Z* loci were incompatible. *In-vitro* pollinated mature stigmas (the testers) in libraries 2 and 4 were single *S* allele incompatible, while in libraries 3 and 5 single *Z*-allele incompatible (Table 4.2.1). Both immature and mature unpollinated stigmas were used as drivers in the construction of *S* and *Z* SI SSH libraries. Libraries 2 and 3 were developed using mature stigmas as drivers; libraries 4 and 5 were developed using immature stigmas as drivers (Table 4.2.2). As a result of the incompatibility response induced by *in-vitro* pollinations, the five libraries were enriched with transcripts involved in the SI response in *Lolium perenne* as well as those related with pollen-pistil interactions. However, the rapid incompatibility reaction in *Lolium* (Heslop-Harrison, 1982) implies that the SI components are likely to be present in the mature stigma and pollen before the first pollen-stigma contact. Furthermore, in all SI systems analysed so far, stigmatic SI gene expression is initiated about two days prior to anthesis (Anderson *et al*., 1986; Nasrallah *et al*., 1988). Therefore, candidate SI components should present in the libraries developed by subtracting *in-vitro* pollinated stigmas (SI genes expressed) with unpollinated immature stigmas (no SI genes expression), that is library 4 and 5 (Table 4.2.2). As a result of *in-vitr*o pollination, the five developed SSH

cDNA libraries should include pollen-specific transcripts and pollen responsive genes during pollen-stigma interactions. For the purpose to identify stigmatic SI components and responsive related genes, one additional pollen specific library (library 6) was created (described in section 4.2.2) to eliminate non-stigma specific transcripts generated by pollen through *in-vitro* pollination from the other five stigma-developed libraries.

Table 4.2.1 Outcomes of *in-vitro* pollinations according to the genotypes of ILGI plants used. Type A is a self-pollination: all *S* and *Z* alleles are incompatible; Type B: allele S_1 is incompatible; and Type C: allele Z_1 is incompatible. Incompatible alleles are shown in bold type.

Type A		♂			
	allele	$\boldsymbol{S_1Z_1}$	$\boldsymbol{S_1Z_2}$	$\boldsymbol{S_2Z_1}$	$\boldsymbol{S_2Z_2}$
♀	$\boldsymbol{S_1Z_1}$	×			
	$\boldsymbol{S_1Z_2}$		×		
	$\boldsymbol{S_2Z_1}$			×	
	$\boldsymbol{S_2Z_2}$				×
Type B		♂			
	allele	$\boldsymbol{S_1Z_1}$	$\boldsymbol{S_1Z_3}$	S_2Z_1	S_2Z_3
♀	$\boldsymbol{S_1Z_1}$	×		$S_{12}Z_{11}$	$S_{12}Z_{13}$
	$\boldsymbol{S_1Z_3}$		×	$S_{12}Z_{13}$	$S_{12}Z_{33}$
	S_3Z_1			$S_{23}Z_{11}$	$S_{23}Z_{13}$
	S_3Z_3			$S_{23}Z_{13}$	$S_{23}Z_{33}$
Type C		♂			
	allele	$\boldsymbol{S_1Z_1}$	S_1Z_3	$\boldsymbol{S_3Z_1}$	S_3Z_3
♀	$\boldsymbol{S_1Z_1}$	×	$S_{11}Z_{13}$		$S_{13}Z_{13}$
	S_1Z_2		$S_{11}Z_{23}$		$S_{13}Z_{23}$
	$\boldsymbol{S_3Z_1}$		$S_{13}Z_{13}$	×	$S_{33}Z_{13}$
	S_3Z_2		$S_{13}Z_{23}$		$S_{33}Z_{23}$

Table 4.2.2 Schemes and outcomes of subtracted libraries. Libraries 1 to 5 were developed from stigmas of genotyped ILGI family plants and incompatibility responses were induced after *in-vitro* pollination of stigmas. Library 6 was a pollen specific control library developed from subtraction pollen-specific genes with stigma specific genes.

library	**Driver stigma**	**Tester *in-vitro* pollinated stigma**		
	♀ genotype	**♀ genotype**	**♂ genotype**	**Incompatibility outcome** (Table 4.2.1)
1	mature $S_1S_2Z_1Z_2$	$S_1S_2Z_1Z_2$	$S_1S_2Z_1Z_2$	Incompatible: Type A
2	mature $S_1S_3Z_1Z_3$	$S_1S_3Z_1Z_3$	$S_1S_2Z_1Z_3$	Semi-compatible (S): Type B
3	mature $S_1S_3Z_1Z_2$	$S_1S_3Z_1Z_2$	$S_1S_3Z_1Z_3$	Semi-compatible (Z): Type C
4	immature $S_1S_3Z_1Z_3$	$S_1S_3Z_1Z_3$	$S_1S_2Z_1Z_3$	Semi-compatible (S): Type B
5	immature $S_1S_3Z_1Z_3$	$S_1S_3Z_1Z_3$	$S_1S_3Z_1Z_2$	Semi-compatible (Z): Type C
6	Pollen control library (Driver: unpollinated stigma; Tester: pollen)			

After cloning of the subtracted cDNA libraries, the white colonies were picked up and the insert cDNAs were amplified with PCR (Figure 4.2.1). The products were cDNA clones enriched in the subtracted cDNA libraries and were subjected to subsequent differential screening.

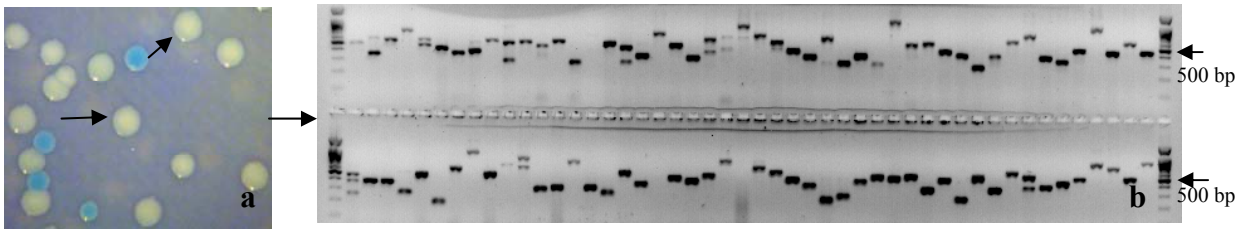


Figure 4.2.1 Cloning and amplification of cDNAs. The white colonies (indicated with arrows in a) generated after cloning of subtracted cDNAs contained inserts and a random numbers of them were picked up for insert analysis.PCR amplified inserts on a 1.5% 0.5X TBE agarose/EtBr gel alongside a 100 bp DNA ladder is shown in b.

4.2.1.2 Differential screening and candidate sequence analysis

A total of 2112 clones were generated from the five SSH cDNA libraries and the differential screening was carried out by hybridizing duplicate clone blots separately to the labelled subtracted unpollinated and *in-vitro* pollinated stigma cDNAs. After detection, 724 clones hybridized more strongly with cDNAs derived from subtracted pollinated stigma rather than with cDNAs derived from subtracted unpollinated stigma cDNAs (Figure. 4.2.2). These

clones were sequenced. The sequences of the clones were generated and subjected to BLAST searches. 218 stigma-specific genes were identified in libraries 1 to 5, based on homology to rice orthologs (Figure 4.2.3).

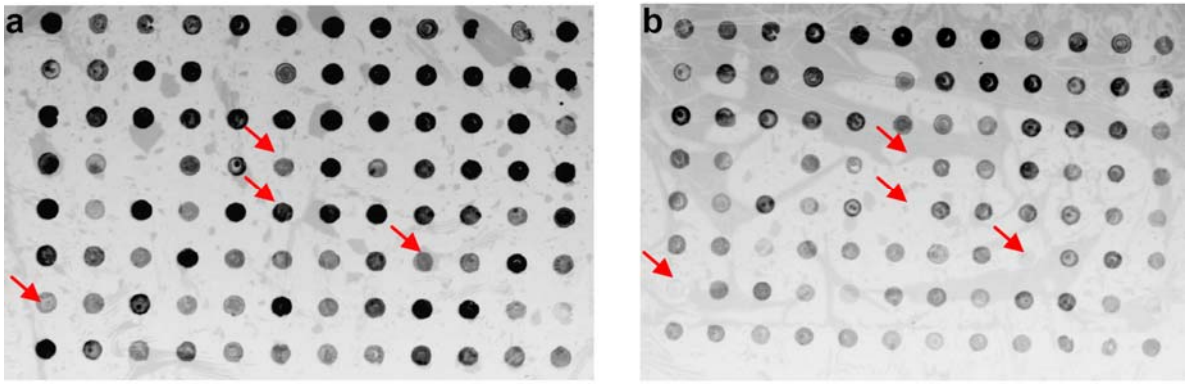


Figure 4.2.2 Chemiluminescence image of duplicate blots representing one of the 96-well plates for the SI SSH cDNA libraries. a Hybridized with subtracted pollinated stigma cDNAs. b Hybridized with subtracted unpollinated stigma cDNAs. The arrows indicate clones hybridize strongly to the pollinated stigma cDNAs but not to the unpollinated stigma cDNAs.

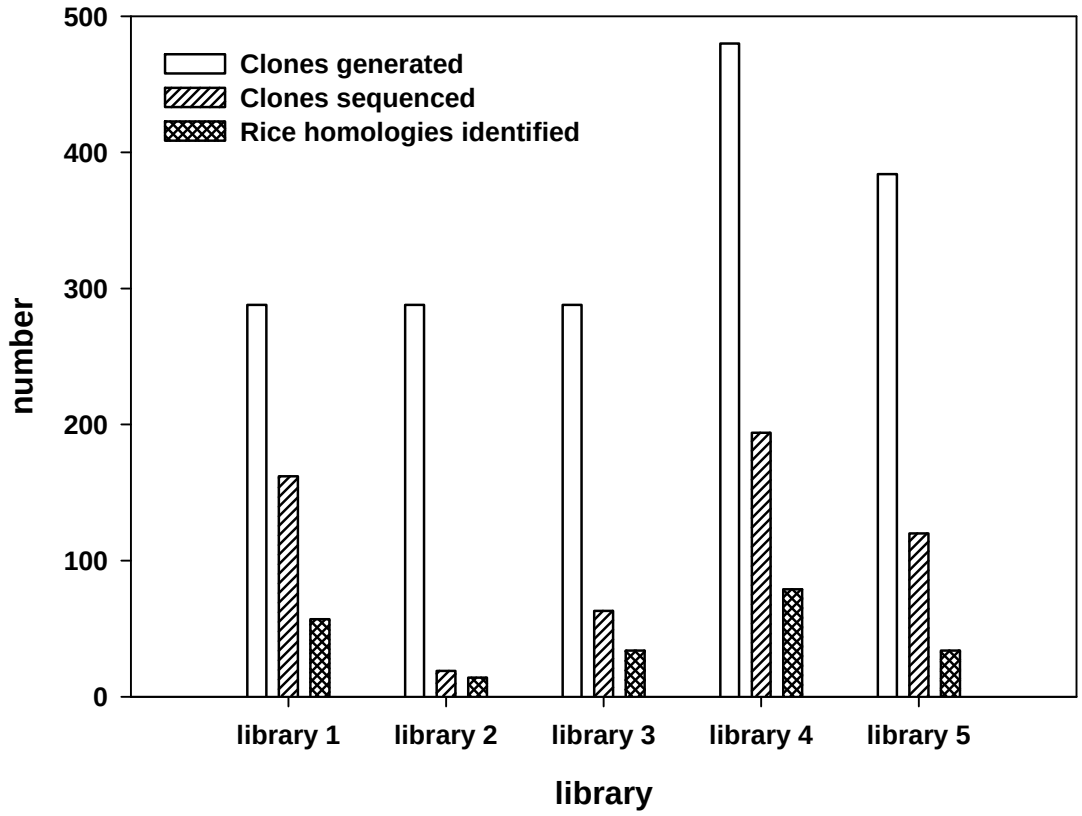


Figure 4.2.3 Results of SI SSH cDNA libraries development. Because of sequence redundancy, the number of rice homologies identified in each library is less than the number of clones sequenced. A total number of 218 individual rice homologies were identified in the five SI SSH cDNA libraries.

The rice sequence homologies indicate that the transcripts identified in the SI SSH cDNA libraries include protein kinases, protein regulation genes, cellular components, signal transduction related proteins, transport proteins and genes with unknown function. They are potential SI components, related genes or other pollen-pistil interactions related gene candidates. According to the Gene Ontology (GO) classification, these transcripts belong to a variety of function groups, such as intracellular (17%), kinase activity (15%), protein modification (11%), membrane (7%), signal transduction (7%), nucleotide binding (6%), transcription (6%), transport (5%), protein binding (4%), electron transport (2%), protein

biosynthesis (2%), response to stress (1%), cytoskeleton (1%), lipid metabolism (1%) and unclassified genes (15%) (Figure 4.2.4).

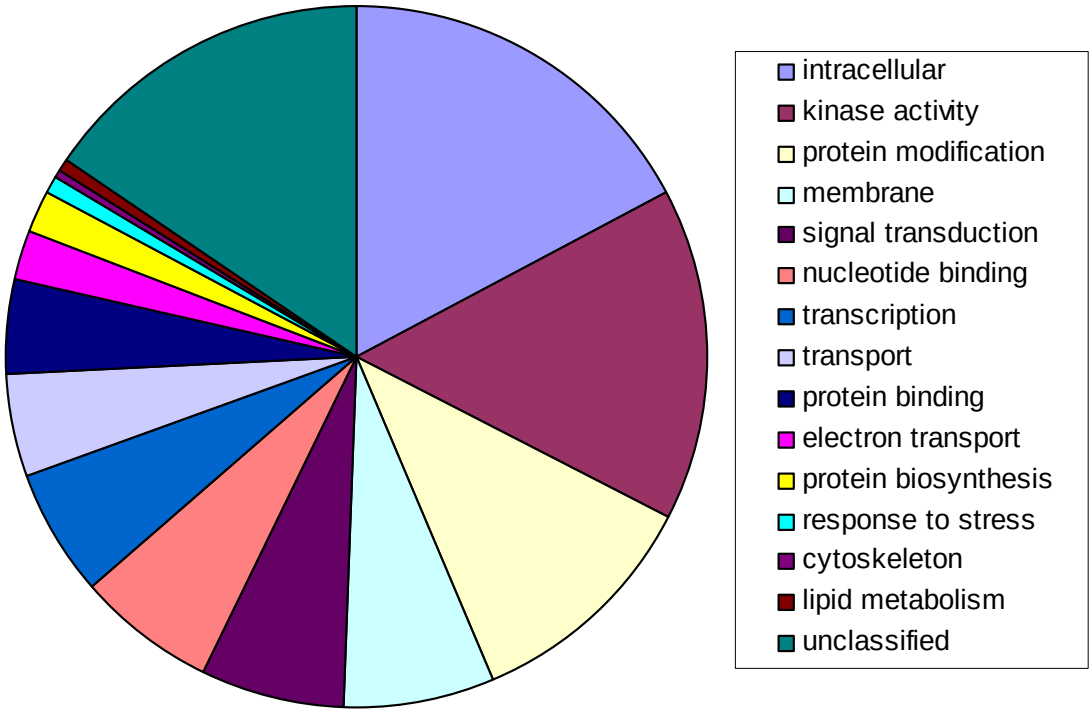


Figure 4.2.4 Groups of transcripts identified in the SI SSH cDNA libraries displayed according to the GO classification for rice homologies.

4.2.2 Pollen specific library

As a result of *in-vitro* pollination, in addition to SI related genes, all the subtracted SI libraries should contain pollen specific genes, which were not present in unpollinated stigma. Therefore, a pollen specific library was developed from pollen cDNA subtracted with mature stigma cDNA. The library was enriched with pollen specific genes and the identified transcripts were used to identify SI components and SI response related candidate genes through library comparison (section 4.2.3.3). A total of 384 clones were generated and the differential screening was carried out by hybridizing duplicate clone blots separately to the labelled subtracted pollen cDNA and unpollinated stigma cDNA probes. After detection, 166

clones hybridized more strongly with cDNAs derived from pollen rather than with cDNAs derived from unpollinated stigma cDNAs. They were sequenced and 69 individual rice homologies were identified through BLAST searches (Appendix C). According to GO classification, the putative pollen specific genes include a wide variety of function groups: intracellular (42%), kinase activity (5%), protein modification (1%), membrane (8%), signal transduction (3%), nucleotide binding (1%), transcription (6%), protein binding (1%), electron transport (5%), response to stress (7%), and unclassified genes (21%) (Fig 4.2.5).

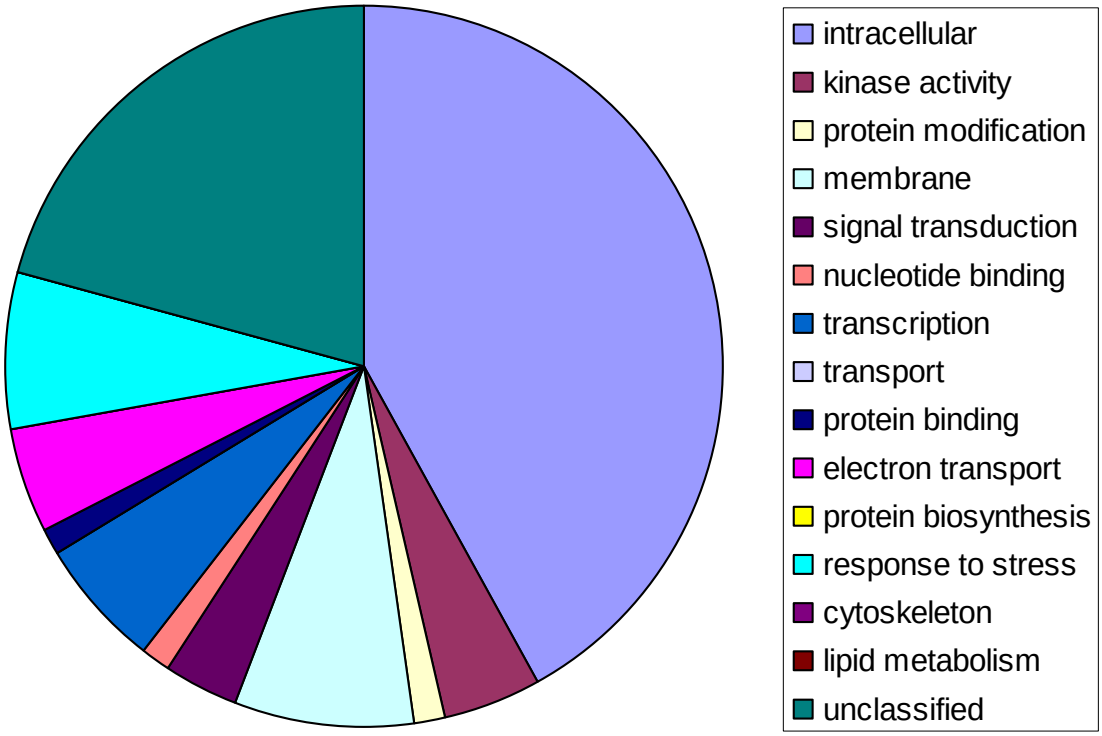


Figure 4.2.5 Groups of transcripts identified in the pollen specific libraries displayed according to the GO classification for rice homologies.

4.2.3 Library comparison for gene function analysis

4.2.3.1 Pollen-pistil interaction related genes

In all the SI SSH libraries developed, *in-vitro* pollinated stigma cDNAs (the tester) were subtracted with unpollinated stigma cDNAs (the driver). As a result of pollination, all the

subtracted cDNA libraries should contain pollen specific genes, which were not present in unpollinated stigma, and pollen-pistil interaction related genes. In library 1, where the self-pollination outcome was fully incompatible, only incompatibility response related pollen-pistil interaction genes were present. In the other four libraries, *in-vitro* pollination induced both incompatible and compatible crosses (Table 4.2.1), therefore additional genes involved in compatible pollination/pollen-pistil interactions were contained. After the comparison of all five SI SSH cDNA libraries, the common genes among them were possible pollen specific and incompatibility response related genes (Table 4.2.3). Pollen specific genes were identified and eliminated by comparison with the pollen control library. However, the pollen control library might not be comprehensive and it is possible that some pollen-specifc genes are still present in the stigmatic libaries after the comparison. The common genes among the libraries 2 to 5 and not expressed in library 1 were genes potentially involved in compatible pollen-pistil interactions (Table 4.2.4). The genes that were only present in library 1 were potential incompatible pollen-pistil response related genes. Altogether, 41 library 1 specific expressed genes were identified (Appendix D) and they fell into different function groups: catalytic activity (21%), nucleic acid binding (12%), intracellular (10%), DNA metabolism (7%), carbohydrate metabolism (7%), translation regulator activity (5%), protein modification (5%), protein metabolism (5%), response to stress (2%), electron transport (2%), transport (2%), kinase activity (2%) and unclassified (20%) (Figure 4.2.6).

Table 4.2.3 Rice homologies of the incompatibility response related genes common in the five SI SSH cDNA libraries. They were listed in the order of the physical appearance on the rice genome: according rice sequence and rice gene annotation information. BLAST searches for rice homology were carried out through the BLASTN search function in the Rice Genome Annotation Project with the Osa1 rice genome coding sequences (CDS) database. No pollen specific genes were identified after comparison with the pollen control library.

Rice Sequence	rice annotation
LOC_Os01g21970	Protein kinase domain containing protein, expressed
LOC_Os01g43910	Protein kinase domain containing protein, expressed
LOC_Os01g57940	Serine/threonine-protein kinase RLCKVII, putative, expressed
LOC_Os01g57974	retrotransposon protein, putative, expressed
LOC_Os04g21340	expressed protein
LOC_Os04g21710	expressed protein
LOC_Os04g32670	exostosin family protein, putative, expressed
LOC_Os05g41950	protein kinase APK1B, chloroplast precursor
LOC_Os05g44940	protein kinase domain containing protein
LOC_Os07g27140	AT hook motif family protein
LOC_Os07g34130	RabGAP/TBC domain-containing protein, putative, expressed
LOC_Os11g07916	nitrogen fixation protein, putative, expressed
LOC_Os11g37690	RabGAP/TBC domain-containing protein, putative, expressed

Table 4.2.4 Rice homologies of the genes potentially involved in compatible pollen-pistil interactions: according rice sequences and rice gene annotation information. BLAST searches for rice homology were carried out through the BLASTN search function in the Rice Genome Annotation Project with the Osa1 rice genome coding sequences (CDS) database. No pollen specific genes were identified after comparison with the pollen control library.

Rice Sequence	rice annotation
LOC_Os01g57420	diacylglycerol kinase variant B, putative, expressed
LOC_Os02g05680	disulfide oxidoreductase/electron carrier/oxidoreductase
LOC_Os02g24430	SEC14 cytosolic factor, putative, expressed
LOC_Os02g49340	nitrate-induced NOI protein, putative, expressed
LOC_Os04g45010	pollen-specific protein SF3
LOC_Os04g54200	diacylglycerol kinase
LOC_Os04g47620	protein kinase APK1B, chloroplast precursor
LOC_Os05g35400	luminal-binding protein 5 precursor
LOC_Os05g38530	heat shock cognate 70kDa protein
LOC_Os05g38770	protein kinase APK1B, chloroplast precursor
LOC_Os05g46720	phosphatidylinositol transfer-like protein III
LOC_Os10g17660	Profilin A, putative, expressed
LOC_Os10g27480	expressed protein

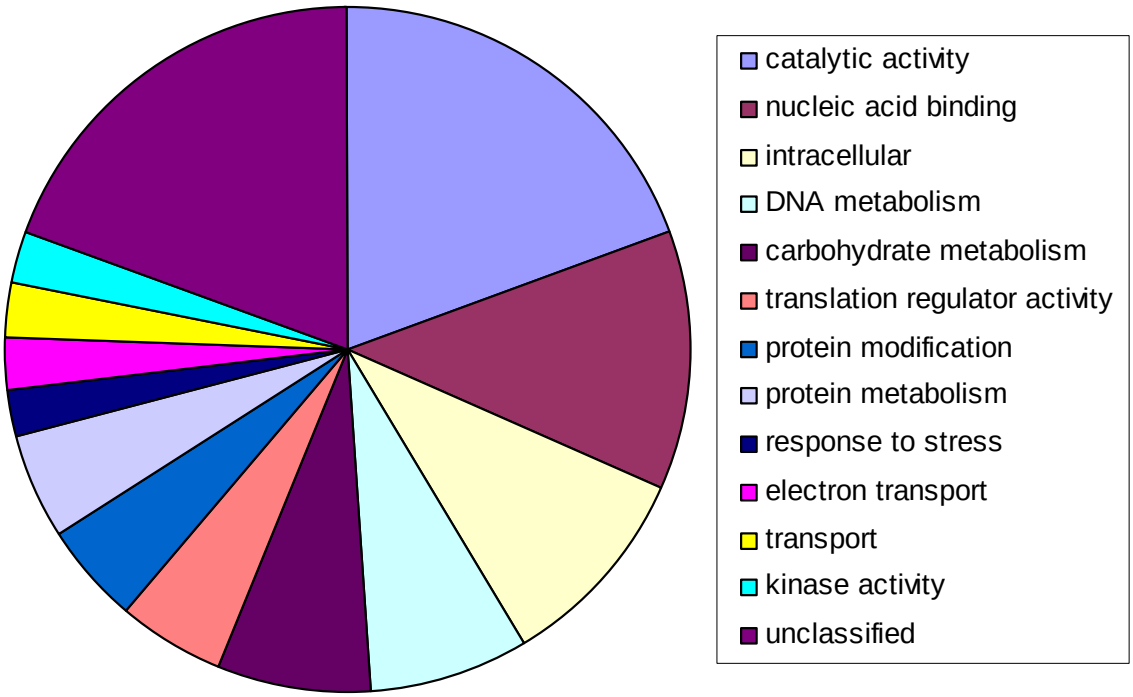


Figure 4.2.6 Groups of library 1 specifically expressed genes displayed according to the GO classification for rice homologies. These genes are potentially involved in incompatibility responses.

4.2.3.2 Stigma development related genes

The SI libraries developed by subtraction with immature stigma cDNAs as driver were anticipated to contain stigma development related genes, absent in other libraries. Therefore, through comparison between libraries 4 and 5 with libraries 1, 2 and 3 (Table 4.2.2), genes specific to libraries 4 and 5 possibly include stigma maturation related genes, as well as those involved in pollen-pistil interactions. Together 82 genes (Appendix E) were identified only in libraries 4 and 5. According to GO classification, they belong to a wide variety of function groups: catalytic activity (23%), protein metabolism (14%), intracellular (7%), membrane (6%), kinase activity (6%), protein binding (5%), transporter activity (5%), extracellular region (5%), protein modification (5%), nucleotide binding (4%), reproduction (2%), signal transduction (2%), transcription (2%), nucleic acid binding (2%) and unclassified (12%) (Figure 4.2.7).

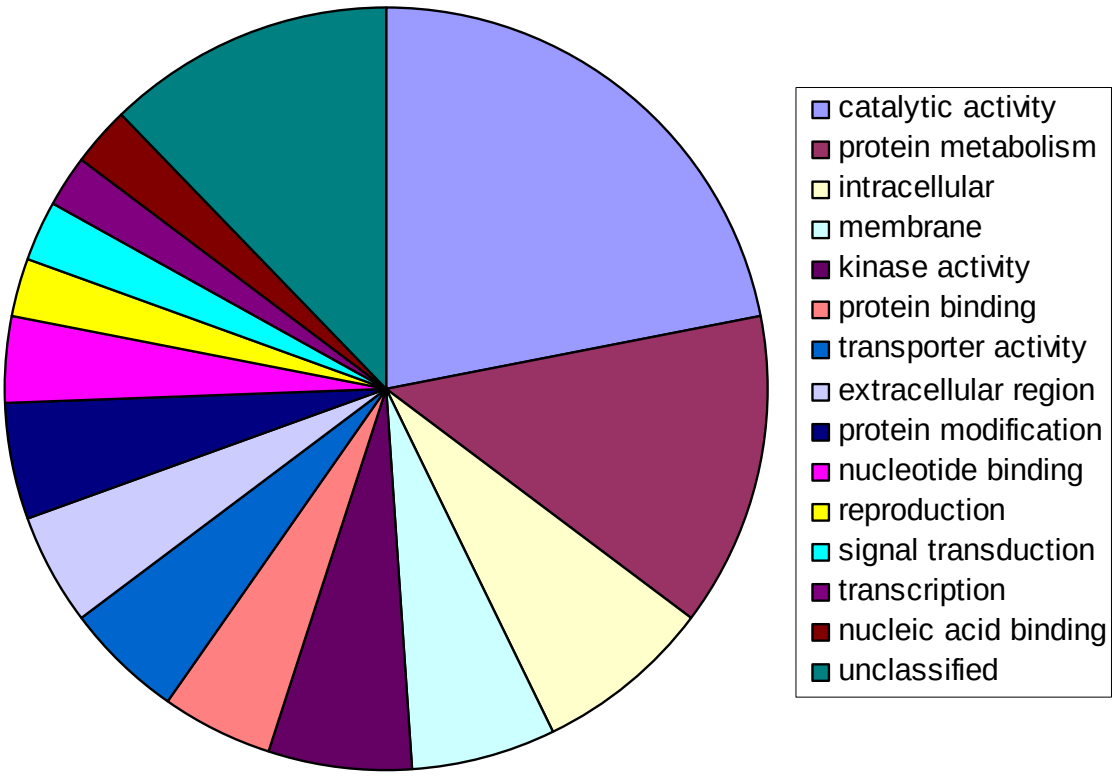


Figure 4.2.7 Groups of library 4 and 5 specific genes displayed according to the GO classification for rice homologies. They are potentially involved in stigma maturation, but also possibly contain some pollination response related genes.

4.2.3.3 Comparative genetics as a screen to select SI component candidates

Previous comparative mapping work (Sim *et al*., 2005; Armstead *et al*., 2002; Jones *et al*., 2002b) and novel rice sequences based STS markers (section 3.2.1) identified genomic regions for *L. perenne S* and *Z* loci on LG1 and LG2, which were syntenic with regions on rice R5 and R4, respectively (Yang *et al*., 2008, Figure 4.2.8). A total of 82 genes isolated in the overall screen were found to correspond to rice genes within the syntenic regions. Based on the knowledge of the physiology of the SI response in grasses and knowledge of other SI systems, it seems likely that the SI genes will be expressed in mature stigma tissue but will be absent at earlier developmental stages. Hence, SI libraries developed by subtracting with

immature stigma material were of particular interest as these might contain SI component candidate genes. To explore this, cDNAs were compared between immature stigma as driver developed libraries (libraries 4 and 5) and mature stigma as driver developed libraries (libraries 1, 2 and 3) (Figure 4.2.9). cDNAs shared among these libraries were possibly involved in a non-SI related stigmatic response upon pollen-stigma contact (as in Table 4.2.4), or alternatively they might be involved in the downstream reactions of the SI response in *L. perenne* (as in Table 4.2.3). Libraries 4 and 5 which were developed using immature stigma cDNA as the driver should potentially include putative SI component genes, SI response genes, stigma development regulation genes and also pollen specific genes. After comparing them with transcripts of the pollen specific library, pollen specific genes found in libraries 4 and 5 were excluded as putative SI component genes (Figure 4.2.9). 22 out of 82 genes from syntenic rice regions remained (Figure 4.2.8) which could be (1) *S* or *Z* gene candidates (2) SI response related genes or (3) stigma development genes, depending on their preliminary expression patterns (Table 4.2.5 and Figure 4.2.9). These transcripts were subjected to more detailed expression analysis.

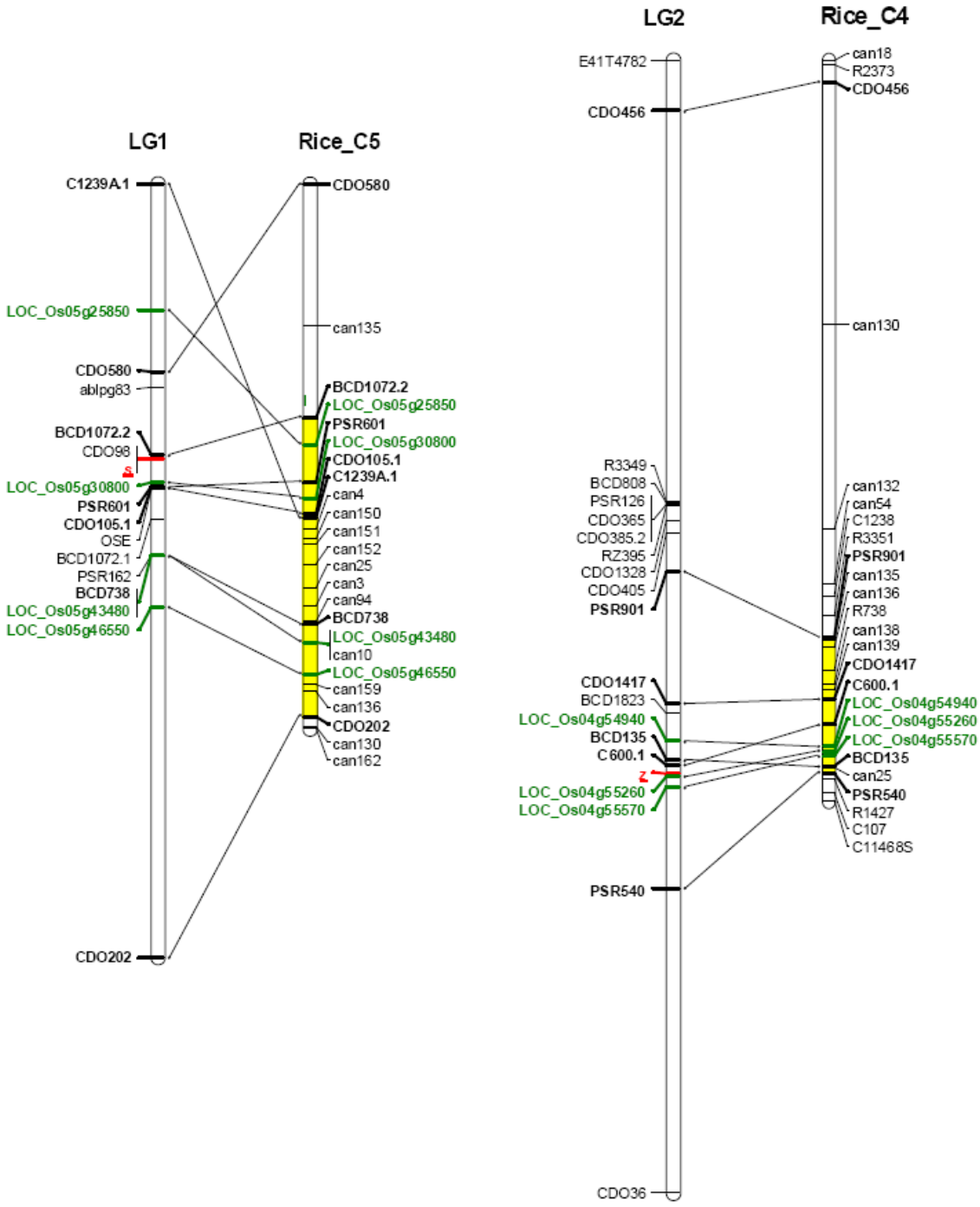


Figure 4.2.8 Comparative maps of *L. perenne* LG1 and LG2 for regions containing *S* and *Z* in relation to rice (*Oryza sativa*) chromosome (C) 5 and C4. Markers on *L. perenne* C1 and 2 were linked to their physical position on rice chromosomes. Positions of rice homologies of the SI candidates and the developed *S* & *Z* flanking markers are shown on rice C4 (*Z*) and C5 (*S*), drawn with the software MapChart V2.2. Novel STS markers (section 3.2.1) are highlighted in green colour. Conserved regions between *L. perenne* and the rice genome are highlighted in yellow colour. 22 candidates (named as canX) in the flanking regions of *S* and *Z* are shown on rice C4 and C5.

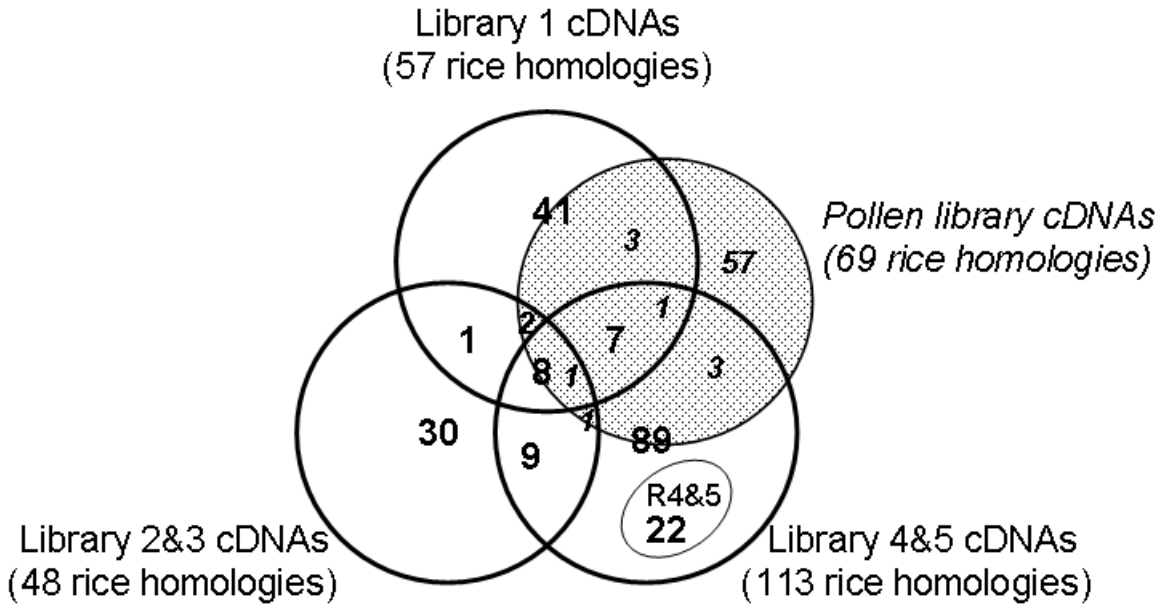


Figure 4.2.9 Distribution of transcripts among the three types of SI cDNA libraries of *S&Z* incompatible outcomes (for library 1), libraries developed from subtracting with mature stigma as driver (for libraries 2&3) and libraries developed from subtracting with immature stigma as driver (for libraries 4&5). Transcripts identified in the pollen control library are indicated in dotted filling for comparison. Numbers in two or more circles in the Venn diagram indicate the overlap of transcripts identified in each library. Numbers in italics indicate pollen-specific transcripts. Within the immature-stigma library-specific transcripts, 22 of them are orthologous to sequences on rice chromosome 4 (R4) and R5.

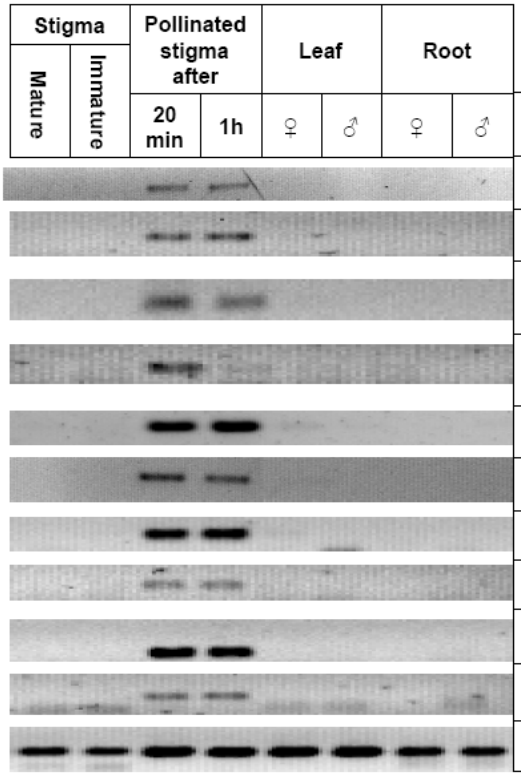
Table 4.2.5 Rice homologies of the 22 candidate genes on rice chromosomes 4 (for *Z*-locus) and 5 (for *S*-locus) in the order of the physical appearance on the rice genome: candidate gene name, according rice sequence, p-value and rice gene annotation information. BLAST searches for rice homologies were carried out through the BLASTN search function in the Rice Genome Annotation Project with the Osa1 rice genome coding sequences (CDS) database.

Name	Rice Sequence	p-value	rice annotation
Can18	LOC_Os04g01150	6.70E-95	phagocytosis and cell motility protein ELM01, putative,expressed
Can130	LOC_Os04g22230	2.60E-05	SCP-like extracellular protein
Can132	LOC_Os04g37780	1.00E+00	Core histone H2A/H2B/H3/H4 family protein
Can54	LOC_Os04g42090	1.40E-53	S-adenosylmethionine decarboxylase proenzyme, putative, expressed
Can135	LOC_Os04g46630	1.60E-11	beta-expansin 2 precursor
Can136	LOC_Os04g47170	1.10E-08	pollen-specific kinase partner protein
Can138	LOC_Os04g49954	4.90E-07	expressed protein
Can139	LOC_Os04g50216	1.50E-80	gtk16 protein
Can25	LOC_Os04g56450	6.80E-38	Protein phosphatase 2C containing protein, Expressed
Can135	LOC_Os05g15690	2.50E-12	beta-expansin 2 precursor
Can4	LOC_Os05g33080	4.10E-29	serine/threonine-protein kinase NAK
Can150	LOC_Os05g33820	1.00E-46	Lipase
Can151	LOC_Os05g34110	5.70E-05	myb-like DNA-binding domain, SHAQKYF class family protein
Can152	LOC_Os05g35860	9.30E-01	retrotransposon protein
Can25	LOC_Os05g38290	3.60E-10	Protein phosphatase 2C containing protein, Expressed
Can3	LOC_Os05g39870	3.60E-17	CBL-interacting serine/threonine-protein kinase 15, putative,expressed
Can94	LOC_Os05g41270	6.80E-51	Calcium-dependent protein kinase, isoform 2, putative,expressed
Can10	LOC_Os05g43540	2.60E-40	expressed protein
Can159	LOC_Os05g47940	1.90E-06	transposon protein
Can136	LOC_Os05g48640	2.10E-36	pollen-specific kinase partner protein
Can130	LOC_Os05g51680	1.20E-06	SCP-like extracellular protein
Can162	LOC_Os05g51790	1.90E-02	ATP binding protein

4.2.4 Expression analysis of SI candidates with reverse transcriptase PCR

In order to verify differential expression of the candidate SI genes during the SI response, primers were designed for the 22 candidate genes in the flanking regions of *S* and *Z* and their RNA accumulation was tested by reverse transcriptase PCR in various tissues: unpollinated

stigma, pollinated stigma at two pollination time points (20 min and 1 hour after *in-vitro* pollination), leaves and roots. Ten out of 22 tested candidate genes showed a tissue specific expression pattern that transcripts were only found in pollinated stigmas (Figure 4.2.10). The expected expressions in unpollinated mature stigmas (as the ten candidates were identified from libraries derived using immature stigma as driver) might have been too weak to be detected on agarose gels. The remaining tested candidates showed no differential expression pattern between tissues (results not shown).



Name	Homology to rice
Can135	beta-expansin 2 precursor
Can4	serine/threonine-protein kinase NAK
Can151	myb-like DNA-binding domain, SHAQKYF family protein
Can3	CBL-interacting serine/threonine-protein kinase 15
Can94	Calcium-dependent protein kinase
Can10	expressed protein
Can136	pollen-specific kinase partner protein
Can130	SCP-like extracellular protein
Can18	ELMO domain-containing protein 2
Can139	gtk16 protein
Control gene: Elongation factor 1α	

Figure 4.2.10 Expression pattern analysis of SI candidate genes by reverse transcriptase PCR. Gene expression of ten candidate genes was detected on agarose gel only in pollinated stigma materials but not in unpollinated stigma, leaf and root tissues. *Elongation factor 1-α* was used as control housekeeping gene.

4.2.5 Gene structure analysis with full length cDNA sequence

Full length cDNA amplification of the ten genes (Figure 4.2.10) was carried out to analyse their gene structure and function domains. Full length sequences of six SI candidates, *Can3*, *Can10*, *Can94*, *Can130*, *Can135* and *Can136*, were obtained (Table 4.2.6) and more convincing homologies with a higher gene identity to other rice genes were found (Table 4.2.7).Their protein domains and function sites were identified using InterProScan tool (http://www.ebi.ac.uk/Tools/InterProScan/) (Table 4.2.8). Both *Can3* and *Can94* have a protein kinase domain and a calcium calmodulin-dependent protein kinase-related domain. *Can3* has a serine/threonine protein kinase domain while *Can94* has a calcium-binding EF-hand domain (Table 4.2.8). These findings imply the involvement of protein kinases and calcium regulation in the SI response in *L. perenne*.

Table 4.2.6 Results of full length cDNA amplifications: candidate gene name, EMBL accession number, rice gene homology and length of the rice cDNA, length of amplified *L. perenne* cDNA, and percentage of identity between candidate gene's rice homology and *L. perenne* full length cDNA.

Name	**Accession No.**	**Rice cDNA homology**		***Lolium perenne* cDNA (bp)**	**Identity (%)**
		Sequence	Length (bp)		
Can3	AM991123	LOC_Os05g39870	1944	1835	56
Can10	AM991120	LOC_Os05g43540	816	922	26
Can94	AM991124	LOC_Os05g41270	1569	1115	27
Can130	AM991119	LOC_Os05g51680	999	826	44
Can135	AM991118	LOC_Os05g15690	795	1088	37
Can136	AM991121	LOC_Os05g48640	1710	1587	59

Table 4.2.7 Comparisons of rice homologies of the SI candidate genes identified with EST fragments (accession number given) and the full length cDNA sequences. BLAST search for rice homology were carried out through the BLASTN search function in the Rice Genome Annotation Project with the Osa1 rice genome coding sequences (CDS) database. The rice gene with the most similarity (lowest P value) to the query sequence was listed and the rice genome annotation information was given.

Candidate	Sequence	Homology to rice	P value	Rice annotation
Can3	EST (AM991123)	LOC_Os05g39870	3.60E-17	CBL-interacting serine/threonine-protein kinase 15, putative, expressed
	Full length cDNA	LOC_Os01g10890	6.80E-232	CBL-interacting serine/threonine-protein kinase 15, putative, expressed
Can10	EST (AM991120)	LOC_Os05g43540	2.60E-40	expressed protein
	Full length cDNA	LOC_Os01g56230	4.40E-67	expressed protein
Can94	EST (AM991124)	LOC_Os05g41270	6.80E-51	calcium-dependent protein kinase, isoform 2,putative,expressed
	Full length cDNA	LOC_Os12g12860	1.60E-133	calcium-dependent protein kinase, isoform AK1, putative, expressed
Can130	EST (AM991119)	LOC_Os05g51680	1.20E-06	pathogenesis-related protein PRB1-3 precursor, expressed
	Full length cDNA	LOC_Os12g43700	6.30E-20	SCP-like extracellular protein, expressed
Can135	EST (AM991118)	LOC_Os05g15690	2.50E-12	beta-expansin 2 precursor, putative, expressed
	Full length cDNA	LOC_Os10g40090	1.40E-17	beta-expansin 1a precursor, putative, expressed
Can136	EST (AM991121)	LOC_Os05g48640	2.10E-36	pollen-specific kinase partner protein, putative, expressed
	Full length cDNA	LOC_Os01g48410	2.40E-166	pollen-specific kinase partner protein, putative, expressed

Table 4.2.8 Function domains identified for the six SI candidates by comparing their full length cDNA sequences with InterPro database for protein function prediction: candidate gene name, InterPro Entry (http://www.ebi.ac.uk/interpro/user_manual.html) and domain description.

Name	InterPro Entry	Function domain
Can3	IPR000719	Protein kinase, core
	IPR002290	Serine/threonine protein kinase
	IPR008271	Serine/threonine protein kinase, active site
	PTHR22982	Calcium/calmodulin-dependent protein kinase-related
Can10	IPR006634	TRAM, LAG1 and CLN8 homology
	IPR010283	Protein of unknown function DUF887, TLC-like
	PTHR13439	CT120 protein
Can94	IPR000719	Protein kinase, core
	IPR002048	Calcium-binding EF-hand
	IPR011992	EF-Hand type
	PTHR22982	Calcium/calmodulin-dependent protein kinase-related
Can130	IPR001283	Allergen V5/Tpx-1 related
	IPR014044	SCP-like extracellular
	PTHR10334:SF7	Pathogenesis-related protein 1
Can135	IPR005795	Major pollen allergen Lol pl
	IPR007117	Pollen allergen/expansin, C-terminal
Can136	IPR005512	Protein of unknown function DUF315, plant

4.3 Discussion

By constructing SSH libraries with self-pollinated or cross-pollinated stigmas (with different *S* or *Z* incompatible outcomes, Table 4.2.2) as testers and unpollinated mature or immature stigmas as drivers, the libraries we developed were enriched with genes involved in the *L. perenne* SI response and other pollen-pistil interactions. We identified 218 transcripts displaying homology to rice genes within a wide variety of function groups (Figure 4.2.4), which might be involved in the incompatible as well as the compatible pollination processes. As the SI response in ryegrass is extremely rapid, all SI determinants should be present in a preformed state before the first pollen-stigma contact. By using unpollinated immature stigmas as drivers to subtract *in-vitro* pollinated stigmas (as in libraries 4 and 5, Table 4.2.2), the developed SSH cDNA libraries were designed to contain SI component genes and related

downstream regulation genes. Through comparisons between libraries with different incompatibility outcomes, the non-SI-related transcripts can be distinguished from those potential SI determinants and SI-related transcripts, such as the common genes among the five stigmatic SI cDNA libraries (Table 4.2.3) and the library 1 specific genes (Figure 4.2.6). 22 potential SI component genes were identified by a comparative genetics approach through scaling down the candidates to the flanking regions of the *S* and *Z* loci (Figure 4.2.8). Ten of the 22 transcripts showed an incompatible pollination related expression pattern in the preliminary expression analysis (Figure 4.2.10). However, the pollen specific library might not be comprehensive for all pollen specific genes and some pollen expressed genes would still be present in the SI cDNA libraries after library comparison. Additional pollen expression analysis of the identified ten SI candidate genes would clarify their tissue specific identity. Furthermore, their expression patterns during a compatible pollination have not been examined. If their expression patterns were the same during the compatible pollination and the incompatible pollination, then the ten candidates would not be SI response specific. Therefore, the ten candidates for possible SI determinants require further investigation. Using another differential expression approach, cDNA-AFLP, Van Daele et al. (2008b) recently reported identification of TDFs involved in the SI response in *L. perenne*. Genome-wide expression profiling of cDNA-AFLP identified TDFs such as actin, a GTP-binding protein and a ubiquitin-like protein were also present in the transcripts identified by the SSH approach (Appendix D and E). Beside these genes, also allele-specific TDFs with serine/threonine protein kinase activity were identified (Van Daele *et al*., 2008b), showing similarity to candidates Can3 and Can4 identified in our SI cDNA libraries using the SSH approach (Figure 4.2.10).

Three different types of incompatibility outcomes were obtained after *in-vitro* pollination (Table 4.2.1) and based on the type of unpollinated stigma used as driver, mature or immature, for developing SSH libraries, three types of SI cDNA libraries were achieved (Figure 4.2.9). Through comparison of these groups of libraries, genes involved in different responses were identified (section 4.2.3).

4.3.1 Pollen-pistil interaction related genes

Genes involved in *L. perenne* pollen-pistil incompatible and compatible interactions were identified from the SSH cDNA libraries (section 4.2.3.1). Protein kinases and RabGAP/TBC domain-containing proteins were identified in all the five stigmatic SI cDNA libraries and they might be involved in the incompatibility response between pollen and pistil (Table 4.2.3). Protein kinases are known to be involved in different signal transduction mechanisms and play crucial roles in a wide variety of cellular functions including proliferation and differentiation (Clark *et al*., 2001). RabGAP/TBC domain-containing protein acts as a GTPase activator in G protein-mediated signalling pathways, which may regulate pollen germination and pollen tube growth (Clark *et al*., 2001). In compatible pollen-pistil interactions, glycerol metabolism related kinases (diacylglycerol kinase and diacyglycerol kinase variant B) and a heat shock protein were identified (Table 4.2.4). Diacylglycerol (DAG) is developed through the hydration of phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase C, during which process inositol 1,4,5-triphosphate (IP3) is developed as well (Dowd *et al.*, 2006). It has been suggested that PIP2 regulates actin deploymerizaing factor (ADF), IP3 regulates internal Ca^{2+} stores and DAG act as a precursor for phosphatidic acid (PA), while ADF, Ca^{2+} and PA are all known involved in regulating pollen tube growth (Cheung and Wu, 2008). Therefore DAG kinases might play a role in the signaling of the

PIP2 hydration process, which in turn regulates pollen growth. Heat shock proteins are expressed under stress conditions, but some are also expressed under normal growth conditions and act as molecular chaperones to assist the folding, assembly and transport of other proteins (Parsell and Lindquist, 1993). Possible cross talk between pollination and stress/defence response is proposed (Li *et al*., 2007a), implying the possible involvement of heat shock proteins in compatible pollen-pistil interactions.

Gene groups involved in incompatibility responses in *L. perenne* were identified in the self-incompatible cDNA library 1 (Figure 4.2.6). Gene groups of SI cDNA libraries 4 and 5 specifically expressed transcripts include pollen-pistil interaction related genes and downstream pollination process related genes (Figure 4.2.7). One function group of interest is protein modification, which consists of ubiquitination pathway related cDNAs, such as ubiquitin-conjugating enzymes and F-box domain containing proteins (Appendix E). Protein degradation mediated by the ubiquitination process has been proposed in S-RNase SI response in Solanaceae (Entani *et al*., 2003). The role of ubiquitination has also been suggested in SI response in *Brassica* for the U-box motif identified in ARC1, a component of the signalling cascade in the SSI system (Azevedo *et al*., 2001). Through the effort of mapping the *Z* locus in *Secale cereale* L., Hackauf and Wehling (2005) identified a putative UBP gene shown pistil specific expression pattern and cosegregation with the *Z* locus (Hackauf and Wehling, 2005). Whether this UBP gene is a component of the *Z* locus or a linked gene with suppressed recombination around the *Z* locus is still undetermined. However, considering the role of ubiquitination in other SI systems and the lack of specificity associated domain, it is suggested that this UBP gene is more likely to be involved in the downstream SI reactions (Yang *et al*., 2008). The identification of ubiquitination related genes from our SI

SSH libraries confirmed the possible involvement of protein ubiquitination in the *Lolium* incompatibility response.

4.3.2 Stigma development related genes

Different function groups identified in immature stigma as driver developed SSH cDNA libraries 4 and 5 include genes potentially involved in stigma development (Figure 4.2.7). Genes with catalytic activity and protein metabolism function are the two most enriched groups. Intracellular proteins, membrane proteins and kinases also display important function groups (Figure 4.2.7). Several classes of genes have been shown to be specifically expressed in stigma (Gasser and Robinson-Beers, 1993). Recently, Li et al. (2007a) identified stigma specific or predominant genes in rice by using a genome-wide gene expression profiling approach. The function category analysis reveals that groups of stigma genes include cell wall-related, cell-cell communication, signal transduction, auxin-responsive components, transcription functions and stress/defence response related genes (Li *et al*., 2007a). One class of genes specifically expressed in stigma are plant receptor-like kinases involved in self-incompatibility responses, such as the *S*-locus receptor protein kinase (*SRK*) in *Brassica* (Nasrallah, 2002). In our developed SI SSH libraries, several protein kinases were also identified (Appendix E), e.g., a calcium-dependent protein kinase and a serine/threonine-protein kinase, implying their possible roles in *Lolium* incompatibility response. Other stigmatic genes identified so far include pectinases, esterases, lipid metabolism related, glycerol metabolism related, pectin metabolism related, extension-like protein and chitinase (reviewed in Gasser and Robinson-Beers, 1993; Swanson *et al*., 2005; Li *et al*., 2007a). Apart from esterases and chintinase, the other genes were identified in our cDNA libraries as

involved in stigma development (Appendix E), providing evidences of those stigmatic genes in grass species.

4.3.3 Involvement of protein kinases in Lolium SI response

Candidates with protein kinase functions are present in all five SI SSH cDNA libraries, implying their roles in pollen-pistil interactions. Protein kinase candidates were also identified as putative SI components in *Lolium* (Table 4.2.5*)* and protein kinase domains were identified in full length cDNA sequences (Table 4.2.6). The tissue specific expression patterns of protein kinases genes *Can3*, *Can4* and *Can94* (Figure 4.2.11), expressed only in pollinated incompatible stigmas, suggest the role of protein kinases in the *Lolium* SI response. The classification of plant protein kinases have been reviewed by Hardie (1999), including the calcium-dependent protein kinase (CDPK) subfamily, the SNF1-related protein kinase (SNRK) family, the receptor-like kinase subfamily, the MAP kinase (MAPK), MAP kinase kinase (MAPKK), and MAP kinase kinase kinase (MAPKKK) subfamilies, the cyclin-dependent kinase (CDK) subfamily, the casein kinase 1 (CK1) and casein kinase II (CK2) subfamilies, the GSK3/SHAGGY subfamily and other subfamiles and miscellaneous kinases. Their functions have also been discussed (Hardie, 1999), stipulating the role of different protein kinase subfamilies in a wide variety of processes in plant growth and development, including pollen tube growth and self-incompatibility responses. Studies in pollen-pistil interactions and signalling in pollination have shown supporting evidence for the involvement of protein kinases in self-incompatibility responses (reviewed in Franklin-Tong, 1999; Wheeler *et al*., 2001; Swanson *et al*., 2004). In SSI species *Brassica*, the female determinants of SI have been identified to be the SRK, a single-pass transmembrane serine/threonine kinase (Nasrallah, 2002). The hyper-variability regions within its receptor domain were predicted to

be responsible for the *S*-specificity and SI response is activated when the *S*-receptor domain of SRK is encoded by the same *S* haplotype as the pollen ligand *SCR* (Kachroo *et al*., 2001; Takayama *et al*., 2001; Hiscock & Tabah, 2003). In the gametophytically controlled *Papaver* SI system, involvement of a MAPK p56 has been reported (Rudd *et al*., 2003), probably leading to PCD. In the grass species rye (*S. cereale* L.), the involvement of protein kinases was proposed based on evidence that kinase inhibitors inhibit the SI response (Wehling *et al*., 1994), though the component has not been identified. Our SSH approach also identified possible involvement of a kinase partner protein (Can136) in the *Lolium* SI response (Table 4.2.5). A kinase partner protein has been reported to play a role in polarized pollen tube growth in *Arabidopsis thaliana* through the interaction with membrane proteins in pollen (Kaothien *et al*., 2005). Depolarized pollen tube growth and irregular actin arrangement in the tip region of pollen were observed through an over-expressing kinase partner protein. The finding that *Can136* was only expressed in pollinated stigmas (Figure 4.2.10), where the incompatibility response takes place, implicates the possible involvement of this protein kinase partner in the *Lolium* incompatibility response. It should be noticed here that though the above mentioned kinase candidates are expressed in incompatibly pollinated stigmas, their expression patterns in pollen material and compatible pollination are uncharacterised. Therefore further experiments are needed to validate their specificity in the *Lolium* SI response through exluding the possible pollen-specific and compatibility-related features.

4.3.4 Involvement of Ca^{2+} in SI responses

Calcium-dependent protein kinases and calcium related protein domains were identified in SI component candidates (Table 4.2.6). The role of Ca^{2+} in regulation of pollen tube growth has been well characterised (Taylor and Hepler, 1997). It has been shown that Ca^{2+} acts as a

second messenger in the self-incompatibility response in *Papaver* (Rudd & Franklin-Tong, 2003) and inhibition of the incompatible pollen is mediated by the activation of a Ca^{2+}-dependent signalling cascade. Franklin-Tong et al (1993, 1995, 1997) revealed that a rapid increase of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) is induced by the SI response in incompatible pollen, leading to the loss of high apical $[Ca^{2+}]_i$ which is a key characteristic of growing pollen tubes. Ca^{2+} signalling related actin reorganization and likely depolymerization has also been found in Papaver (Geitmann *et al.*, 2000; Snowman *et al.*, 2002) and recently reported in the papilla cells of *Brassica rapa* after self-pollination (Iwano *et al*., 2007). Wehling et al. (1994) reported a possible role of Ca^{2+} in the SI response in rye where the SI response in *in-vitro* self-pollinations was inhibited by application of Ca^{2+} antagonists to isolated stigma. In a similar preliminary experiment carried out in *L. perenne* (data not shown), the Ca^{2+} antagonists verapamil and lanthanum (La^{3+}) chloride were shown to inhibit or delay the SI response, supporting the role of Ca^{2+} regulation during the incompatibility response.

This study described the cloning and sequencing of transcripts from SI cDNA libraries that were generated using the SSH technique. The developed stigmatic libraries have led to the identification of transcripts putatively involved in pollen-stigma interactions (compatible and incompatible), stigma development and those that may be pollen-specific. Through comparing with the transcripts identified in the pollen-specifc library, the non-stigma specific transcripts were eliminated from subsequent analysis. According to the various incompatibility outcomes among the stigmatic SI libraries, SI-related transcripts were differentiated from non-SI transcripts via library comparisons. Since this study was concentrated on the *Lolium* SI response, those transcripts potentially involved in SI were further investigated. Using a comparative genetics approach, transcripts in the regions of potential SI determinants were

selected. To eliminate false positives, subsequent preliminary expression pattern analysis was carried out to evaluate the differential expression by reverse transcriptase PCR. At the end, ten candidates were identified with an incompatible pollination related expression pattern. In order to determine at what time point during the SI response the differential expression occurred, the ten SI candidates will be subjected to quantitative expression analysis by real-time PCR (Chapter 5). One problem within this study is that the incompleteness of the pollen-specific library might cause the presence of some leaked pollen-transcripts in the candidates. If that was the case, then the induced stigmatic expression of the candidates upon incompatible pollination would be due to the pollen expression introduced by the i*n-vitro* pollination. Therefore, a pollen control reaction should be tested in the RT-PCR expression analysis. Additionally, a compatible pollination control reaction should also be included to confirm the non-SI expression of the identified candidates.

CHAPTER 5

EXPRESSION PATTERN ANALYSIS OF THE IDENTIFIED SI CANDIDATE GENES WITH REAL-TIME PCR

5.1 Introduction

The differential expression technique SSH was used in Chapter 4 to identify transcripts differentially expressed in *Lolium perenne* L. stigmas after *in-vitro* pollination. Using comparative genetics, 22 transcripts in the regions of the *S* and *Z* loci were identified as putative SI components and ten of them showed a tissue specific differential expression pattern after preliminary analysis. However, the results obtained from reverse transcriptase PCR were only semi-quantitative, therefore a more sensitive quantification method is necessary for the clarification and comparison of the candidate gene expression pattern during the *L. perenne* SI response.

Since its introduction more than 10 years ago (Heid *et al*., 1996), real-time PCR has become the standard method for gene quantification. Its large dynamic range, high sensitivity, specificity, accuracy and reproducibility have resulted in a rapidly expanding number of applications, such as molecular diagnostics, quantification of specific transcripts, and detection and quantification of foreign DNA (reviewed in Klein, 2002; Gachon *et al*., 2004). Gene expression from very small amounts of RNA can be analysed and a large number of samples and/or many different genes can be investigated in the same experiment (Willard *et al*., 1999). It is therefore more flexible and rapid than traditional gene quantification methods like northern blotting and in situ hybridization. The principles of real-time PCR include the detection and quantification of a fluorescent reporter whose accumulation is proportional to accumulation of PCR products and the first significant increase in the amount of PCR product (Ct – threshold cycle) correlates to the initial quantity of the target template. As a result of the detection of "amplification-associated fluorescence" at each PCR cycle, real-time PCR data can be obtained just after cycling, and no post PCR processing is required.

5.1.1 Real-time PCR vs traditional PCR

A PCR can be broken up into three stages: (1) Exponential, when exact doubling of product is accumulating at every cycle (assuming 100% reaction efficiency); (2) Linear, when the reaction components are being consumed, the reaction is slowing, and products are starting to degrade; (3) Plateau, which is the end point of a PCR when the reaction has stopped, no more products are being accumulated and if left long enough, the PCR products will begin to degrade. Real-time PCR permits the detection of PCR amplification during the exponential phase of the PCR, which is in contrast to the end point detection of PCR amplification in traditional PCR.

5.1.2 Reverse Transcription

The initial step of real-time quantitative PCR is reverse transcription (RT) of RNA to a single strand cDNA. There are two methods of reverse transcription: (1) one-step RT, where RT and real-time PCR are carried out in the same reaction; and (2) two-step RT, where cDNA is synthesized first and then subjected to real-time PCR in another reaction. One-step RT-PCR has the advantage of detection of rare transcripts but requires sequence-specific primers for cDNA synthesis. Two-step RT-PCR can use sequence-specific primers, random primers or oligo-dT for cDNA synthesis and has the advantage of eliminating primer-dimers and applying the same sample for multiple analyses (Vandesompele *et al*., 2002a). Sequence-specific primers specifically reverse transcribe complementary RNA sequences and are the only suitable primer type for one-step RT-PCR. They can reverse transcribe the most specific cDNA and provide the greatest sensitivity (Lekanne Deprez *et al*., 2002). Oligo-dT primers can reverse transcribe only eukaryotic mRNAs and retroviruses with poly-A tails, but not ribosomal RNA (rRNA). They may have also difficulties transcribing long mRNA transcripts

or transcripts containing hairpin loops and have the tendency towards 3' end transcription. Random primers (e.g. hexamers) can reverse transcribe all mRNAs as well as rRNA at the same time, therefore they are less specific than oligo-dT primers. There is no biased transcription towards 3' ends and they are preferred for transcribing long transcripts or transcripts containing hairpin loops. However, problems may occur when the target mRNA is present at low levels, leading to disproportionate priming and subsequent inaccurate quantification (Zhang and Byrne, 1999). Different reverse transcriptase methods can generate considerable differences in specificity. Therefore, it is important to apply the same priming and reaction conditions to produce comparable results for quantification.

5.1.3 Detection of amplified products

At present, three main methods of fluorescence detection are available and their principles have been described by van der Velden et al. (2003).

(1) SYBR Green: SYBR Green I fluorescence is enormously increased upon binding to double-stranded DNA (Morrison *et al*., 1998). During the extension phase, more and more SYBR Green I will bind to the PCR product, resulting in an increased fluorescence. Consequently, during each subsequent PCR cycle more fluorescence signal will be detected.

(2) Hydrolysis probe: The hydrolysis probe is conjugated with a quencher fluorochrome, which absorbs the fluorescence of the reporter fluorochrome as long as the probe is intact. However, upon amplification of the target sequence, the hydrolysis probe is displaced and subsequently hydrolyzed by the Taq polymerase. This results in the separation of the reporter and quencher fluorochrome and consequently the fluorescence of the reporter fluorochrome becomes detectable. During each consecutive PCR cycle this fluorescence will further increase because of the progressive and exponential accumulation of free reporter

fluorochromes. Hydrolysis probes include different systems like TaqMan (Holland *et al*., 1991), Molecular Beacons (Tyagi and Kramer, 1996) and Scorpion probes (Thelwell *et al*., 2000).

(3) Hybridization probes: In this technique one probe is labelled with a donor fluorochrome at the 3' end and a second adjacent probe is labelled with an acceptor fluorochrome. When the two fluorochromes are in close vicinity (1–5 nucleotides apart), the emitted light of the donor fluorochrome will excite the acceptor fluorochrome, so called fluorescence resonance energy transfer (FRET) (Ha *et al*., 1996). This results in the emission of fluorescence, which subsequently can be detected during the annealing phase and first part of the extension phase of the PCR reaction. After each subsequent PCR cycle more hybridization probes can anneal, resulting in higher fluorescence signals.

In this study, the SYBR Green fluorescent detection system was applied due to its lower cost compared to the other methods. Another advantage is that the reaction conditions can be identical to those optimised with regular PCR (Bustin and Nolan, 2004). The disadvantage of SYBR Green is its ability to bind to any double stranded DNAs, including primer-dimers and non specific amplicons. This can be overcome by running a dissociation curve which detects the melting temperature of the desired amplicon (Ririe *et al*., 1997). In the dissociation curve, a drop in fluorescent signal is related to the dissociation of the amplicon, as a result of the release of the SYBR Green dye from the double stranded DNA. Detection of more than one drop in fluorescent signal indicates the presence of more than one amplicon. In this case, PCR conditions should be adjusted to optimise single amplicon amplification.

5.1.4 Normalisation with reference genes

Normalisation is important for real-time PCR quantification analysis to control several problematic variables such as the amount of original sample, transcriptional activity and PCR efficiencies (Bustin and Nolan, 2004). Strategies for normalising these variations have been reviewed and compared by Huggett et al. (2005), including matching sample size, extraction of good quality RNA, use of a similar quantity of RNA and internal controls with reference genes or artificial molecules. To date, reference gene control is the most popular normalization strategy. It is easy to perform and can control every step of the real-time PCR. Reference genes, also called housekeeping genes, should express at the same level in all study samples. Many studies have used a particular reference gene for normalisation without a validation process. However, some reports later demonstrated that the expression of housekeeping genes can vary considerably in different samples (Bas *et al*., 2004; Brunner *et al*., 2004; Dheda *et al*., 2004; Suzuki *et al*., 2000; Tricarico *et al*., 2002) and wrong/false reference gene selection could lead to altered results. It is therefore crucial to validate reference genes for normalization. The software geNorm, developed by Vandesompele et al. (2002b), is such a program to identify the most appropriate reference genes according to the similarity of their expression profile. The internal reference gene-stability measure M is defined as the average pairwise variation of a particular gene with all other reference genes. The minimum number of reference genes required can be verified through stepwise exclusion of the less stable expressed gene with the highest M value. The geometric mean of the expression of validated reference genes has been demonstrated to be a reliable normalization factor. The program has been applied in many studies for selection of the most appropriate reference genes (Czechowski *et al*., 2005; Zhang *et al*., 2005; Reid *et al*., 2006; Wei *et al*.,

2007; Rieu *et al*., 2008; Sanchez *et al*., 2008). Detailed principles are published in Vandesompele et al. (2002b).

5.1.5 Quantification methods

Real-time PCR results are quantified absolutely and relatively. Absolute quantification determines the exact copy number of the target using an internal or external calibration curve generated by using dilutions of a plasmid containing the specific target gene. This method requires the least amount of validation because the PCR efficiencies of the target genes and reference genes do not have to be equivalent and the sample quantitative values are interpolated from the standard curve. An obvious consideration of this method is the requirement and cost involved to generate standard curves, especially when testing a large number of samples and target genes. Relative quantification compares the change in expression level of a target gene versus a reference gene and the expression level of the same gene in target sample versus reference sample (Pfaffl, 2001). It is more widely applied and more practical as it compares target samples against controls directly. There are two relative quantification models: the efficiency-calibrated model (Pfaffl, 2001) and comparative Ct ($\Delta\Delta$Ct) model (Livak and Schmittgen, 2001). The mathematical model presented by Pfaffl (2001) takes the calculated PCR efficiencies into consideration. PCR efficiencies were calculated by carrying out a dilution series of template cDNA and performing real time PCR. The resulting Ct values are plotted against template concentration and the slope of the line is calculated and used to determine the amplification efficiency (E) with equation $E = 10^{(-1/slope)}$ (Pfaffl, 2001). ΔCt for each gene is calculated by subtracting the Ct number of the target sample from that of the control sample ($Ct_{target} - Ct_{control}$). The ratio of target gene expression is derived from the ratio between E_{target} to the power of ΔCt_{target} and $E_{reference}$ to the power of

$\Delta Ct_{reference}$ (Equation 1). The ΔΔCt method is derived to eliminate the use of standard curves as long as the E_{target} and the $E_{referenc}$ are relatively equivalent (equals 2 to indicate the doubling of product at each cycle), then the result for relative quantification is achieved from $2^{-\Delta\Delta Ct}$ (Equation 2) (Pfaffl, 2001; Livak and Schmittgen, 2001). It is very useful when a high number of targets and/or a large number of samples are tested, e.g. in microarray analysis.

$$Ratio = \frac{\left(E_{t\arg et}\right)^{\Delta Ct_{t\arg et}}}{\left(E_{reference}\right)^{\Delta Ct_{reference}}} \qquad \text{Equation 1}$$

$$Ratio = 2^{-\Delta\Delta Ct} \quad (\Delta\Delta Ct = \Delta Ct_{reference} - \Delta Ct_{t\arg et}) \qquad \text{Equation 2}$$

5.1.6 Real-time RT-PCR data analysis

Most real-time PCR systems allow extraction of Ct values directly through fluorescence measurement and some instruments have built in software programmes for the interpretation of unknown quantities using a standard curve of consecutively diluted known quantities. However, these programmes are inadequate for analysing raw data as meaningful results in normalized and calibrated relative quantities. In addition, limitations such as data processing, sampling numbers and multiple run calibration have also been reported in currently available programmes (reviewed in Hellemans *et al*., 2007). A recently developed tool, qBase (Hellemans and Vandesompele, Ghent University Hospital, Belgium), allows the straightforward and automated analysis of the raw real-time PCR data for relative quantification. It has already been applied successfully in gene expression analysis in *Arabidopsis* (Falcone *et al*., 2007; Remans *et al*., 2008). qBase is based on a modified ΔΔCt method to take multiple reference genes and gene specific amplification efficiencies into account, as well as the errors on all measured parameters along the entire calculation

procedure. An inter-run-calibration algorithm is also included to adjust run-to-run differences. Normalised relative quantities are calculated with the formula $\frac{E_{goi}^{\Delta Ct,goi}}{\sqrt[f]{\prod_{0}^{f} E_{ref_0}^{\Delta Ct,ref_0}}}$. The calculation steps are detailed in Hellemans et al. (2007).

5.1.7 Aims

The aim of this experiment is to verify temporal expression patterns of the *L. perenne* ten potential incompatible response related candidates identified from SI cDNA libraries (Chapter 4). To determine at which time point differential expression occurred, real-time PCR analysis is performed during an incompatible pollination time course series of 0 min, 2 min, 5 min, 10 min, 20 min and 30 min after *in vitro* self-pollination.

5.2 Results

5.2.1 Optimization of the annealing temperature for each primer pair

The optimal annealing temperature for each primer pair of the genes from Table 2.3.2 was determined as described in section 2.3.3. Their values were listed in Table 5.2.1 and were applied in the real-time PCR assay. The SYBR green dye detection system was used in this real-time PCR approach. SYBR green binds to all double stranded DNAs and as a consequence any unspecific amplified products, including primer-dimer, can contribute to the overall fluorescent signal. Therefore, the amplification of a single specific PCR product with an optimized annealing temperature is critical for the success of a quantification analysis using SYBR green dye.

Table 5.2.1 Optimal annealing temperature for each primer pair of the genes in real-time PCR analysis.

Gene	Annealing temperature (°C)
Actin	64
Elf1-α	63.5
GAPDH	66
Tubulin	58
Can3	59
Can4	64
Can10	62
Can18	57
Can94	62
Can130	60
Can135	60
Can136	62
Can139	60
Can151	60

5.2.2 Determination of PCR amplification efficiency

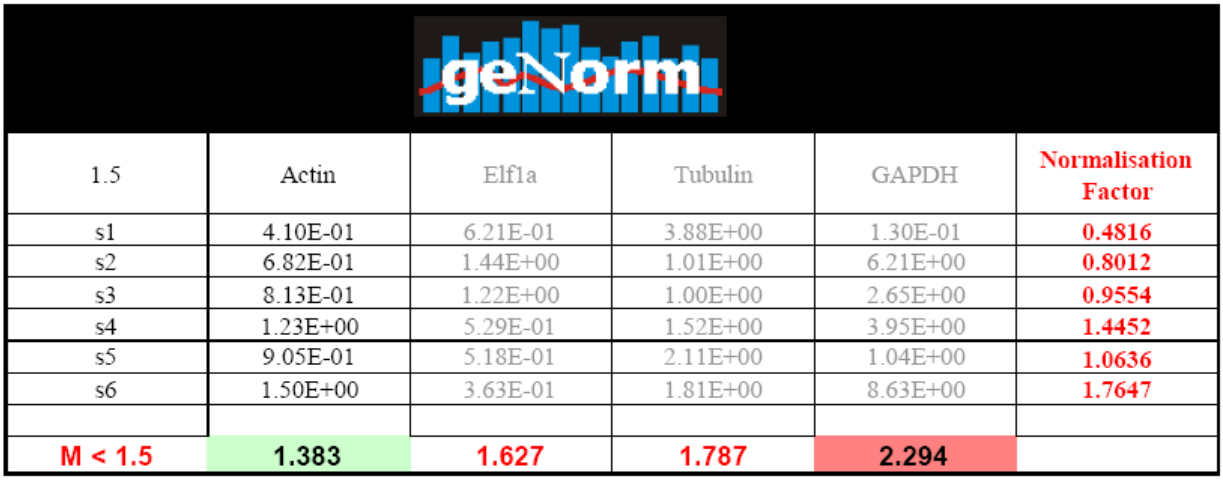
PCR amplification efficiency values for each primer pair from Table 2.3.2 were calculated as described in section 2.3.4.2. The ideal efficiency value for each primer pair would be 2.0, indicating a doubling of PCR product at every cycle during the exponential stage of PCR. The actual efficiency values for each primer pair were listed in Table 5.2.2, with an efficiency ranging from 1.9 to 2.5 and the standard deviation from 0.02 to 0.15. These values were used for relative quantification.

Table 5.2.2 PCR amplification efficiency (E) and its standard deviation (SD) values calculated for each primer pair by qBase. The fact that some E values were larger than 2 is probably caused by the linear regression analysis which does not take into account that the PCR amplification efficiency can maximally be 2.

Gene	E	SD
Actin	1.9191	0.0998
Elf1-α	2.1015	0.0685
GAPDH	2.0000	0.0500
Tubulin	2.0431	0.1264
Can3	2.0656	0.0550
Can4	1.9693	0.0249
Can10	1.9155	0.0361
Can18	1.8886	0.0265
Can94	2.5361	0.1114
Can130	2.1606	0.1033
Can135	2.4928	0.1484
Can136	2.2749	0.0570
Can139	2.0231	0.0314
Can151	2.1027	0.0450

5.2.3 Evaluation of reference genes

The expression stability parameter (M) for each of the four tested reference genes was evaluated using the geNorm programme. Assuming that the reference genes are not co-regulated, the lower the M value the more stable the expression in the particular samples under investigation (Vandesompele *et al*., 2002b). Out of the four evaluated reference genes, *actin* had the lowest M value of 1.383 and *GAPDH* the highest of 2.294 (Figure 5.2.1).



1.5	Actin	Elf1a	Tubulin	GAPDH	Normalisation Factor
s1	4.10E-01	6.21E-01	3.88E+00	1.30E-01	0.4816
s2	6.82E-01	1.44E+00	1.01E+00	6.21E+00	0.8012
s3	8.13E-01	1.22E+00	1.00E+00	2.65E+00	0.9554
s4	1.23E+00	5.29E-01	1.52E+00	3.95E+00	1.4452
s5	9.05E-01	5.18E-01	2.11E+00	1.04E+00	1.0636
s6	1.50E+00	3.63E-01	1.81E+00	8.63E+00	1.7647
M < 1.5	1.383	1.627	1.787	2.294	

Figure 5.2.1 Screenshot of the output from the programme geNorm showing the M value of each reference gene. The six investigated samples were: S1 - unpollinated stigma cDNA; S2 – pollinated stigma cDNA collected at 2 min after *in-vitro* self-pollination; S3 - pollinated stigma cDNA collected at 5 min after *in-vitro* self-pollination; S4 - pollinated stigma cDNA collected at 10 min after *in-vitro* self-pollination; S5 - pollinated stigma cDNA collected at 20 min after *in-vitro* self-pollination; S6 - pollinated stigma cDNA collected at 30 min after *in-vitro* self-pollination.

The average M value after exclusion of the two genes (*GAPDH* and *tubulin*) with the highest M value was 1.235 (Figure 5.2.2). The two genes with the lowest M value after stepwise exclusion were *actin* and *elf1-α*. They would represent a good pair of reference genes for normalization assuming that they are not co-regulated. However, Vandesompele et al. (2002b) recommend the selection of at least three reference genes as an appropriate strategy for normalization. Therefore the reference gene *tubulin*, which has the second highest M value (Figure 5.2.1) was included for evaluation (Figure 5.2.3) and the average M value with three reference genes (*actin*, *tubulin* and *elf1-α*) was 1.251 (Figure 5.2.4). It was then decided to use these three reference genes for normalization to circumvent the risk of inaccurate normalization due to two reference genes being co-regulated.

1.5	Actin	Elf1a	Normalisation Factor	
s1	4.10E-01	6.21E-01	0.6586	
s2	6.82E-01	1.44E+00	1.2915	
s3	8.13E-01	1.22E+00	1.2970	
s4	1.23E+00	5.29E-01	1.0530	
s5	9.05E-01	5.18E-01	0.8933	
s6	1.50E+00	3.63E-01	0.9637	
M < 1.5	1.235	1.235		

Figure 5.2.2 Screenshot of the output from the programme geNorm showing average M values of reference genes calculated after exclusion of the two reference genes with the highest M value, only *actin* and *elf1-α* were evaluated.

1.5	Actin	Elf1a	Tubulin	Normalisation Fac
s1	4.10E-01	6.21E-01	3.88E+00	1.0000
s2	6.82E-01	1.44E+00	1.01E+00	1.0000
s3	8.13E-01	1.22E+00	1.00E+00	1.0000
s4	1.23E+00	5.29E-01	1.52E+00	1.0000
s5	9.05E-01	5.18E-01	2.11E+00	1.0000
s6	1.50E+00	3.63E-01	1.81E+00	1.0000
M < 1.5	1.199	1.295	1.260	

Figure 5.2.3 Screenshot of the output from the programme geNorm showing average M values of reference genes calculated by geNorm programme after exclusion of the reference gene *GAPDH*.

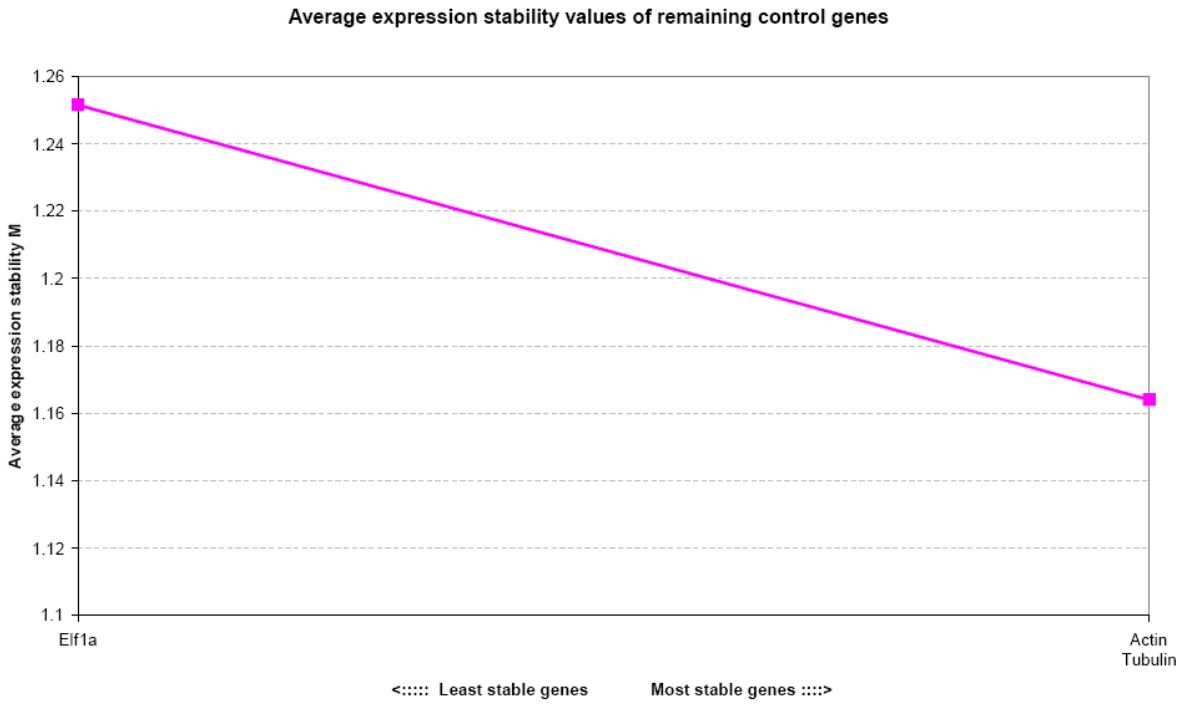


Figure 5.2.4 Screenshot of the output from the programme geNorm showing average expression stability values of the remaining three reference genes (*actin*, *elf1-α* and *tubulin*) calculated after exclusion of the *GAPDH* gene which has the highest M value.

5.2.4 Relative quantification for expression pattern analysis

5.2.4.1 Expression pattern in a pollination time course

The relative quantification was carried out using the programme qBase (Hellemans *et al*., 2007) as described in section 2.3.4.4. The ten potential incompatible response related candidates were analysed and normalised to the three reference genes described in section 5.2.3. Their relative quantities and relevant standard deviations in each stigma cDNA sample collected during the self-pollination time course were shown in Table 5.2.3. These values were transferred to line plots for analysing the expression pattern variations of the candidate genes (Figure 5.2.5).

Table 5.2.3 Results of relative quantification analysed with the programme qBase. The 'Quantity' shown is the "Log10" value of each gene's relative quantity after setting the expression level of reference gene to be "1". The standard deviation (StdDev) was calculated using values from the three replicates (section 2.3.4.1) for each reaction. The six investigated samples were: S1 - unpollinated stigma cDNA; S2 –pollinated stigma cDNA collected at 2 min after *in-vitro* self-pollination; S3 - pollinated stigma cDNA collected at 5 min after *in-vitro* self-pollination; S4 - pollinated stigma cDNA collected at 10 min after *in-vitro* self-pollination; S5 - pollinated stigma cDNA collected at 20 min after *in-vitro* self-pollination; S6 - pollinated stigma cDNA collected at 30 min after *in-vitro* self-pollination.

	S1		S2		S3		S4		S5		S6	
	Quantity	StdDev	Quantity	StdDev	Quantity	StdDev	Quantity	StdDev	Quantity	StdDev	Quantity	StdDev
Actin	0.40969	0.10364	0.68158	0.26996	0.81273	0.30830	1.22937	1.18354	1.50114	0.77553	0.90481	0.19630
Elf1a	0.62146	0.11243	1.43653	0.49468	1.21507	0.28629	0.52948	0.17514	0.36316	0.13862	0.51775	0.09021
Tubulin	3.87863	1.55440	1.00860	0.98366	1.00000	0.57719	1.51711	0.61212	1.81145	0.54451	2.10801	0.38561
Can3	0.00322	0.00082	11.77037	3.84988	12.86185	3.03473	11.84074	4.11471	1.09852	0.25006	0.29555	0.03373
Can4	0.00337	0.00079	5.14826	1.65546	6.77264	1.68525	6.48566	2.11066	1.14412	0.25048	0.25184	0.02881
Can10	0.01513	0.00230	29.71683	9.62096	26.67671	6.76964	18.12131	6.86389	1.13951	0.28321	0.54789	0.06959
Can18	0.00154	0.00046	12.58313	4.05448	15.62307	3.40492	18.34874	5.90225	0.49585	0.10559	0.17287	0.02411
Can94	0.01189	0.00270	7.94648	2.67927	11.92178	3.25876	9.28821	2.95616	1.55415	0.54381	0.34414	0.04687
Can130	0.00727	0.00689	359.46884	145.53477	369.76250	157.42209	547.61866	240.82461	6.37823	1.31138	1.49778	0.15937
Can135	0.00195	0.00055	382.72925	159.06502	887.28689	399.21807	1416.98311	732.75386	2.57439	0.62324	1.07771	0.11603
Can136	0.00538	0.00193	11.59620	3.87103	21.74506	6.21369	11.64496	3.76319	1.93216	0.59809	0.45591	0.04702
Can139	0.01283	0.00206	1.49836	0.49879	2.18406	0.48637	0.58926	0.19024	0.08856	0.02010	0.05052	0.01147
Can151	0.03793	0.00940	21.70437	7.43321	20.52995	4.43195	641.65693	227.22940	2.64673	0.91576	0.95306	0.11898

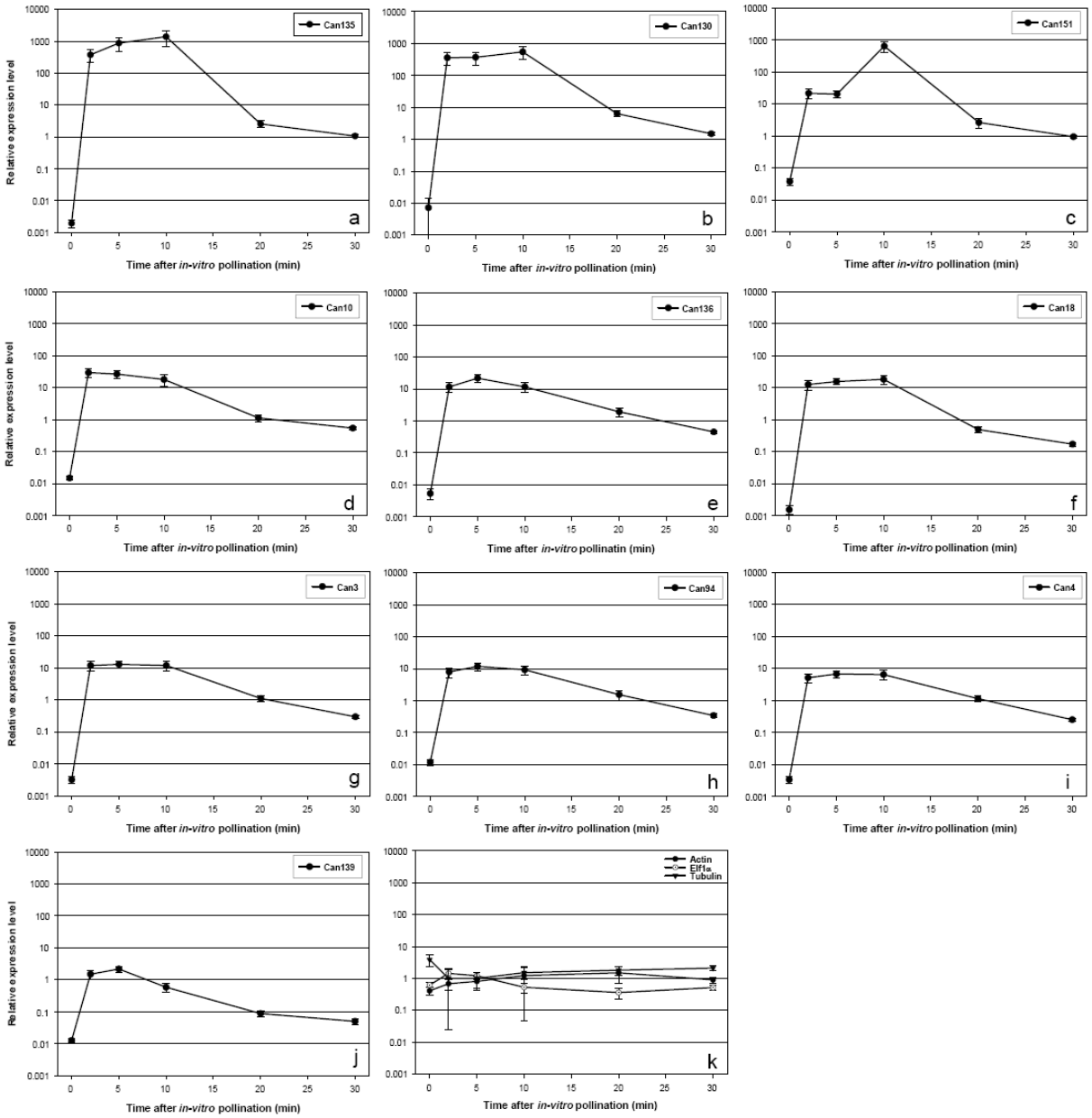


Figure 5.2.5 Graphs showing the expression pattern of the ten SI related candidate genes in stigma during a self-pollination time course of 0 min, 2 min, 5 min, 10 min, 20 min and 30 min after *in-vitro* pollination: a – Can135; b – Can130; c – Can151; d – Can10; e – Can136; f – Can18; g – Can3; h – Can94; i – Can4 and j – Can139. *Actin*, *elf1-α* and *tubulin* genes were used as reference genes and are shown in graph k. On the y axis the relative expression levels are represented using the 'Quantity (Log value)' shown in Table 5.2.3. The standard deviation bar is included in each of the line plot graphs.

The real-time PCR analysis during an incompatible pollination time course confirmed the specific expression pattern for the ten SI related candidate genes (Figure 5.2.5; Table 5.2.4) which were previously identified by reverse transcriptase PCR expression analysis (section

4.2.4). A rapid increase in expression within two minutes after *in-vitro* self pollination was observed, reaching a maximum between two and ten minutes after pollen-stigma contact (Figure 5.2.5), implicating the involvement of those candidate genes in the *L. perenne* incompatibility response. *Can135* has the highest maximum relative expression level of more than a 1000 fold increase. *Can130* and *Can151* have the maximum relative expression level of a little less than a 1000 fold increase. *Can10*, *Can136*, *Can18*, *Can3* and *Can94* have the maximum relative expression level between a 10 and 100 fold increase. *Can4* and *Can139* have lower maximum relative expression level with less than 10 fold increases. All the candidate genes, except *Can151*, displayed a similar expression pattern of a rapid increase followed by decrease during the incompatibility response. *Can151* is different in that a second major increase in expression from five to ten minutes after the pollen-stigma contact was observed (Figure 5.2.5).

Table 5.2.4 Rice homologies of the ten SI candidate genes in the order of the physical appearance on the rice genome: candidate gene name, according rice sequence, p-value and rice gene annotation information. BLAST searches for rice homology were carried out using the SSH sequence results through the BLASTN search function in the Rice Genome Annotation Project with the Osa1 rice genome coding sequences (CDS) database.

Name	**Rice Sequence**	**p-value**	**rice annotation**
Can18	LOC_Os04g01150	6.70E-95	ELMO domain-containing protein 2, putative, expressed
Can139	LOC_Os04g50216	1.50E-80	gtk16 protein
Can135	LOC_Os05g15690	2.50E-12	beta-expansin 2 precursor
Can4	LOC_Os05g33080	4.10E-29	serine/threonine-protein kinase NAK
Can151	LOC_Os05g34110	5.70E-05	myb-like DNA-binding domain, SHAQKYF class family protein
Can3	LOC_Os05g39870	3.60E-17	CBL-interacting serine/threonine-protein kinase 15, putative, expressed
Can94	LOC_Os05g41270	6.80E-51	Calcium-dependent protein kinase, isoform 2, putative, expressed
Can10	LOC_Os05g43540	2.60E-40	expressed protein
Can136	LOC_Os05g48640	2.10E-36	pollen-specific kinase partner protein
Can130	LOC_Os05g51680	1.20E-06	SCP-like extracellular protein

5.2.4.2 Differential expression between candidate genes

The expression levels of the ten potential incompatible response related candidates were further compared at each time point during the incompatible pollination time course (Figure 5.2.6). *Can135* and *Can130* have higher expression levels than the other candidate genes between 2 min and 10 min after the pollen-stigma contact but show similar expression level as the others at the later pollination time point, implicating a faster decrease in expression of these two candidates. *Can139* has a lower expression level than the other candidate genes after the pollen-stigma contact, but a relative high gene expression level in the unpollinated stigma sample. The remaining candidates, *Can10*, *Can151*, *Can136*, *Can18*, *Can3*, *Can94* and *Can4*, displayed similar expression levels after pollen-stigma contact and during the incompatible pollination process, with the exception of the significant higher expression of *Can151* at the 10 min time point of the pollination process. The relative expression levels of the ten candidate genes in unpollinated stigma (at the 0 min time point of the pollination process) were lower compared to the reference genes and also displayed differences between them (Figure 5.2.6). Therefore, to better understand and compare the correlations between the incompatibility response and the expression pattern of the candidate genes, the relative expression level of each candidate gene was compared in fold change between every two time points next to each other during the pollination time course, e.g. using the 'Quantity' value in Table 5.2.3 to evaluate fold change at S2-S1 (0-2 min), S3-S2 (2-5 min), S4-S3 (5-10 min), S5-S4 (10-20 min) and S6-S5 (20-30 min) (Figure 5.2.7). *Can135*, *Can130* and *Can151* had much more significant (hundreds-fold) changes in expression during the incompatible pollination; *Can10*, *Can136*, *Can18* and *Can3* had about ten-fold changes in expression levels while *Can94*, *Can4* and *Can139* had less than ten-fold changes during the time course. For all of the ten candidates, their expression was induced considerably within two minutes of an

incompatible pollination. Continuous expression was observed from 2-5 min except in *Can10* and some of the candidates (*Can135*, *Can130*, *Can151* and *Can18*) showed accumulation from 5-10 min (Figure 5.2.7). Major drops in gene expression were observed from 10-20 min for all candidate genes except for *Can139* at 5-10 min. Some candidates (*Can136*, *Can4*, *Can94* and *Can4*) started decreasing from 5-10 min while for Can10 from 2-5 min (Figure 5.2.7). The different expression patterns of the candidate genes during an incompatible pollination time course imply their different involvement in the *L. perenne* incompatibility response, probably using different mechanisms through divergent pathways. The possible roles of these candidate genes and putative pathways triggered by the SI response are discussed in the Discussion section.

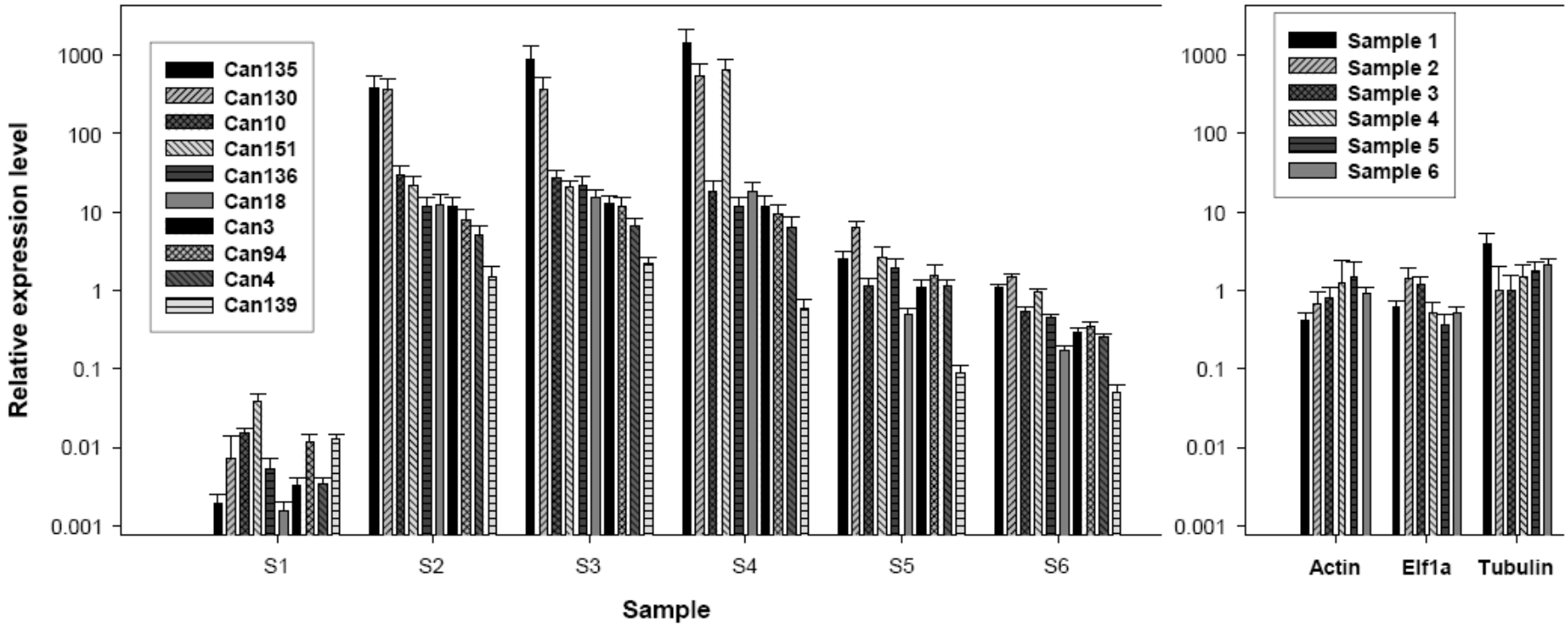


Figure 5.2.6 Graph showing expression levels of the ten candidate genes at each time point during the incompatible pollination process: Sample 1 (S1) – 0 min; Sample 2 (S2) – 2 min; Sample 3 (S3) – 5 min; Sample 4 (S4) – 10 min; Sample 5 (S5) – 20 min and Sample 6 (S6) – 30 min. The expression profiles of the three reference genes in all samples are shown at the right side of the graph.

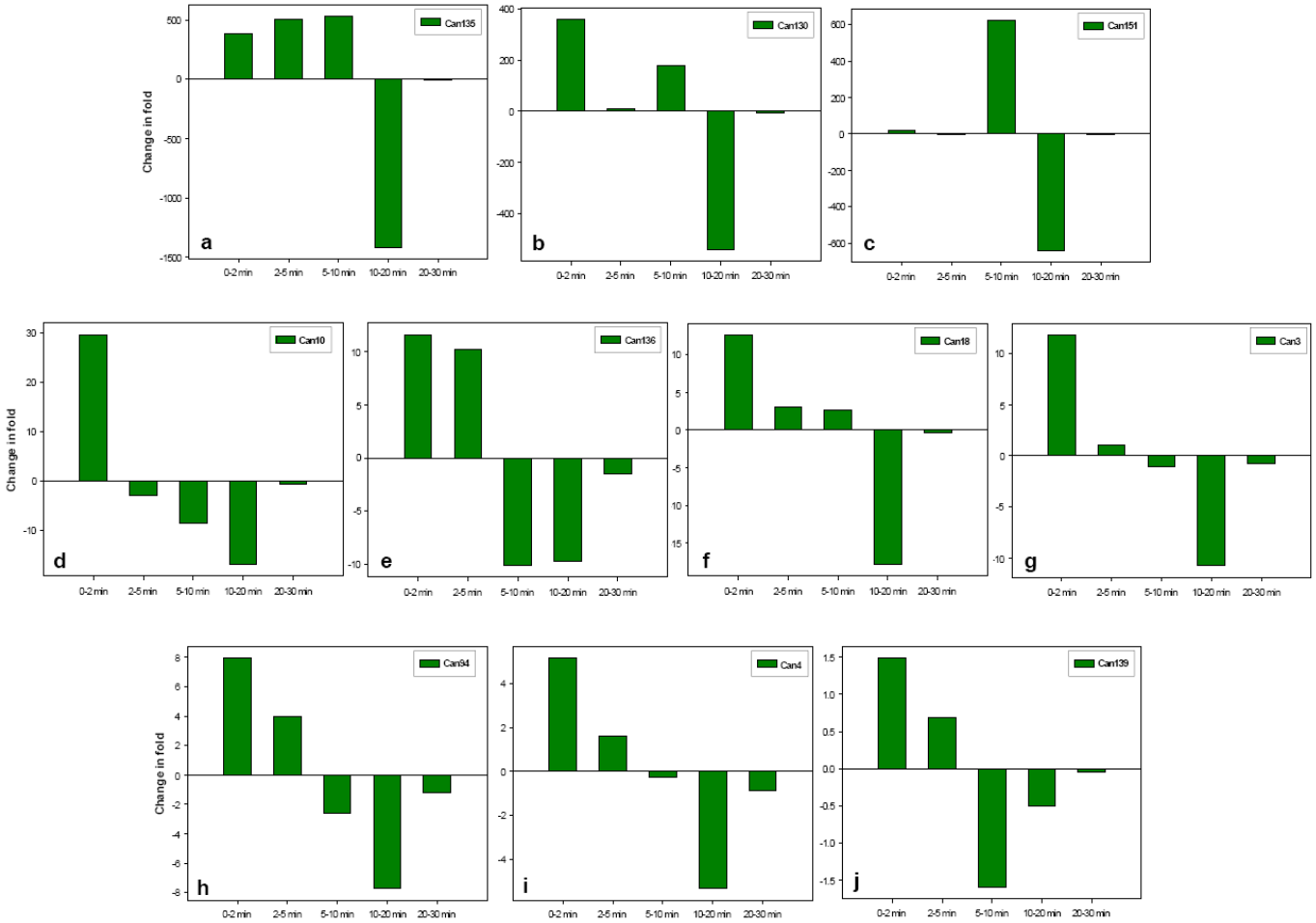


Figure 5.2.7 Graphs showing changes in expression level of each candidate gene during the *in-vitro* self pollination time course between 0-2 min, 2-5 min, 5-10 min, 10-20 min and 20-30 min. Figures a, b and c in the first row reveal 100-fold changes in expression level; figures d to g in the second row include candidate genes which have changes in expression level above ten-fold; and figures h, i and j show fold change in expression level less than ten-fold.

5.3. Discussion

The purpose of carrying out the real-time PCR study was to verify the expression patterns during the incompatibility response in *L. perenne* of the ten potential incompatible response related candidates identified in Chapter 4. All candidates showed a regulated gene expression pattern during the incompatible pollination process. A noticeable general pattern was the rapid up-regulation in expression within two minutes after *in-vitro* pollination, which is consistent with the rapid incompatibility response observed in *Lolium* (Heslop-Harrison, 1982), implying the putative involvement of the candidate genes in the *L. perenne* SI response. Different levels of accumulation and decline in expression were observed for these candidates during subsequent time intervals of the incompatible pollination process. This might shed light on their possible roles during the SI response whether they are true candidate genes for the SI initial response components or if not, whether and how they might be involved in the downstream incompatibility reactions. Additional control experiments, such as during cross-pollination or "mock" pollination process and in pollen material, will reveal the SI specificity of these candidates. Possible roles of the candidate genes in the SI response are now discussed.

Can135 represents a putative expansin protein (Table 5.2.4). Expansins are a class of wall proteins with the ability to promote the extension of plant cell walls (McQueen-Mason *et al*., 1992) through a mechanism that disrupts the adhesion of wall matrix polysaccharides to one another or to cellulose microfibrils (Shcherban *et al*., 1995). It has been suggested that expansins are prominent candidates to facilitate plant cell growth when cell walls are under tension (Shcherban *et al*., 1995). These proposed functions of expansins could explain the expression pattern of *Can135* during the SI response. Upon pollen-stigma contact, the pollen cell walls are under stress and the rapid increase in expression of *Can135* (Figures 5.2.5 &

Figure 5.2.7 a) would induce cell growth, and hence pollen germination. The fact that *Can135* was not identified in the pollen-specific cDNA library (Chapter 4 Appendix C) suggests it is more likely involved in the pollen-stigma interaction and is induced by pollen-stigma contact. The possibility of it being the pistil S determinant could therefore be excluded, however its relation with the incompatibility respsonse implicates a role as a putative downstream SI gene. As a result of the well known rapid incompatibility response in grasses (Heslop-Harrison, 1982), the expression of *Can135* in pollen should decline within minutes in order to inhibit pollen tube growth. The increase of *Can135* in *in-vitro* pollinated incompatible stigma between 2-10 minutes (Figure 5.2.7 a) might be due to its accumulation rather than expression. If it is in the pollen where the expression of *Can135* is induced by the incompatibility response and reaches the maximum within several minutes. Then through pollen-stigma contact, *Can135* might be transported gradually from pollen to stigma somehow, leading to the delayed increase of *Can135* in the stigma with a subsequent significant drop in its gene expression level.

Can130 has a high similarity to a putative sperm-coating glycoprotein (SCP)-like extracellular protein (Table 5.2.4), a member of the pathogenesis-related (PR) protein family which is involved in various signalling processes with a putative Ca^{2+} chelating function (Fernández *et al*., 1997; Milne *et al*., 2003). PR proteins are induced during incompatible host-pathogen interactions or other stress-related responses (Van Loon and Van Strien, 1999). The PRs have been identified in different plant families, being induced by different endogenous and exogenous signalling compounds. They have shown consistent expression levels in the apoplast as well as in the vacuolar compartment during development (Van Loon and Van Strien, 1999). It has been suggested that PRs have important functions other than adaptation

to biotic stress. Similarities between self-incompatibility responses and plant-pathogen interactions have recently been reviewed (Sanabria *et al*., 2008). The induced expression of *Can130* upon pollen-stigma contact (Figure 5.2.7 b) strengthens the parallels between incompatible host-pathogen and incompatible pollen-stigma interactions. PRs are involved in the Ca^{2+}-dependent signalling network in plant innate immunity leading to the programmed cell death. In the SI response, PRs might play the same role in regulating pollen tube growth through a Ca^{2+}-dependent signalling network. The putative function of PRs in signalling transduction suggests the involvement of *Can130* in the downstream SI response.

Can151 is a putative myb-like DNA-binding protein with a SHAQKYF domain (Table 5.2.4). In tomato, a similar protein, LeMYBI, was identified (Rose *et al*., 1999). LeMYBI showed strong similarity to plant myb transcription factors, containing two myb-like domains and a SHAQKYF amino acid motif in the second myb-like repeat. Myb-like proteins have been widely found in eukaryotes and have been shown to act as DNA-binding transcription factors to regulate cell growth and differentiation (Lipsick, 1996). But the function of the SHAQKYF containing LeMYBI-like proteins in gene regulation has not been illustrated. *Can151* has an exceptional increase in expression from 5-10 min after pollen-stigma contact (Figure 5.2.7 c), which implicates its possible role in the downstream SI response as a transcription factor to regulate expressions of other candidate genes. However, further analysis is necessary for a better understanding of the involvement of the myb-like DNA-binding protein in the *L. perenne* SI response.

Can18 is similar to a putative phagocytosis and cell motility protein ELMO (Table 5.2.4). In mammalian cells, ELMO-1 functionally cooperates with Dock180 to stimulate a Rac-guanine

nucleotide exchange factor (GEF), leading to Rac1 activation and cytoskeletal rearrangements. ELMO was identified as an upstream regulator of Rac1 GTPase that affect phagocytosis and cell migration in mammals (Gumienny *et al*., 2001). The regulatory role of Rac-GTPase in actin-based cell motility shows a significant degree of conservation between plants and animals (Cheung *et al*., 2003). In plants, Rac-like GTPases have been shown to have profound effects on the actin cytoskeleton and regulation of pollen tube growth. Over-production of an *Arabidopsis* Rac AtRac2 (Kost *et al*., 1999) and a tobacco Rac NtRac1 (Chen *et al*., 2002) lead to abnormal pollen tube tip growth. The identification of *Can18* and its increase in expression upon pollen-stigma contact suggests its possible function upstream of Rac-like-GTPase, which subsequently regulates pollen tube growth. The expression pattern of *Can18* (Figure 5.2.7 f) during the incompatible pollination process is similar to that of *Can135* (Figure 5.2.7 a), which potentially regulates pollen germination upon pollen-stigma contact, implicating possible cross-talk or co-regulation between *Can18* and *Can135* during the SI response.

Can139 has putative voltage-gated chloride channel activity (http://www.gramene.org/Oryza_sativa_japonica/geneview?gene=LOC_Os04g50216), which is involved in the regulation of cell volume, control of electrical excitability, and transepithelial transport in higher organisms (Schmidt-Rose and Jentsch, 1997). In plants, chloride channels are shown to be involved in a number of plant specific functions (Schroeder, 1995) and are responsible for the generation of action potentials (Hedrich and Becker 1994). It is also suggested that plant chloride channels play an important role in signal perception and transduction by stimulating anion efflux (Assmann, 1993). Chloride ions have been associated with pollen tube tip growth and increased cell volume of pollen tubes (Zonia *et al*., 2002).

The Cl^- flux was shown to be regulated by inositol 3,4,5,6-tetra*kis*phosphate, which is phosphorylated in a Ca^{2+} dependent manner (Zonia *et al*., 2001). Wehling et al. (1994) reported in rye the involvement of Ca^{2+}-induced signal transduction during the self-incompatibility response. The identification of *Can139* in the *Lolium* SI response implies a putative ion channels-related signal transport mechanism leading to pollen tube inhibition. The expression of *Can139* is less regulated compared to other candidate genes during the *L. perenne* SI response, about 1.5 fold differences (Figure 5.2.7 j), implicating its more likely role as a secondary factor.

Can10 represents a putative expressed protein (Table 5.2.4) and has been identified to contain a TRAM, LAG1 and CLN8 (TLC) homology function domain (Chapter 4, Table 4.2.6). TLC-domain containing proteins have been reported in yeast, mammalians and humans with various lipid metabolism functions such as lipid synthesis activation, protecting proteins from proteolysis, lipid transport and lipid sensing (Winter and Ponting, 2002). Lipids have been shown to be essential for successful pollen development on the stigma (Elleman *et al*., 1992; Wolters-Arts *et al*., 1998). The expression of *Can10* started to decline after 2 min upon pollen-stigma contact (Figure 5.2.7 d), implying possible lipid degradation during the incompatibility response in *L. perenne*, which in turn inhibits pollen development.

Three of the SI candidate genes (*Can3*, *Can4* and *Can94*) with protein kinase functions were identified (Table 5.2.4), implying the role of protein kinases in the *L. perenne* SI response (Figure 5.2.5). These three candidate genes also displayed the same expression patterns during the incompatible pollination time course (Figure 5.2.7 g, h, i). The classification and functions of plant protein kinases have been reviewed by Hardie (1999), stipulating the role of different protein kinase subfamilies in a wide variety of processes in plant growth and development,

including pollen tube growth and self-incompatibility responses. Ca^{2+}-dependent protein kinase (CDPK), such as Can94, is proposed to control pollen germination and growth through regulating cytoskeletal dynamics (Estruch *et al.*, 1994). Studies in pollen-pistil interactions and signalling in pollination have shown supporting evidence for the involvement of protein kinases in self-incompatibility responses (reviewed in Franklin-Tong, 1999; Wheeler *et al*., 2001; Swanson *et al*., 2005). In *Nicotiana alata*, a soluble pollen-expressed protein kinase Nak-1, similar to Can4, has been identified as sharing some features of CDPKs and plays a role in the phosphorylation of the stylar S-RNases involved in SI in *N. alata* (Kunz *et al.*, 1996). This is of interest as it has been shown to be involved in a signalling interaction between pollen and a pistil component known to be involved in SI. In the SSI species *Brassica*, the female SI determinant SRK is a single-pass transmembrane serine/threonine kinase (Nasrallah, 2002). In the gametophytically controlled *Papaver* SI system, a MAPK p56 (Rudd *et al*., 2003) has been reported to lead to programmed cell death (Li *et al*., 2007b). Thus in several different SI systems, kinases play a key role in the SI response. In the grass species *S. cereale* L., an involvement of protein kinases was proposed based on evidence that kinase inhibitors inhibit the SI response (Wehling *et al*., 1994), though the component itself has not been identified. In our study, also a putative kinase partner protein (Can136) was identified (Table 5.2.4). A kinase partner protein has been reported to play a role in polarized pollen tube growth in *A. thaliana* through the interaction with membrane proteins in pollen (Kaothien *et al*., 2005). Depolarized pollen tube growth and irregular actin arrangement in the pollen tube tip region were observed through over-expression of a kinase partner protein (Kaothien *et al*., 2005). The expression of candidate gene *Can136* (Figure 5.2.7 e) during the SI reaction was very similar to the three protein kinase candidates (*Can3*, *Can4* and *Can94*) (Figure 5.2.7 g, h, i), implying the role of a kinase partner protein in the inhibition of pollen

tube growth, probably through the interaction with protein kinases in a cascade reaction. Therefore, *Can3*, *Can4* and *Can94* are promising candidates for the *Lolium* SI determinants.

Besides their protein kinase functions, Can3 (putatively interacts with CBL, a calcineurin B-like calcium sensor protein) and Can94 (a putative calcium-dependent protein kinase) are also calcium related proteins (Table 5.2.4) where calcium/calmodulin-dependent function domains are identified (see Chapter 4 Table 4.2.6), implicating the involvement of Ca^{2+} in the *L. perenne* SI response. Studies have shown that calcium is involved in SI responses of the inhibition of pollen germination and tube growth. Studies on pollen tubes undergoing the SI response in *Papaver* have revealed the involvement of Ca^{2+} signalling cascades. Studies using ratiometric Ca^{2+} imaging quantified the alterations in $[Ca^{2+}]_i$ and confirmed that the SI response stimulates an influx of extracellular Ca^{2+} and increased amounts of $[Ca^{2+}]_i$ are located in the subapical and shank regions of the pollen tube leading to the inhibition of pollen tube growth (Franklin-Tong *et al*., 1997; Franklin-Tong *et al*., 2002). Ca^{2+} signalling related actin reorganization and likely depolymerization have been reported showing involvement in the SI responses in *Papaver* (Geitmann *et al.*, 2000; Snowman *et al.*, 2002) and *Brassica rapa* (Iwano *et al*., 2007). A potential involvement of Ca^{2+} in the grass SI system has been identified in rye (*Secale cereale*) where calcium antagonists had overcome the SI response (Wehling *et al*., 1994). In a preliminary experiment carried out in *L. perenne* (data not shown), the Ca^{2+} antagonists verapamil and $LaCl_3$ were shown to inhibit or delay the SI response where pollen tube growth was observed in self-pollinated stigmas after incubation on verapamil or $LaCl_3$ containing medium. The next step would be to identify the components involved in the Ca^{2+} mediated pathways and their possible communications in the *L. perenne* SI response.

The identification of these genes suggests the possibility that several events are induced by an incompatible pollination in *L. perenne*. A model of possible events are shown in Figure 5.3.8. A possible scenario for an SI response might be the following: Ca^{2+} related signalling networks involving protein kinases and ion channel activities inhibit pollen tube growth by regulating extracellular Ca^{2+} influx, leading to the loss of high apical $[Ca^{2+}]_i$ which is crucial for pollen tube growth. Down-regulation of an expansin protein and a myb-like protein may reduce cell growth and differentiation, which will lead to tube growth inhibition. The actin cytoskeleton will likely be influenced through a Rac-GTPase pathway to cause the arrest of pollen tube growth. Down-regulation of lipid metabolism may result in inhibited pollen tube germination/growth. Together these events could contribute to the eventual inhibition and death of incompatible pollen tubes. However, it is unclear whether these events occur in parallel or if they are interlinked by a signalling cascade. Furthermore, as the pathways proposed are based on the stigmatic differential gene expressions of the ten candidates during the *Lolium* SI response, it is unclear how the signals are transduced from stigma to pollen in order to regulate pollen tube growth. Thus many questions remain to be answered.

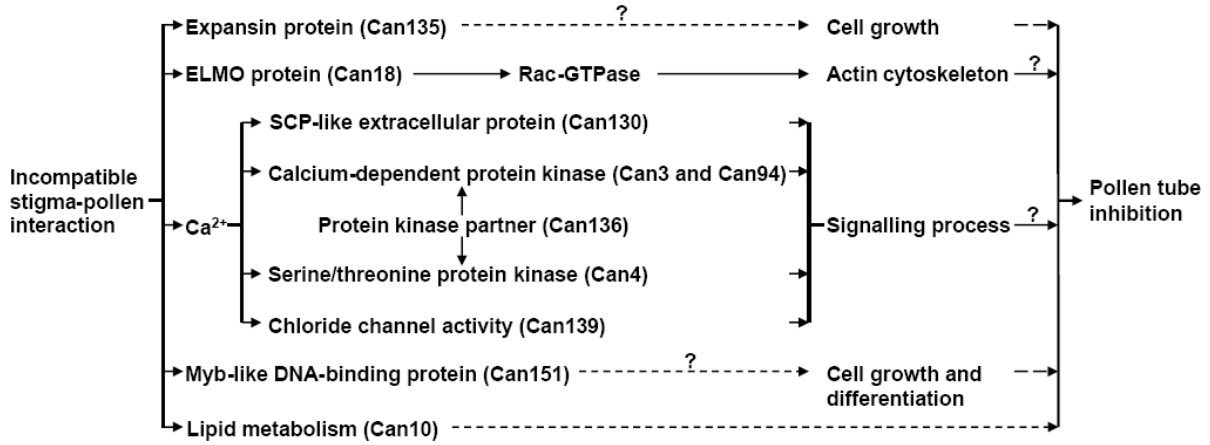


Figure 5.3.8 Putative events in the *L. perenne* self-incompatibility response. An incompatible pollination may be postulated to trigger Ca^{2+} related signalling networks, involving protein kinases and ion channel activities; regulates expression of a expansin protein and a myb-like protein which are associated with cell growth and differentiation; affects actin cytoskeleton through a Rac-GTPase pathway and regulates lipid metabolism involved in pollen development. These events may play a role leading to ultimate pollen tube growth inhibition. Up-regulated processes are illustrated with solid arrow; down-regulated processes are illustrated with dashed arrows. Unidentified mechanisms are indicated with question marks.

There are similarities between the grass GSI and SSI systems (reviewed by Yang *et al*., 2008), including the trinucleate pollen at the time of dispersal, short-lived pollen with a high respiratory rate, dry stigma, rapid SI response at the stigma surface, Ca^{2+} signalling participation and the involvement of protein kinases. The identification of candidate protein kinases in the *L. perenne* SI response in this study (Figure 5.2.8) adds weight to the relationship between SSI and grass SI. Those candidate genes (*Can3*, *Can4*, *Can94* and *Can136*) are of particular interest for further investigations. Identification of the SI specificity of these genes and determination of their genetic map positions in relation to *S* or *Z* will lead to a better understanding of their involvement in the grass SI system.

Up to date, little is known about the *L. perenne* SI signalling networks and the downstream targets (Figure 5.3.8). Future work combining molecular genetics and proteomics would lead

to the identification of signalling components and facilitate the subsequent functional analysis (Reddy and Reddy, 2004). Approaches such as a yeast two-hybrid screening, fluorescence resonance energy transfer (FRET) (reviewed in Mendelsohn and Brent 1999) and bioluminescence resonance energy transfer (BRET) (Xu *et al*., 2007) could also be applied for identifying protein interaction partners.

Chapter 6 will describe work carried out for determining the genetic map distances between these candidate genes and *S* or *Z* loci through mapping on the ILGI family and newly developed *S* and *Z* fine mapping populations of *L*. *perenne* L.

CHAPTER 6

LINKAGE ANALYSIS OF THE IDENTIFIED SI CANDIDATES AND MAPPING OF THE *S* & *Z* LOCI ON THE FINE MAPPING POPULATIONS

6.1 Introduction

6.1.1 Genetic mapping and its applications

The construction of detailed genetic maps with high levels of genome coverage is the basis for applications of molecular markers in plant genetics and breeding practices, such as identifying genes or QTL associated with traits of agronomical and economical importance, comparative mapping between different species, MAS for desirable traits and map-based cloning of genes of interest. These strategies require no prior knowledge of the gene products responsible for the traits of interest, providing suitable means for investigating the *S* and *Z* two loci self-incompatibility system in grass species where the gene products have not been identified so far. For MAS and map-based cloning, markers which co-segregate or are tightly linked to the gene of interest are essential (Ribaut *et al*., 1998; Gupta *et al*., 1999). High-resolution mapping such as flanking marker analysis (Dixon *et al*., 1995), pooled sample mapping (Churchill *et al*., 1993) and physical mapping (King *et al*., 2007a) are used to search for tightly linked markers. Once these have been found, the inheritance of the gene can be traced with MAS for breeding programs and help with map-based cloning.

6.1.2 Mapping functions

Genetic mapping determines the order and relative genetic distances between markers along a chromosome. Genetic map distances between two markers are calculated based on recombination or crossovers events in that region between two non-sister chromatids of each pair of homologous chromosomes during meiosis. The observed recombination frequency between two makers is about half the number of crossover events between them since only two of the four chromatids participate in the crossover (Ott, 1999). Unlinked markers have a

recombination frequency of c. 50% and the closer the linkage between two markers, the lower the recombination frequencies. The map distance between two markers is proportional to the total number of crossover events rather than to the recombination frequency. When double crossover events take place between two markers, the distance between those two markers is underestimated regarding the recombination frequency. However, when the map distance between two markers is small enough to avoid double crossover events, all recombinants are the result of single crossover events and recombination frequencies are therefore suitable for measuring map distances. There are two genetic mapping functions frequently used, Haldane (Haldane, 1931) and Kosambi (Kosambi, 1944), to convert recombination fractions into map distance in centiMorgans (cM). Haldane's mapping function predicts the number of crossovers from recombination frequencies and assumes that there is no interference which would increase or decrease the fraction of double crossovers. Kosambi's mapping function makes moderate allowances for crossover interference in neighbouring locations and is based on experimental data regarding the fraction of double crossovers as the map distance varies (Kosambi, 1944). Several computer packages are currently available for automated genetic mapping (Ott, 1999). A widely used commercial programme is JoinMap (Van Ooijen and Voorrips, 2001), which incorporates both Haldane and Kosambi mapping functions. This programme was applied in this study to perform linkage analysis and to calculate map distances.

6.1.3 Mapping strategy for outbreeding plants

Development of appropriate mapping populations is required for genetic mapping. Linkage analysis and map construction with molecular markers have been applied mostly to populations such as backcross (BC_1), second filial generation (F_2), recombinant inbred lines

(RILs), double haploids (DHs) and near isogenic lines (NILs), which are derived from the F_1 of a cross between two fully homozygous diploid parents (Burr *et al*., 1988; He *et al*., 2001; Doerge, 2002). These mapping pedigrees are suitable for most agronomically important crops that are self-fertile. Linkage analysis based on these populations is simple and straightforward as genetic segregation is the result of recombination from a single F_1 genotype (Maliepaard *et al*., 1997). However, for many outbreeding grass species including *L. perenne*, the gametophytic self-incompatibility system limits breeding by severe inbreeding depression, hampering the production of inbred lines and F_1 hybrids. The heterozygous character of the mapping parents complicates the linkage analysis for outbreeding species, because as many as four alleles may segregate at a single locus, and markers can segregate in two (1:1), three (1:2:1) or four (1:1:1:1) genotypic classes. Therefore, other mapping strategies are developed for these heterozygous populations. One method is to perform a "two-way pseudo-testcross" procedure (Ritter *et al*., 1990; Grattapaglia and Sederoff, 1994; Hemmat *et al*., 1994). Another method is to produce a BC_1-type progeny in order to simplify the segregation; however, incompatibility might prevent the backcross or cause severe selection in the progeny (Maliepaard *et al*., 1997). The pseudo-testcross strategy has been generally applied for mapping in outbreeding species (Grattapaglia and Sederoff, 1994; Hemmat *et al*., 1994; Sewell *et al*., 1999; van der Voort *et al*., 1999; Butcher and Moran, 2000; Wu *et al*., 2000; Graham *et al*., 2004) and has been used mostly for the construction of mapping populations in *Lolium* spp. (Table 6.1.1). The theoretical background for linkage analysis using this approach has been explained by Ritter et al. (1990). No genetic information is available for the heterozygous parents and the genetic segregation of a marker is obtained after analysing the parents and the progeny, therefore the so called "pseudo-testcross" (Grattapaglia and Sederoff, 1994). It allows the construction of individually independent linkage maps. If one of the

parents is homozygous or near-homozygous, a one-way pseudo-testcross can be established (Ritter *et al*., 1990; DeSimone *et al*., 1997). When using the segregation data for each heterozygous parent, in this way through a two-way pseudo-testcross procedure, two independent linkage maps can be developed. It is possible to construct a genetic linkage map combining the two independent maps with the use of codominant markers segregating in both parents (Maliepaard *et al*., 1998). The ILGI mapping population p150/112 used in this study was derived from a cross between an anther culture-derived double haploid plant as female parent and an unrelated multiple heterozygous parent of complex descent as pollinator (Bert *et al*., 1999; Jones *et al*., 2002b), following the one-way pseudo-testcross strategy. This population has been extensively applied in linkage mapping in *L. perenne* and marker loci of different types have been reported (Table 6.1.1). It also has been phenotyped and genotyped for the *S* and the *Z* loci (Thorogood *et al*., 2002), facilitating the identification of tightly linked *S* and *Z* markers, which could be further used for MAS and map-based cloning.

6.1.4 Development of mapping populations for fine mapping S and Z loci

Map-based cloning provides a means of identifying the *S* and the *Z* loci gene components. However, this approach requires screening of a large population for the precise mapping of a target gene (Tanksley *et al*., 1995; Ferreira *et al*., 2006). Hence, fine mapping populations of over one thousand genotypes for constructing genetic maps covering the *S* and the *Z* loci and to assist future map-based cloning were developed by Dr. Daniel Thorogood at IBERS specifically for this purpose. The strategy for the construction of the *L. perenne S* and *Z* loci fine-mapping populations is as described by Baumann et al. (2000) and has been applied in the fine mapping of the *S* and *Z* loci in *Phalaris coerulescens* (Bian *et al*., 2004) and *Hordeum bulbosum* (Kakeda *et al*., 2008). The *S* mapping population of *P. coerulescens* contained 862

individuals and the *Z* mapping population contained 213 individuals (Bian *et al*., 2004). The *S* and *Z* mapping populations of *H. bulbosum* consisted of 662 and 400 individuals, respectively (Kakeda *et al*., 2008). The advantages of using these fine mapping populations are the ability to detect contaminants in the progeny and no requirement to genotype individuals in large populations. Only a single *S* or *Z* genotype will be obtained in the relative mapping population (Figure 6.1.1). The parents are homozygous at one locus (hom) and heterozygous at the other (het), as referred to hom/het or het/hom in accordance with the genotypes of *S* and *Z* (e.g. $S_2S_2Z_1Z_2$.is hom/het type and $S_2S_3Z_1Z_1$ is het/hom type). The homozygous locus of the female parent is the target mapping locus. For the hom/het x het/hom cross ($S_2S_2Z_1Z_2$ x $S_2S_3Z_1Z_1$), the *S* mapping population, one pollen gamete (S_2) is self-incompatible and only the other pollen gamete (S_3) will be able to succeed fertilization. Therefore, all individuals in the progeny should be heterozygous at the target mapping locus *S* (S_2S_3). The genotypes of the other locus, *Z* in this population, would be either homozygous (Z_1Z_1) or heterozygous (Z_1Z_2). For the het/hom x hom/het cross ($S_2S_3Z_1Z_1$ x $S_2S_2Z_1Z_2$), the *Z* mapping population, all descendants should have the identical heterozygous genotype (Z_1Z_2) at the *Z* locus and either homozygous (S_2S_2) or heterozygous (S_2S_3) at the *S* locus (Figure 6.1.1). Consequently, no genotyping is required for the *S* and *Z* loci during mapping. If there is a recombination between a marker and the mapping locus, the recombinant will be revealed as homozygous for the marker locus and heterozygous for the mapping locus (Figure 6.1.2). The recombination frequency between the marker and the mapping locus can be calculated by dividing the number of homozygous individuals with the total number of genotypes in the mapping population (Leach, 1988).

***Z*-population**

	♂ $S_2S_2Z_1Z_2$ pollen		
	gamete	S_2Z_1	S_2Z_2
♀ $S_2S_3Z_1Z_1$	S_2Z_1	/	$S_2S_2\mathbf{Z_1Z_2}$
	S_3Z_1	/	$S_2S_3\mathbf{Z_1Z_2}$

***S*-population**

	♂ $S_2S_3Z_1Z_1$ Pollen		
	gamete	S_2Z_1	S_3Z_1
♀ $S_2S_2Z_1Z_2$	S_2Z_1	/	$\mathbf{S_2S_3}Z_1Z_1$
	S_2Z_2	/	$\mathbf{S_2S_3}Z_1Z_2$

Figure 6.1.1 Scheme for construction of *S* and *Z* fine-mapping populations. The genotypes for each parent and gamete are given. The *Z* population is produced through het/hom (♀) x hom/het (♂) cross, while the *S* population is the reciprocal cross, hom/het (♀) x het/hom (♂). For each cross, only a single pollen gamete is able to effect fertilization: S_2Z_2 to pollinate the pistil with the genotype $S_2S_3Z_1Z_1$ for the *Z*-population and S_3Z_1 to pollinate the pistil with the genotype $S_2S_2Z_1Z_2$ for the *S*-population. The genotypes of all progeny from each population at the target locus will be the same heterozygous type: Z_1Z_2 for the *Z*-population and S_2S_3 for the *S*-population (indicated with bold type). The other locus is expected to segregate in a ratio of 1:1 for two different genotypes: S_2S_2 and S_2S_3 for the *S* locus in the *Z*-population; Z_1Z_1 and Z_1Z_2 for the *Z* locus in the *S*-population.

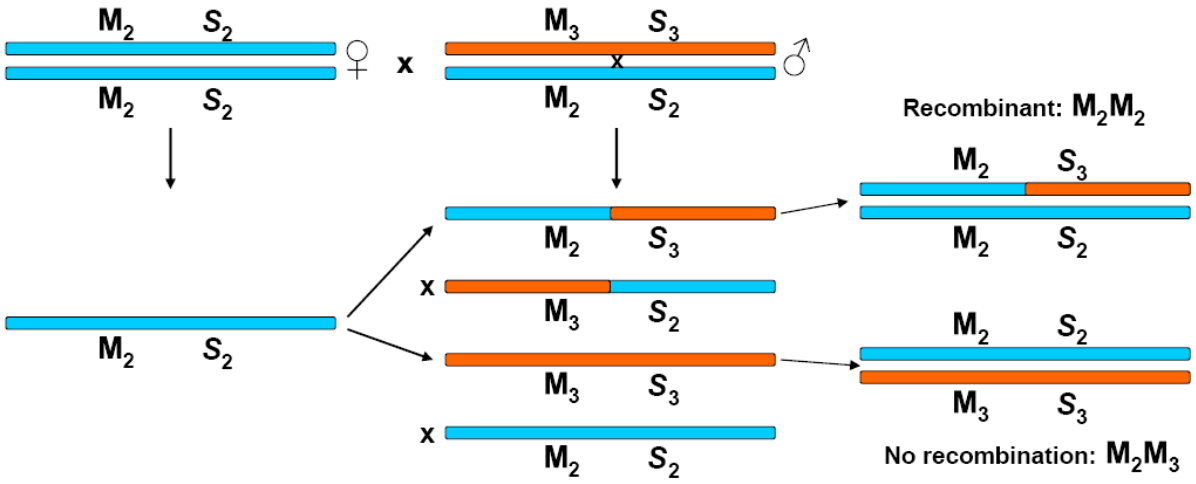


Figure 6.1.2 Scheme for the detection of recombinants between the *S* locus and a linked marker (M). Assuming that two marker alleles M_2 and M_3 are closely linked to the S_2 and S_3 alleles, respectively, in this hom/het x het/hom cross ($S_2S_2Z_1Z_2$ x $S_2S_3Z_1Z_1$), the paternal parent generates pollen gametes of four different genotypes (M_2S_3, M_3S_2, M_3S_3, M_2S_2) as a result of recombination, and only a single genotyped gamete (M_2S_2) is produced by the maternal parent. However, only the pollen gametes with the S_3 genotype (M_2S_3 and M_3S_3) can effect fertilization on the S_2 genotyped stigma (M_2S_2), therefore all the progeny are heterozygous at the *S* locus (S_2S_3). The individuals resulting from a recombination event between M loci and the *S* locus (M_2S_3) are homozygous at M loci (M_2M_2), while those with no recombination between M loci and the *S* locus (M_3S_3) are heterozygous at M loci (M_2M_3). Detection of recombinants between the *Z* locus and a linked marker is carried out using the same scheme.

6.1.5 Genetic mapping in L. perenne

Genetic linkage maps for *L. perenne* and closely related species have been developed over the last ten years and a number of the published genetic linkage maps of *Lolium* spp. and its allies were summarised in Table 6.1.1. These maps were developed from an assortment of molecular markers including isozymes, RFLPs, RAPDs, AFLPs, SSR markers, inter simple sequence repeat (ISSR) markers, telomeric repeat associated sequence (TAS) markers, CAPS, SNPs and STSs. Several maps, specially those developed most recently, were based predominantly on SSR markers (Jones *et al*., 2002a; Warnke *et al*., 2004; Jensen *et al*., 2005b; Hirata *et al*., 2006; Gill *et al*., 2006; King *et al*., 2008; Anhalt *et al*., 2008). SSRs are tandem repeated sequences comprised of small sequence motif of 1 to 6 bp, as mono-, di-, tri-, tetra-, penta-, or hexa-nucleotide (Ellegren, 2004). Polymorphisms generated with SSRs are due to variation in the number of repeated units. By designing PCR primers in the conserved non-repetitive flanking regions of SSRs, polymorphisms can be easily detected as length variations. SSRs are codominant markers, highly reproducible, multiallelic, widely dispersed throughout the genome in both coding- and non-coding regions and amenable to automated analysis (Powell *et al*., 1996a). They have become an ideal tool for genotyping plant material, surveying genetic diversity, molecular mapping, MAS as well as identifying genes of interest (Varshney *et al*., 2005). The PCR-based character of SSRs makes them transferable between and within species. A degree of conservation in SSR motifs has been shown in closely related species (Asp *et al*., 2007), making SSR markers a valuable resource for comparative genetic mapping and tagging genes of interest. SSR markers are expensive and time consuming to develop (Squirrell *et al*., 2003), however, development and information on SSR markers for *Lolium* has increased rapidly in recent years, making them partially publicly available to research groups and being widely used for genetic and breeding studies (Faville *et al*., 2004;

Kubik *et al*., 2001; Lauvergeat *et al*., 2005; Jensen *et al*., 2005a; King *et al*., 2008; Studer *et al*., 2008a).

Table 6.1.1 Published genetic maps of *Lolium* spp and allies. The pedigree and the size of mapping populations, the map length, marker types and references are given.

Species	Population	Genotypes	Map length	Markers	Reference
Interspecific	One-way pseudo-testcross Doubled haploid x interspecific hybrid F1 (*L. perenne* x *L. multiflorum*)	89	692 cM	41 RFLPs, 48 RAPDs, 17 isozymes	Hayward *et al*., 1998
L. perenne L.	One-way pseudo-testcross p150/112 Di-haploid (♀) x hybrid F1 (♂)	95	930 cM	463 AFLPs, 3 isozymes, 5 ESTs	Bert *et al*., 1999
L. perenne L.	One-way pseudo-testcross p150/112	155	814 cM	258 loci (93 SSRs, 86 AFLPs, 74 RFLPs, 5 isozymes, STSs)	Jones *et al*., 2002a
L. perenne L.	One-way pseudo-testcross p150/112	183	811 cM	240 loci (RFLP, AFLP, isozyme, EST markers)	Jones *et al*., 2002b
L. perenne L.	BC_1-type p150/112	156	515 cM (327 cM*)	133 loci	Armstead *et al*., 2002 (*developed with common loci)
	F2 (self-pollinating a single hybrid plant Perma x Aurora)	180	565 cM (446 cM*)	74 loci	
	Integrated		516 cM (361 cM*)	133 RFLPs, 13 AFLPs, 4 isozymes, 2 SSRs	
Festuca pratensis Huds.	Two-way pseudo-testcross F1 (B14/16 ♀ x HF2/F ♂)	138	Maternal 602.3 cM	223 loci	Alm *et al*., 2003
			Paternal 581.5 cM	237 markers (RFLPs, AFLPs, Isozymes, SSRs)	
			Combined 658.8 cM	466 markers	
L. perenne L.	Two-way pseudo-testcross F1 (NA_6 x AU_6)	157	NA_6 963 cM	88 EST-RFLPs, 71 EST-SSRs	Faville *et al*., 2004
			AU_6 757 cM	67 EST-RFLPs, 8 EST-SSRs	
Interspecific	Three-generation *L. multiflorum* x *L. perenne*	91	Female 712 cM Male 537 cM	235 AFLPs, 106 SSRs, 81RAPDs, 16 RFLPs, 2 isozyme loci, 2 morphological markers	Warnke *et al*., 2004
L. multiflorum Lam.	Two-way pseudo-testcross F1 (Nioudachi x Nigatawase)	82	1244.4 cM	199 RFLPs, 116 AFLPs, 70 TASs	Inoue *et al*., 2004
L. perenne	Two-way pseudo-testcross F1 (heterozygous SB2 x TC1)	252	744 cM	227 loci (AFLPs, SSRs, RFLPs, STSs)	Muylle *et al*., 2005

Species	Population		Genotypes	Map length	Markers	Reference
L. perenne L.	Two-way pseudo-testcross F2 VrnA (Synthetic variety "Veyo" x ecotype "Falster")		184	490.4 cM	59 AFLPs, 33 SSRs, 1 CAP	Jensen *et al*., 2005a
L. perenne L.	One-way pseudo-testcross p150/112		87	Consensus 772 cM	317 markers (65 SSRs, AFLPs, RFLPs, STSs)	Jensen *et al*., 2005b
	Two-way pseudo-testcross F2 VrnA		184			
	SB2TC1 (Muylle *et al*., 2005)		252			
	Pop8490		147			
L. multiflorum Lam.	Two-way pseudo-testcross F1 (11S2 ♀ x 11F3 ♂)		60	Female 887.8 cM	236 SSRs, 69 AFLPs, 34 RFLPs	Hirata *et al*., 2006
				Male 795.8 cM	251 SSRs, 78 AFLPs, 32 RFLPs	
L. perenne L.	Two-way pseudo-testcross F1 (NA_6 X AU_6)		157	NA_6 1070.1 cM	79 gene associated SNPs (integrated to Faville *et al*., 2004)	Cogan *et al*., 2006
				AU_6 868.6 cM		
L. perenne	F2 (selfing F1 hybrid between two partially inbred parental lines) (same as Armstead *et al*., 2002)		94	675.6 cM (framework map)	376 gene-tagged SSRs, 9 RFLPs	Gill *et al*., 2006
L. multiflorum Lam.	Two-way pseudo-testcross F1 (11S2 ♀ x 11F3 ♂)		60	Female 799.8 cM	69 EST-CAPS, AFLPs, RFLPs, SSRs	Miura *et al*., 2007
				Male 906.4 cM		
L. perenne	BC_2 type (intercrossing and backcrossing of forage and amenity genotypes)	Forage	100	433.8 cM	118 SSR loci	King *et al*., 2008
		Amenity	79	438.5 cM	94 SSR loci	
L. perenne	F2 biomass		360	592.3 cM	65 SSRs, 10 AFLPs	Anhalt *et al*., 2008
	F1 late flowering		182	225.7 cM	55 SSRs, 5 CAPS	
L. perenne	Two-way pseudo-testcross F2 VrnA (same as Jensen *et al*., 2005ab)		92	250.7 cM	27 ISSRs	Pivoriene *et al*., 2008

6.1.6 Aims

In this chapter, the development of *L. perenne* fine mapping populations for the *S* and *Z* loci is described. The objectives were to (1) generate linkage maps for the *S* and *Z* loci on the fine mapping populations; (2) identify tightly linked markers for the *S* and *Z* loci; (3) compare linkage maps between the ILGI population and *S* and *Z* fine mapping populations with common markers; (4) attempt linkage analysis of the candidate genes identified in the SI SSH libraries with the *S* or *Z* locus. Genetic mapping was carried out with available SSR markers (published and licensed), and STS, EST and CAPS markers collected during this study.

6.2 Results

6.2.1 Selection of SSR markers

A total of 51 SSR markers that have been mapped on *Lolium* LG1 and LG2 were tested on the parental DNA of the *S*- and *Z*-population in order to detect polymorphism as described in section 2.4.3. All markers showed amplification and fifteen of them were identified to be polymorphic between the parents, p235/40/13 and p232/88/10. Three of the polymorphic SSRs showed poor amplification when tested on the mapping progeny and were omitted from further analysis (LPSSRHXX238, LpACT26F10 and M15185). At the end, twelve polymorphic SSR markers were used to analyze recombination frequencies (Table 6.2.2), first on a subset of the fine mapping populations, an average of 200 genotypes in each of the *S*- and *Z*-population. The SSR markers showing close linkage with *S* or *Z* loci were further screened on the entire fine mapping populations and as well on the ILGI population. The results of SSR markers selection was summarised in Table 6.2.1. The results of SSR markers analysis was described in section 6.2.4.

Table 6.2.1 Summary of tested SSRs, polymorphic SSRs and selected SSRs for mapping in the fine mapping populations.

Linkage group	Number of tested SSRs	Polymorphic SSRs	Final selected SSRs	% polymorphic
LG1	23	11	9	39%
LG2	28	4	3	11%

Table 6.2.2 List of polymorphic SSR markers selected on *L. perenne* LG1 and LG2 for fine mapping the *S* and *Z* loci. The source of each marker is given.

LG1		LG2	
Source	**Marker**	**Source**	**Marker**
ViaLactia	rv0252	ViaLactia	rv0122
	rv0033	CRC	LPSSRK12E03
Kubik *et al*., 2001	PR25	Jensen *et al*., 2005	LpSSR112
	PR8		
	PRE		
CRC	LPSSRK12D11		
Jensen *et al*., 2005	LpSSR085		
	LpSSR057		
Lauvergeat *et al*., 2005	B3-B7		

6.2.2 Development of mapping markers

Markers for the ten SI candidate genes identified in SSH libraries were designed as described in section 2.4.4 and summarised in Table 6.2.3. Markers for four candidate genes were developed.

Table 6.2.3 Summary of marker development for mapping candidate genes. 'Mono-' refers to monomorphic genotype on ILGI plants; 'Poly-' refers to polymorphic genotype on ILGI plants. 'Y' refers to Yes; 'N' refers to No or None. '/' means not available.

Name	EST marker	SNPs	Restriction Enzyme	Marker for mapping
Can3	Mono-	Y	N	/
Can4	Mono-	N	N	/
Can10	Poly-	-	-	EST marker
Can18	Mono-	Y	N	/
Can94	Mono-	N	N	/
Can130	Mono-	Y	*BstZ*I	CAPS marker
Can135	Mono-	N	N	/
Can136	Mono-	Y	*Mse*I	CAPS marker
Can139	Mono-	Y	N	/
Can151	Mono-	N	N	/
Lolium G10	Mono-	Y	*Bcc*I	CAPS marker

6.2.2.1 EST markers

Only the EST marker developed for *Can10* (Table 2.4.5) amplified polymorphic products on the ILGI population (Figure 6.2.1) and was applied directly for linkage analysis for the *S* and *Z* loci. Primer pairs of the other nine candidate genes (Table 2.4.5) amplified in ILGI genotypes, but showed no evident polymorphism on the agarose gel (results not shown) and were thus subjected for SNPs detection.

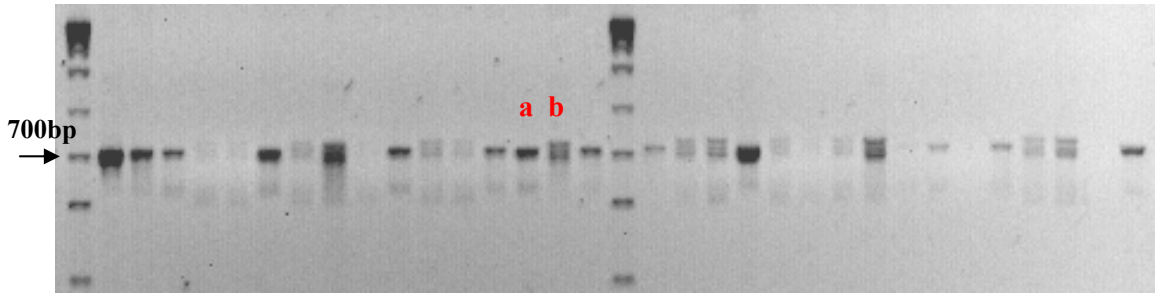


Figure 6.2.1 The EST marker of *Can10* amplified polymorphic products on the ILGI population. The gel picture shows amplification of 32 ILGI genotypes. The two genotypes for scoring segregation patterns of the marker are indicated with 'a' and 'b'. A single band of the 1 kb DNA ladder (Metabion) is indicated.

6.2.2.2 CAPS markers

After comparing the sequences of the amplified products from ILGI genotypes with different *S* and *Z* genotypes, SNPs were identified for *Can3*, *Can18*, *Can130*, *Can136* and *Can139* (Table 6.2.3). Except for *Can139*, CAPS markers were developed from the SNPs and the markers for *Can130* and *Can136* were applicable for linkage analysis on the ILGI population (Figure 6.2.2 and Figure 6.2.3). The CAPS marker for the *Lolium* G10-protein gene was also developed and analyzed on the ILGI population (Figure 6.2.4).

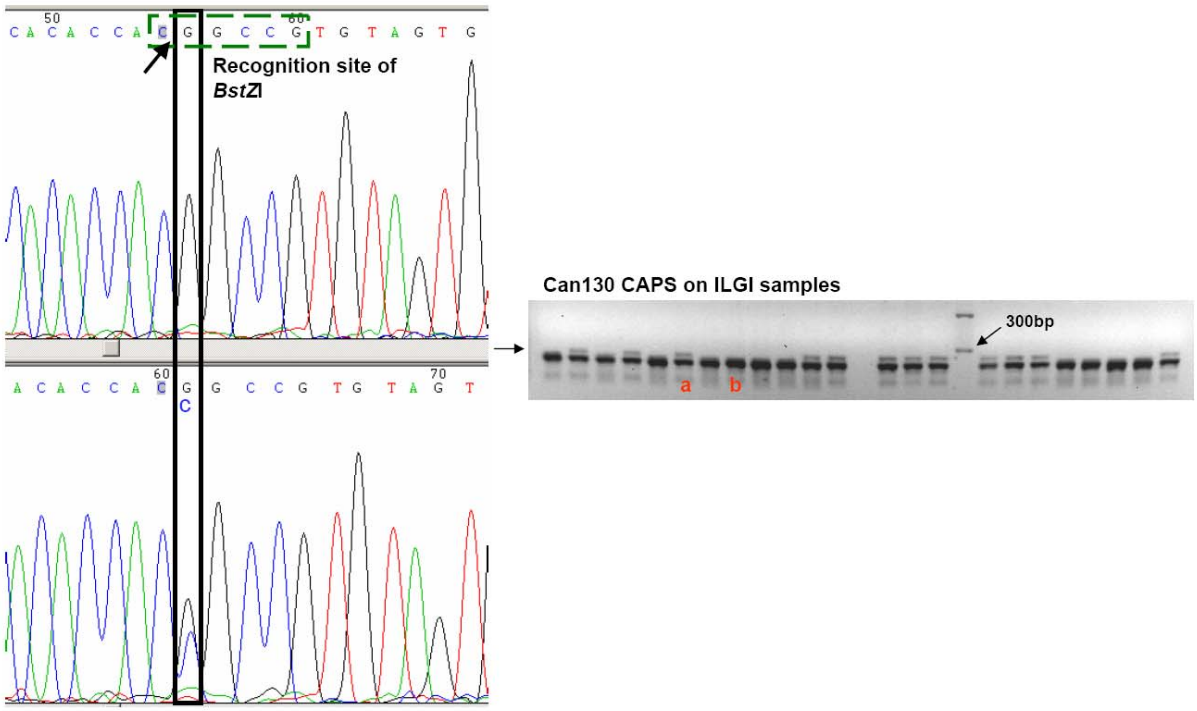


Figure 6.2.2 Mapping of *Can130* with a CAPS marker. The EST marker of *Can130* was used to amplify ILGI DNAs with different *S* and *Z* genotypes and the resulting PCR products were sequenced. A G/C SNP (indicated with a black box on the chromatographs) was detected between amplified products of two different *S* genotypes (S_1S_2 and S_1S_3). The SNP resided within the recognition site (indicated with a green dot box) of the enzyme *BstZ*I (the cutting site is indicated with an arrow). For a random selection of ILGI genotypes PCR products were amplified and the products digested with *BstZ*I (shown on the gel). The two genotypes for scoring segregation patterns of the CAPS marker are indicated with 'a' (with the G/C SNP revealing two bands) and 'b' (without SNP revealing one band after digestion). A single band of the 100 bp DNA ladder is indicated on the gel. The CAPS marker was applied to the entire ILGI population after verifying polymorphism. Sequencing results were visualised using Chromas Version 2.13.

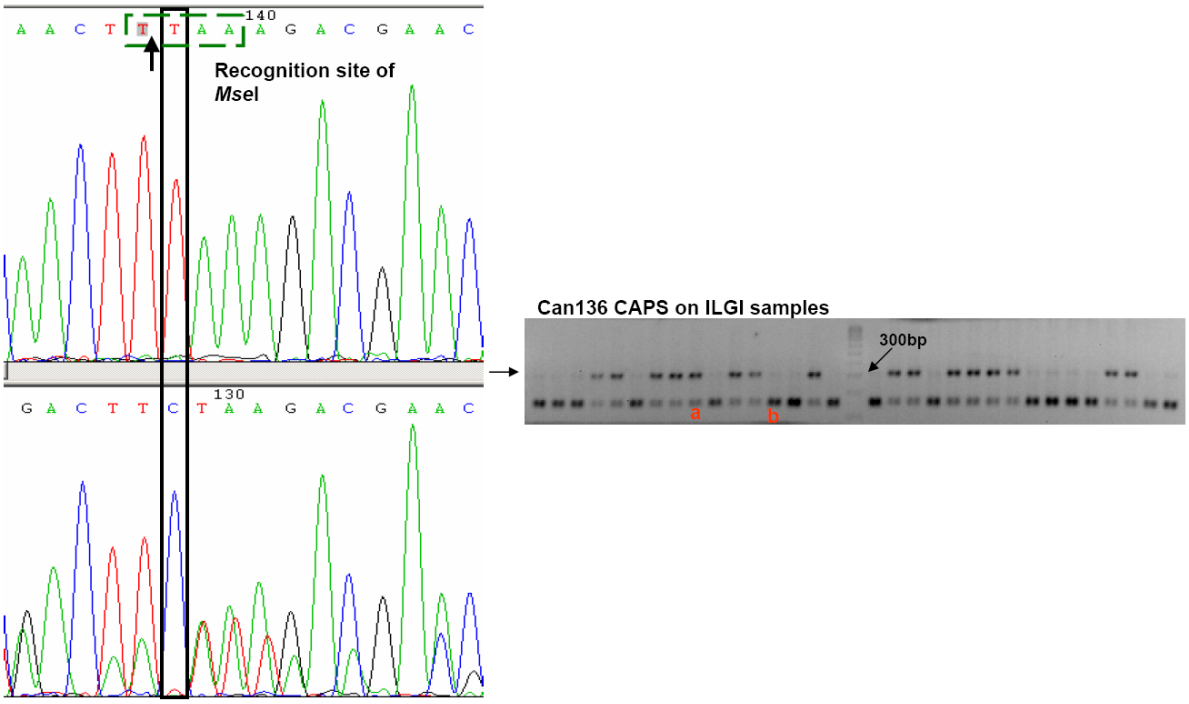


Figure 6.2.3 Mapping of *Can136* with the CAPS marker. The EST marker of *Can136* was used to amplify ILGI DNAs with different *S* and *Z* genotypes and the resulting PCR products were sequenced. A T/C SNP (indicated with a black box on the chromatographs) was detected between amplified products of two different *S* genotypes (S_1S_2 and S_1S_3). The SNP resided within the recognition site (indicated with a green dot box) of the enzyme *Mse*I (the cutting site is indicated with an arrow). A random selection of DNAs of ILGI plants were amplified and the products were digested with *Mse*I (shown on the gel). The two genotypes for scoring segregation patterns of the CAPS marker are indicated with 'a' (with the T/C SNP revealing two bands) and 'b' (without SNP revealing one band after digestion). A single band of the 100 bp DNA ladder is indicated on the gel. The CAPS marker was applied to the entire ILGI population after verifying polymorphism. Sequencing results were visualised using Chromas Version 2.13.

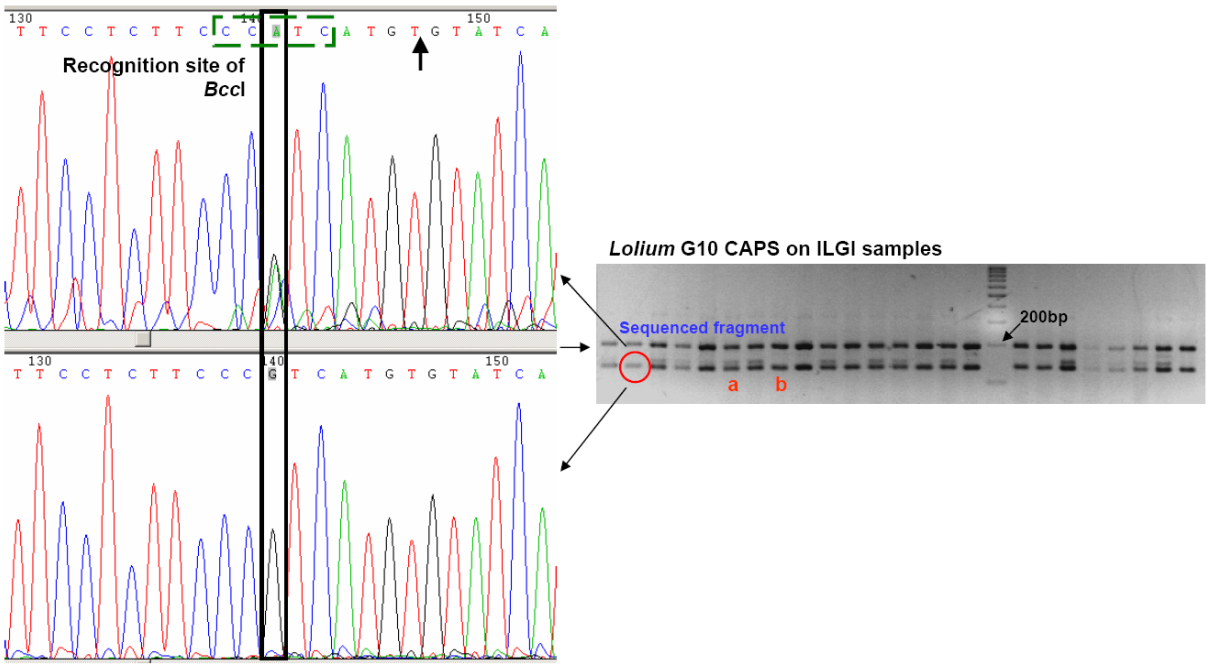


Figure 6.2.4 Mapping of the *Lolium* G10-protein gene with a CAPS marker. The EST marker of *Lolium* G10 was used to amplify ILGI DNAs with different *S* and *Z* genotypes. Two fragments were obtained for each amplicon. They were gel purified and sequenced separately. The amplified fragment (indicated with a red circle on the gel picture) containing an A/G SNP (indicated with a black box on the chromatographs) was identified between two different *S* genotypes (S_1S_2 and S_1S_3). The SNP resided within the recognition site (indicated with a green dot box) of the enzyme *Bcc*I (the cutting site is indicated with an arrow). A random selection of ILGI plants were amplified and the products digested with *Bcc*I were shown on the gel. The two genotypes for scoring segregation patterns of the CAPS marker are indicated with 'a' (with the A/G SNP revealing two lower bands) and 'b' (without SNP revealing one lower band after digestion). A single band of the 100 bp DNA ladder is indicated on the gel. The CAPS marker was applied to the entire ILGI population after verifying polymorphism. Sequencing results were visualised using Chromas Version 2.13.

No applicable markers for mapping were developed for the remaining seven candidate genes. Mapping of these seven SI candidate genes with a BAC sequencing approach will be the subject of future research.

6.2.2.3 STS marker

Using a comparative genetics approach, as described in Chapter 3, STS markers were developed from the rice genome in the regions flanking the *S* and *Z* loci. One STS marker

(05g33100) derived from the gene sequence on rice chromosome 5 was identified showing segregation patterns on the ILGI population (Figure 6.2.5). It was included in linkage analysis for the determination of the genetic distance of the candidate genes on the ILGI population.

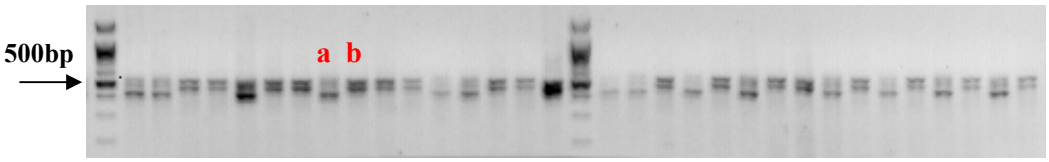


Figure 6.2.5 The STS marker 05g33100 amplified polymorphic products on the ILGI population. The gel picture shows amplification of 32 ILGI plants. The two genotypes for scoring segregation patterns of the marker are indicated with 'a' and 'b'. A single band of the 100 bp DNA ladder is indicated.

6.2.3 Mapping of markers on the ILGI population

A total of 19 markers were subjected to linkage analysis using the ILGI population. They were listed in Table 6.2.4. The SSR markers were selected based on their close linkage with the *S* or *Z* loci identified by mapping on the fine mapping populations (see section 6.2.4). 15 markers were mapped on the *L. perenne* ILGI population on LG1 and LG2 (Figure 6.2.6).

Table 6.2.4 List of markers used in the linkage analysis on the ILGI population. Information on the type of each marker is given.

Name	Type of marker
LpGK1* LpGK2* LpCadelp* *Can10*	EST *from Dr Bruno Studer
Can130 *Can136* *Lolium G10*	CAPS
04g54940 04g55260 04g55290 05g30800 05g33100 05g43480	STS
Rv0122 Rv0252 LpSSR057 LpSSR112 LPSSRK12D11 LPSSRK12E03	SSR

Four markers, *Can10* (EST), *Can130* (CAPS), 05g43480 (STS) and LpSSR112 (SSR), were not mapped on *L. perenne* LG1 and LG2, displaying no linkage with the *S* or *Z* locus. CAPS markers for *Can136* and the *Lolium* G10-protein gene were mapped on LG1, showing linkage with the *S* locus, consistent with the expected segregation patterns of these two markers (Figure 6.2.3 and Figure 6.2.4). However, they were mapped 38 cM and 20 cM away from the *S* locus, respectively, implying that *Can136* and the G10-protein are probably not directly involved in the *L. perenne* SI response. The STS marker 04g55290 showed previously linkage with the *S*-distorted locus on LG3 (refer to section 3.2.1) and using this mapping strategy was mapped on *L. perenne* LG1, located at a map distance of 32 cM away from the *S* locus (Figure 6.2.6). For the *Z* locus, two STS markers (04g54940 and 04g55260) and three EST markers (LpGK1, LpGK2 and LpCadelp) were mapped at distances less than 8 cM (Figure

6.2.6), showing relatively close linkage with the *Z* locus. Therefore, they were further fine mapped on the entire *Z*-population and the results were described in section 6.2.4.3.

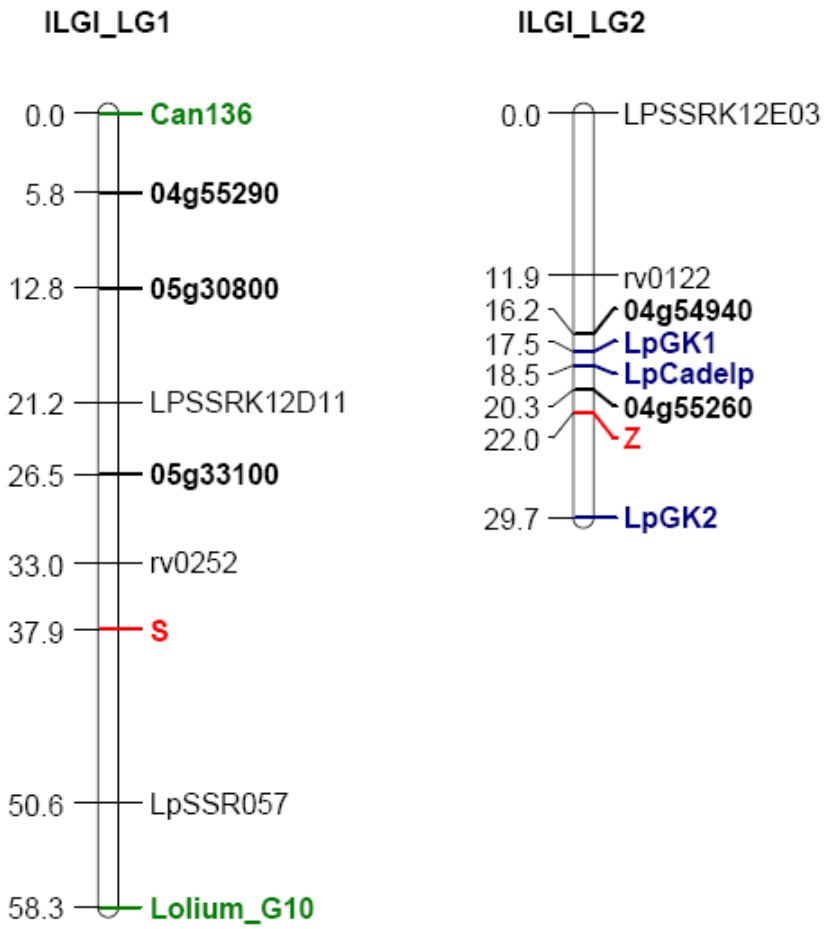


Figure 6.2.6 Genetic map developed from the ILGI population displaying the genetic distance between markers on *L. perenne* linkage groups 1 and 2, respectively. Genetic distances are given in centimorgans (cM). *S* and *Z* loci are marked in bold and red color. CAPS markers for *Can136* and *Lolium* G10-protein gene are highlighted in green color. STS markers are indicated in bold type and EST markers are highlighted in blue color. The other markers on the linkage groups are SSR markers.

6.2.4 Marker analysis on the fine mapping populations

6.2.4.1 Selected screening for selfs progeny or contaminants

SSR marker Rv1112 was used to screen for self progeny or contaminants in the *S*- and *Z*-populations as described in section 2.4.3. The results were listed in Table 6.2.5. The entire *Z*-

population was screened with marker Rv1112 for fine mapping of tightly linked markers identified in section 6.2.3. For the *S*-population, about a quarter of the progeny was screened initially for verifying SSRs analysis. The mapping results on the ILGI population (Figure 6.2.6) showed that only one SSR marker Rv0252 was linked rather close to the *S* locus, at a distance of ~5 cM; while all the other markers were mapped more than 10 cM away from the *S* locus. As a result, the screening and fine mapping analysis were not carried on for the remaining individuals of the *S*-population.

Table 6.2.5 Information of the fine mapping populations used for linkage analysis. The numbers listed for each population are: plants screened, identified selfs progeny or contaminants, and the genotypes used for marker analysis.

Population	Screened	Selfs or contaminants	Contamination level	Genotypes
S fine mapping	340	3	0.88%	**337**
Z fine mapping	1196	14	1.17%	**1182**

6.2.4.2 SSR markers analysis on a subset of the fine mapping populations

Twelve SSR markers were selected (Table 6.2.1). Nine SSRs on *L. perenne* LG1 were analyzed on a population of 337 genotypes from the *S*-population and the three SSRs on LG2 were analyzed on a population of 239 genotypes from the *Z*-population. Four STS markers were also included in the analysis: 05g33100 and 05g30800 on the *S*-population; 04g55260 and 04g54940 on the *Z*-population. Data were scored as described in section 2.4.5 to determine the distance between markers and the *S* or *Z* loci. Recombination frequencies (Rf) for each marker were calculated from an average of 200 (ranging from 113 to 299) genotypes in the *S*-population and 180 (ranging from 159 to 200) genotypes in the *Z*-population. The mapping results of LG1 SSR markers were shown in Table 6.2.6 and that of LG2 in Table 6.2.7.

Table 6.2.6 Recombination frequencies (Rf) of SSR markers on LG1 in the *S*-population. The SSR markers were listed in order of Rf value.

Marker	Genotypes	Recombinants	Rf %
LPSSRK12D11	291	4	1.374570
Rv0252	299	10	3.344482
LpSSR057	199	15	7.537688
Rv0033	151	14	9.271523
05g33100	292	43	14.726027
PRE	122	24	19.672131
05g30800	178	37	20.786517
B3-B7	124	28	22.580645
LpSSR085	128	29	22.656250
PR25	113	27	23.893805
PR8	140	43	30.714286

Table 6.2.7 Recombination frequency (Rf) of SSR markers on LG2 in the *Z*-population. The SSR markers were listed in order of Rf value.

Marker	Genotypes	Recombinants	Rf %
04g55260	175	1	0.57142857
LPSSRK12E03	200	14	7.00000000
04g54940	181	13	7.18232044
LpSSR112	196	15	7.65306122
Rv0122	159	21	13.20754717

The LG2 SSR markers and three of the LG1 SSR markers (LPSSRK12D11, Rv0252 and LpSSR057), which were mapped close to the *S* locus with smaller recombination frequencies on the *S*-population, were subjected to linkage analysis on the ILGI population as shown in section 6.2.3. Differences in the order and distances between common markers on different populations were observed (Figure 6.2.7). The value of map distance between markers derived from the *S*-population and *Z*-population was smaller than that of the ILGI population. The size differences of the mapping populations might be a reason, as the analyzed *S*- or *Z*-populations contained twice the genotypes compared to the ILGI population, which could as well explain the altered order of markers on the linkage maps between different populations: LPSSRK12D11 on LG1 was mapped at a distance of 1.4 cM away from the *S* locus on the *S*-population but on the ILGI population with a map distance of 16.8 cM from the *S* locus; and

LPSSRK12E03 on LG2 was mapped to the *Z* locus at a distance of 7 cM on the Z-population but 22 cM on the ILGI population. Technical problems concerning the ILGI population might also disturb genetic mapping results, because there was a possible mix up of genotypes of the ILGI population at the time of DNA extraction (with a second batch of individuals after propagation). The genotype mix-up might cause false scores for the markers LPSSRK12D11 and LPSSRK12E03 and lead to the consequent disorder of their locations on the linkage maps of the ILGI population compared to the fine mapping populations.

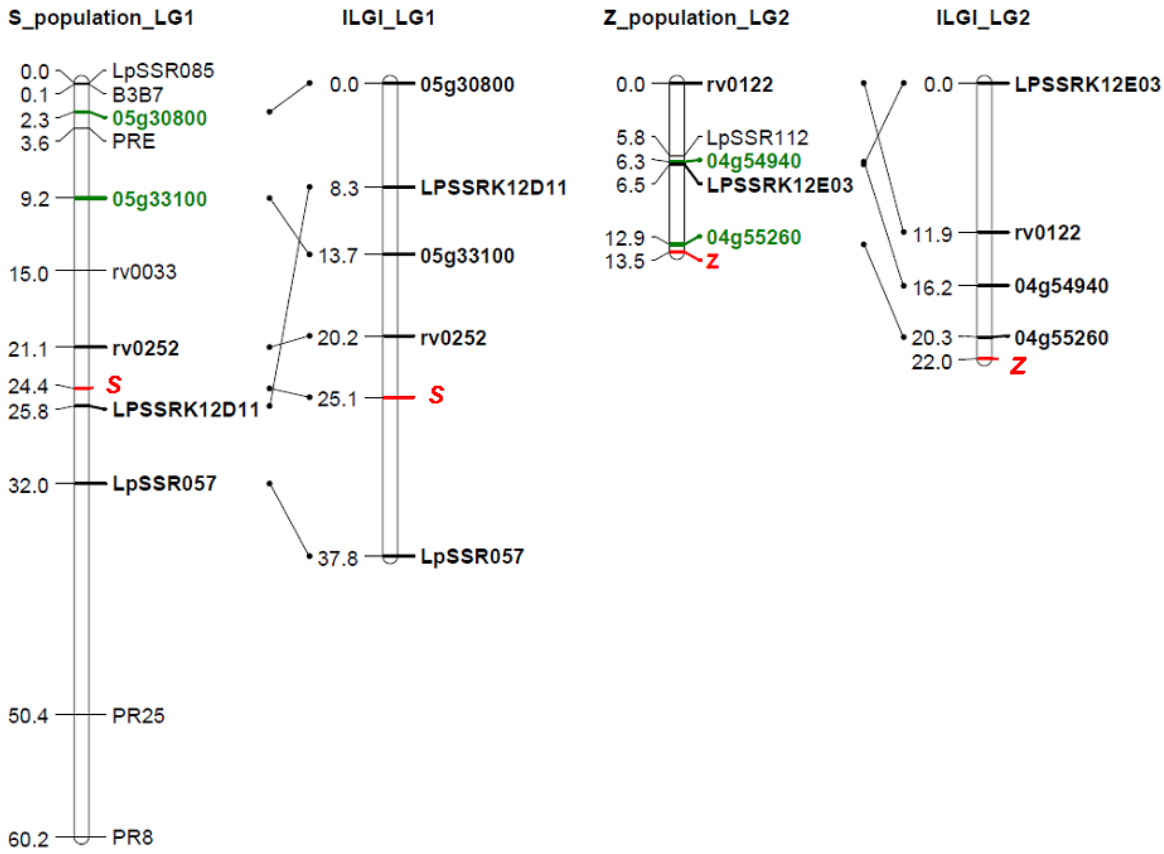


Figure 6.2.7 Comparison of linkage group 1 and 2 genetic maps derived from SSR markers and STS markers (highlighted in green color) between the *S*-population or the *Z*-population and the ILGI population. The order of SSR markers on the fine mapping population was determined by scoring recombinants. The markers which shared common recombinants were linked to the same chromosome ends while those with no common recombinants were mapped to the opposite chromosome ends of the *S* or *Z* loci. Map distances were given in cM to the left of linkage group bars.

6.2.4.3 Fine mapping on the *Z*-population

Five markers which showed close linkage to the *Z* locus have been identified (section 6.2.3). They were subjected to linkage analysis on the entire *Z*-population to determine their map distances with the *Z* locus. The *Z*-population contained 1182 genotypes in total (Table 6.2.5). Recombination frequencies of the five markers were calculated from an average of 1020 genotypes, ranging between 951 and 1094. Mapping results for these closely linked *Z* markers on the *Z*-population are shown in Table 6.2.8. The linkage map was developed after scoring the recombinants and compared with the map derived from the ILGI population (Figure 6.2.8).

Table 6.2. 8 Recombination frequency (Rf) of closely linked *Z* markers. The markers were listed in order of Rf value.

Marker	Genotypes	Recombinants	Rf %
LpGK2	1094	10	0.914076782
04g55260	989	16	1.617795753
LpGK1	1058	19	1.79584121
LpCadelp	1049	22	2.192564347
04g54940	951	61	6.414300736

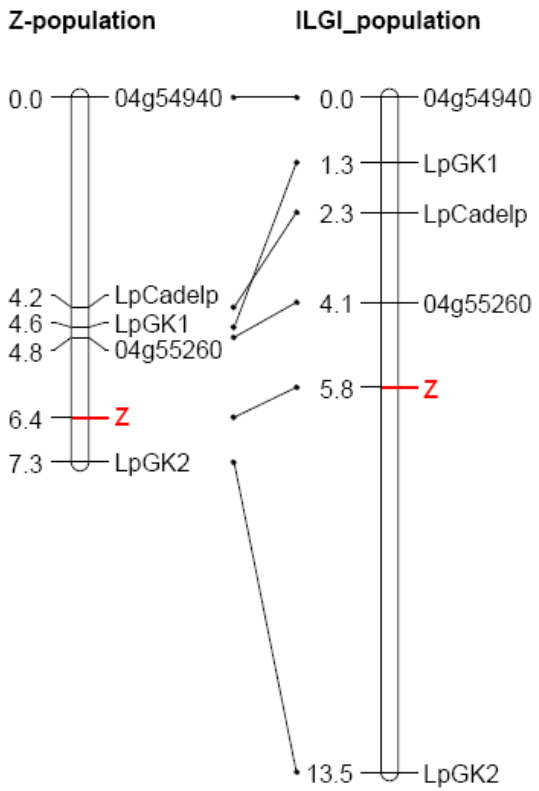


Figure 6.2.8 Comparison of the linkage maps derived from tightly linked *Z*-markers between the *Z*-population and the ILGI population. The order of markers on the *Z*-population was determined by scoring recombinants. Map distances were given in cM at the left of linkage group bars.

The closest mapped *Z*-marker on the ILGI population was the STS marker 04g55260. This marker mapped at a distance of 1.7 cM from the *Z* locus; while LpGK2 on the *Z*-population was mapped in a distance of 0.9 cM and was the closest marker to the *Z* locus. On the *Z*-population, a comparable result was obtained for 04g55260, which was mapped at a distance of 1.2 cM from the *Z* locus. Mapping of the other STS marker 04g54940 on the two populations also revealed similar map distances with the *Z* locus, 5.8 cM on the ILGI population and 6.4 cM on the *Z*-population, respectively. The three EST markers (LpGK1, LpGK2 and LpCadelp) mapped closer to the *Z* locus on the Z-population than on the ILGI population: LpGK1 with a map distance of 1.8 cM compared to 4.5 cM; LpGK2 with a map distance of 0.9 cM compared to 7.7 cM; and LpCadelp with a map distance of 2.2 cM compared to 3.5 cM. LpGK1 showed close linkage with 04g55260 on the *Z*-population, with

a map distance interval of 0.2 cM, but were 2.8 cM away from each other on the ILGI linkage map. The order of LpGK1 and LpCadelp was altered comparing the two maps, which could be the effect of different types and sizes of mapping populations.

6.3 Discussion

Genetic maps covering the *L. perenne S* and the *Z* loci regions were developed on the ILGI population for the purpose to determine the relationships among identified candidate SI genes (Chapter 5) and the *S* or *Z* loci. Two candidate genes, *Can136* and the *Lolium* G10-protein gene, were mapped on LG1, but at a distant position to the *S* locus, which excludes them being the SI components. No linkage relationships were identified between *Can10*, *Can130* and the *S* or *Z* loci as the two candidates were not mapped on LG1 or LG2. These results can be explained by the findings based on the full length sequences of the candidate genes. BLAST search was carried out to identify the rice homologies using the full length cDNA sequences obtained for six candidate genes (*Can3*, *Can10*, *Can94*, *Can130*, *Can135* and *Can136*). After the full length sequences of the six candidate genes became available, better fitting homologies with a higher gene identity to other rice genes were found (Table 4.2.7). Compared to the rice homologies identified using their EST in the SI cDNA libraries, each of the new rice homologies for the candidate genes was shown to have the almost same function but at a different location on the rice genome (Table 4.2.7). In contrast to the original identified rice homologies on rice R5, the candidate genes *Can3*, *Can10* and *Can136* have better fitting rice homologies on rice R1; *Can94* and *Can130* have better fitting rice homologies on rice R12; and that of *Can135* on rice R10. Since the syntenic region of the *S* locus is on rice R5 and that of the *Z* locus is on rice R4, it is not surprising that *Can10* (on rice R1) and *Can130* (on rice R12) were not linked with the *S* or *Z* loci. The relationship of

Can136 (on rice R1) and the *S* locus was interesting. Thorogood et al. (2002) reported a locus on *L. perenne* LG3 (orthologous to rice R1) showing distorted segregation with the *S* locus, implying the possible association of *Can136* with the *S*-distorted locus. Though not directly linked with *S* or *Z*, the possible roles of these candidates in the downstream SI reactions cannot be ruled out. The genetic positions of the remaining candidate genes in relation to the *S* or *Z* locus remain undetermined. If any of those would show close linkage with the *S* or *Z* loci, the function of that candidate would be of particular interest for further investigation and the marker could be used for future MAS and map-based cloning.

The construction of detailed linkage maps of the *S* and *Z* loci can assist in the identification of tightly linked markers and the determination of the map positions of *S* and *Z*, and also provides a start point for map-based cloning of the SI genes. In this study, large segregating populations were developed for fine mapping the *S* and *Z* loci in *L. perenne*. Populations generated from the same principle for the respective species have been developed for the fine mapping of the SI loci in *P. coerulescens* (Bian *et al*., 2004) and *H. bulbosum* (Kakeda *et al*., 2008). Because of the possible effects of pseudo-self-compatibility (Wricke, 1978; Hayman, 1992) and other environmental influences during pollination, contaminants are normally obtained in the progenies. For fine mapping purposes, it is important to detect contaminating individuals in the population, as even only a few of them would lead to inaccurate results to a great extent. The contamination levels of the fine mapping populations developed in this study were 0.88% for the *S*-population and 1.17% for the *Z*-population (Table 6.2.5). Compared with the fine mapping populations in *P. coerulescens*, an average contamination level of 2.6% was reported for the *S*-population and 0.5% for the *Z*-population (Bian *et al*., 2004). The different level of contamination observed here appear to be related to the size of progeny in the fine mapping population. The *S*-population of *L. perenne* contained less than

half of the progeny as in *P. coerulescens* (340 vs 862) combined with a lower contamination level; while the progeny in the *Z*-population of *L. perenne* was more than five times of that in *P. coerulescens* (1196 vs 213), hence the higher contamination level. The ease of distinguishing recombinants from contaminants in this type of fine mapping population is therefore an evident and essential advantage to obtain reliable data for mapping.

The detailed *S* and *Z* loci genetic maps developed in this study are based on SSR markers (Figure 6.2.7). For outbreeding species as *L. perenne*, SSR markers have been revealed as an ideal tool for MAS breeding, genotyping for the purpose of varietal identification and seed-purity certification, and isolation of genes of interest via map-based cloning (Powell *et al*., 1996a; Gupta and Varshney, 2000; Varshney *et al*., 2005). SSR markers and SSR-based genetic linkage maps for *Lolium* spp. are increasingly under development since the last few years (Kubik *et al*., 1999, 2001; Jones *et al*., 2001; Warnke *et al*., 2004; Lauvergeat *et al*., 2005; Jensen *et al*., 2005b; Studer *et al*., 2006; Gill *et al*., 2006; King *et al*., 2008; Studer *et al*., 2008a), which facilitates in this study the selection of SSR markers mapped on *Lolium* LG1 and LG2, where the *S* and *Z* loci are located, respectively (Thorogood *et al*., 2002). Around twenty-five SSR markers each on LG1 and LG2 were tested on the relative fine mapping populations, of which 39% from LG1 were utilizable on the *S*-population and 11% from LG2 were utilizable on the *Z*-population. The low percentage of polymorphic SSR markers on the *S* and *Z* loci fine mapping populations could be explained by the effect of incompatibility when producing mapping progenies. Only 50% of the pollinations between the parents were compatible (Figure 6.1.1), leading to a putative under-representation of some areas of the genome with markers. The closest SSR marker for the *S* locus had a map distance of 1.4 cM on the *S*-population, while the closest SSR on the *Z*-population was mapped 7 cM away from the *Z* locus. Compared with their map locations on the ILGI population,

discrepancies were observed between the two different populations (Figure 6.2.7), e.g. the closest *S* marker on the *S*-population was mapped on the ILGI population LG1 more than 15 cM away from the *S* locus and the closet *Z* marker on *Z*-population was mapped on ILGI population LG2 more than 20 cM away from the *Z* locus. The differences could be explained by the relative smaller size of the ILGI population (96 genotypes) compared to the fine mapping populations (337 genotypes for the *S*-population and 239 genotypes for the *Z*-population). Different mating schemes (Liu, 1998) and different genetic backgrounds (Barth *et al*., 2001) may also be the reason for variation in recombination frequency. However, the degree of discrepancies obtained between the fine mapping populations and the ILGI population was intriguingly high. Therefore it is more likely that the mix up of genotypes of the ILGI population could cause a major effect, leading to false scoring during the mapping on the ILGI population and leading to differences on the genetic linkage maps.

Mapping of the SI loci has been performed in several grass species including *L. perenne*. The most close linked marker or co-segregating markers for the *S* or the *Z* loci have been reported in each species. They were summarized in Table 6.3.1, including the results for *L. perenne* in this thesis. Up to date, co-segregating markers have been reported for the *S* locus in *P. coerulescens* (Bian *et al*., 2004) and *H. bulbosum* (Kakeda *et al*., 2008), and for the *Z* locus in *Secale cereale* (Hackauf and Wehling, 2005) and *H. bulbosum* (Kakeda *et al*., 2008). In *L. perenne*, *S* or *Z* co-segregating markers have not been identified so far, while the closest markers were developed on the ILGI population through a comparative mapping approach as described in Chapter 3. Interestingly, the closest *Z* marker on the ILGI population, 04g55260 with a map distance of 0.2 cM, was mapped 1.6 cM away from the *Z* locus on the fine mapping population (Figure 6.2.8), implicating that the marker LpGK2, with a map distance of 0.9 cM on the *Z*-population, might have the closest linkage with the *Z* locus in *L. perenne*.

For the *S* locus in *L. perenne*, the fine map was based on SSR markers, therefore no data were available for the ILGI *S* RFLP marker CDO98 on the fine mapping population. However, the identification of CDO98 as being the closest *S* linked marker was consistent with a previous mapping result in *L. perenne* (Thorogood *et al*., 2002).

Rice genome regions that are orthologous to the *S* and *Z* loci can be identified through searching for rice homologies of the *S* and *Z* linked markers. This comparative approach has been suggested as having the potential of identifying the SI candidate genes with the aid of rice genome sequence information (Bian *et al*., 2004). Relationships between the *S* linkage maps of different grass species and the rice genome were shown in Figure 6.3.1, while these of the *Z* linkage maps were shown in Figure 6.3.2. The linkage maps of *L. perenne* were developed in Chapter 3 and the fine linkage maps were developed in this chapter. The linkage maps from other grass species were obtained from previous reported studies (Table 6.3.1). It can be observed that for the *S* locus (Figure 6.3.1), close linked markers on *H. bulbosum*, *S. cereale* and *P. coerulescens* span a wide physical region on rice chromosome 5. It has been suggested that reduced recombination around the *S* locus is due to the near-centromeric location of the *S* locus (Korzun *et al*., 2001; Bian *et al*., 2004; Kakeda *et al*., 2008). Recombination suppression near the SI locus has also been reported in other SI species, such as *Brassica* (Casselman *et al*., 2000), *Petunia inflate* (Wang *et al*., 2004), *Ipomoea trifida* (Rahman *et al*., 2007) and *Antirrhinum* (Yang *et al*., 2007). In *P. inflata*, the suppression of recombination is caused by the centromeric location of the *S* locus (Entani *et al*., 1999; Wang *et al*., 2003), but for the other species, heterogeneity features of the *S* locus have been shown to be the explanation (Boyes *et al*., 1997; Cui *et al*., 1999; Shiba *et al*., 2003; Entani *et al*., 2003; Yang *et al*., 2007). The *S* markers on *L. perenne* were better physically linked compared to the findings in other grass species. The closest marker for the *S* locus, CDO98, is

located near the centromere in *L. perenne* LG1 (Jones *et al*., 2002b). Low recombination between the centromere and the nucleolar organizer region has been revealed on *L. perenne* LG1 (King *et al*., 2002). However, recombination suppression has not been observed for the *S* locus region in *L .perenne*. This implies that map-based cloning for the *S* gene in *L. perenne* is still applicable compared to the other grass species, where recombination suppression could hinder this approach.

For the *Z* locus (Figure 6.3.2), in contrast to the *S* locus, close linked markers have been identified within a short physical distance on rice chromosome 4, implying a more promising map-based cloning approach for studying the *Z* locus. The relatively high recombination frequency around the *Z* locus is consistent with its location at the long arm of the chromosome indicated by the positions of markers in *S. cereale*, *P. coerulescens* and *L. perenne* (Korzun *et al*., 2001; Bian *et al*., 2004; Jones *et al*., 2002b). By sequencing the orthologous regions on rice chromosome 4, a putative *Z* candidate gene, TC116908, was reported for *S. cereale*, showing pistil-specific expression pattern and co-segregation with the *Z* locus (Hackauf and Wehling, 2005). The real identity of this candidate as a SI component has yet to be determined. Nevertheless, the map-based approach in investigating the *Z* locus in *S. cereale* provides a model for identifying the *Z* gene in *L. perenne*, given that closely linked *Z* markers have been identified within a distance of 1 cM (04g55260 and LpGK2) on *L. perenne* LG2.

The use of rice contigs to identify SI genes in grasses has challenges. First, rice is a self-compatible grass; therefore the SI genes might not be retained in the rice genome and no homologues of the *S* or *Z* gene may present. Second, in Brassicaceae, it is suggested that the *S* locus is a dynamic locus undergoing repeated rearrangement, deletions and insertions and the *S* locus of *Arabidopsis lyrata* is not syntenous with the *Brassica S* locus, but translated as a unit between two distant chromosomal locations (Kusaba *et al*., 2001). Therefore if the *S* and

Z genes are present in the rice genome, they might be located somewhere else. However, such rearrangement is not present in *Prunus S* locus (Vieira *et al*., 2008b). Even if the rice genome does not contain homologues for the *S* and *Z* genes, a cross species approach could be applicable between other grass species. The rice orthologous regions for the *S* loci identified from different grass species, though spanning a large physical distance, were very similar between species (Figure 6.3.1). The almost same rice orthologous regions were identified for the *Z* loci from different grass species (Figure 6.3.2). These comparable results imply a possible degree of conservation of the regions around the *S* and *Z* loci within the grass species. The conservation around the *S* and *Z* loci regions has also been reported between *Lolium* and *Secale* (Thorogood *et al*., 2002) as well as *Phalaris* and *Secale* (Bian *et al*., 2004). It is therefore possible to transfer *S* or *Z* markers between species and allow for cross species gene isolation, if similar rates of recombination are observed around these physical regions. Furthermore, the generation of allele specific *S* and *Z* markers will assist in MAS for traits that are associated with the SI loci. Additionally, seed yield losses caused by SI might be circumvented (Thorogood *et al*., 2002; Studer *et al*., 2008b).

Table 6.3.1 Summary of the closest makers for the *S* and *Z* loci in grass species. The map distances between the marker and the *S* or *Z* locus are given in centimorgans (cM). Co-segregating markers have a map distance of 0 cM. The type of each marker is included.

Reference	**Species**	***S* locus**	***S*-marker**	**Type**	***Z* locus**	***Z*-marker**	**Type**
Langridge *et al*., 1999	*P. coerulescens*	2 cM	Bm2	EST	/	/	/
Korzun *et al*., 2001	*S. cereale*	/	Bm2	EST	/	BCD266	RFLP
Thorogood *et al*., 2002	*L. perenne*	0.6 cM	Cdo98	RFLP	2.5 cM	Bcd1823	RFLP
Bian *et al*., 2004	*P. coerulescens*	0.13 cM Co-segregation	Wg811, Psr168 Bm2, Bcd762	EST/RFLP	0.9 cM	Bcd266	RFLP
Hackauf and Wehling, 2005	*S. cereale*	/	/	/	0.5 cM Co-segregation	TC89057 TC116908	STS
Kakeda *et al*., 2008	*H. bulbosum*	Co-segregation	HTL, HAS31, HAS175	STS	Co-segregation	Bcd266	RFLP
Chapter 2 (ILGI population)	*L. perenne*	0.1 cM	Cdo98	RFLP	0.22 cM	04g55260	STS
Chapter 5 (fine mapping populations)	*L. perenne*	1.4 cM	LPSSR12D11	SSR	0.9 cM	LpGK2	EST

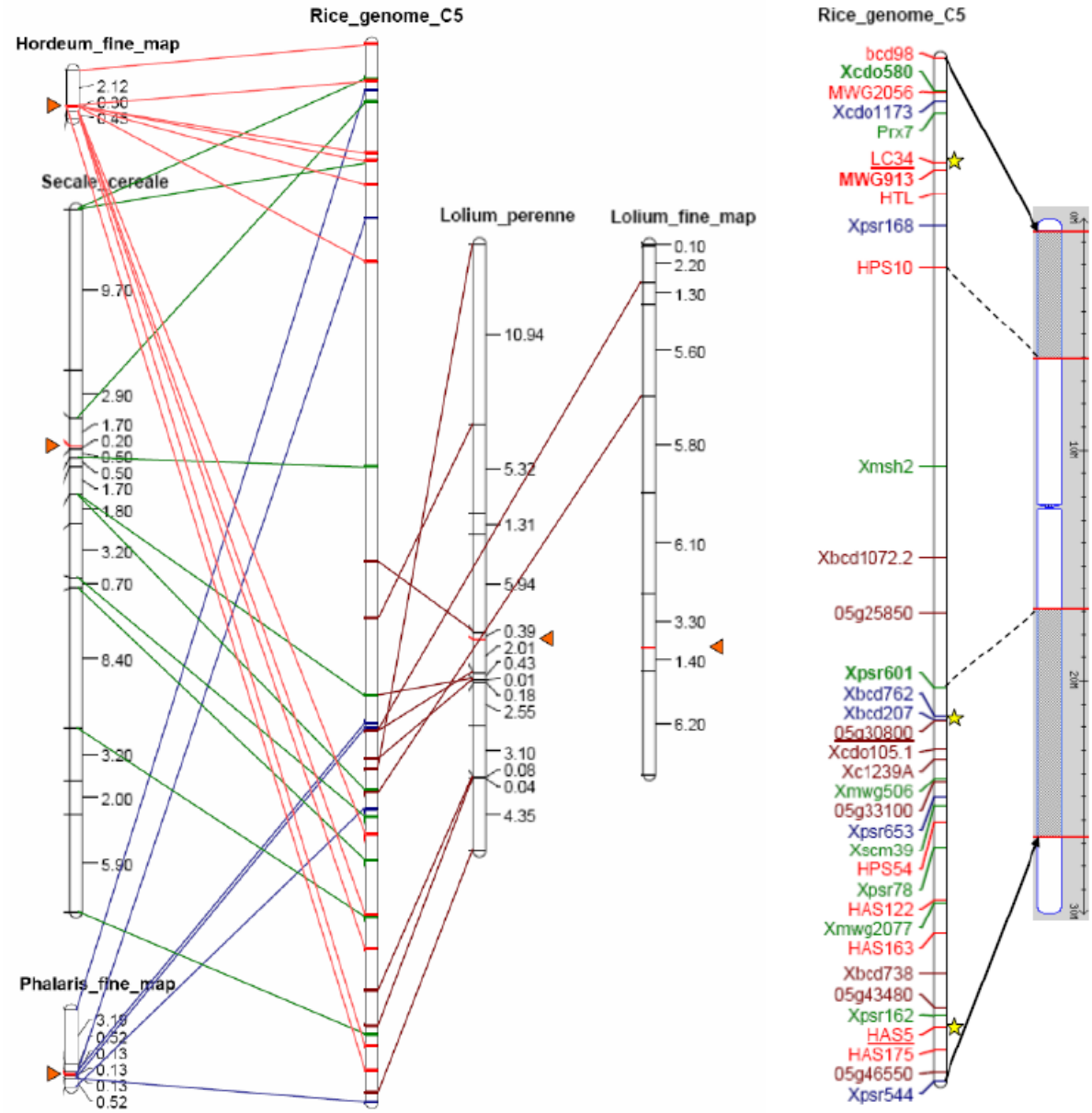


Figure 6.3.1 Relationship of the *S* linkage maps with the rice genome chromosome 5 (C5). Markers were indicated in different colors for different species: *Hordeum* in red color; *Secale* in green color; *Phalaris* in blue color and *Lolium* in dark red color. The *S* locus on each linkage map was indicated with an orange arrow. Markers from which rice homologs have been identified were linked to their physical positions on rice C5. Names of the markers and the physical locations of the rice homologs were shown on rice C5, linked with the simplified diagram of the intact chromosome where the position in Mb was indicated. The region on the rice genome with high density of *S* markers from different species was indicated with dots on the diagram. The markers most closely linked to the *S* locus were underlined and their physical positions on rice C5 were indicated with yellow stars. The schematic diagram of rice C5 was obtained from the International Rice Genome Sequencing Project (IRGSP) genome sequence build 4 in Rice annotation project database (RAP-DB) (http://rapdb.dna.affrc.go.jp/).

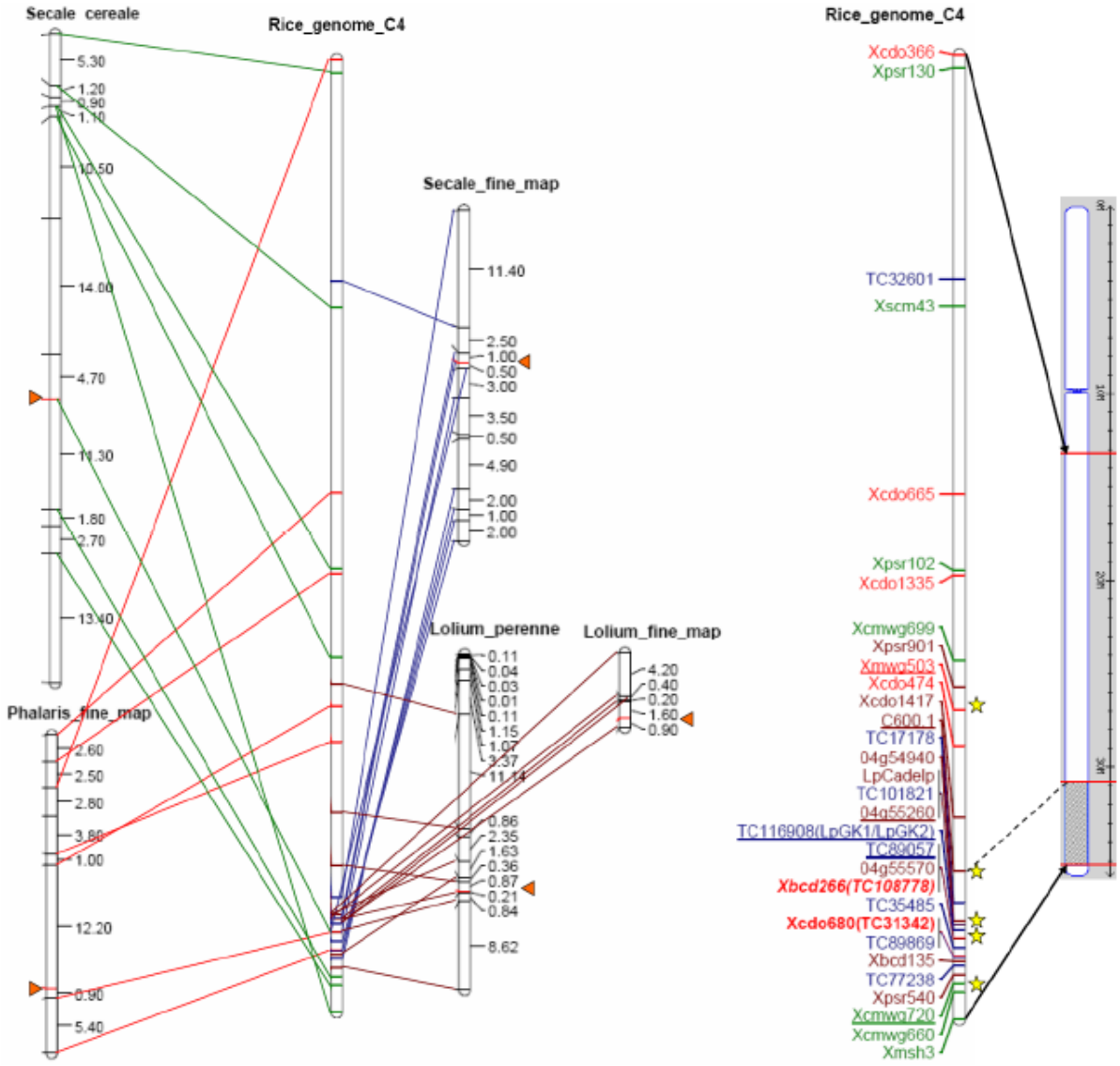


Figure 6.3.2 Relationship of the *Z* linkage maps with the rice genome chromosome 4 (C4). Markers were indicated in different colors for different maps: *Secale* in green color; *Phalaris* in red color, *Secale* fine map in blue color and *Lolium* in dark red color. The *Z* locus on each linkage map was indicated with an orange arrow. Markers from which rice homologs have been identified were linked to their physical positions on rice C4. Names of the markers and the physical locations of the rice homologs were shown on rice C4, linked with the simplified diagram of the intact chromosome where position in Mb was indicated. The region on rice genome with high density of *Z* markers from different species was indicated with dots on the diagram. The markers most closely linked to the *Z* locus were underlined and their physical positions on rice C4 were indicated with yellow stars. The schematic diagram of rice C4 was obtained from IRGSP genome sequence build 4 in RAP-DB (http://rapdb.dna.affrc.go.jp/).

CHAPTER 7

GENERAL DISCUSSION

7.1 General discussion

The components that specifiy the SI systems in several species have been identified. For the single-locus controlled SI systems, both the stigma and the pollen determinants were first identified in the *Brassica* SSI system. Through the use of isoelectric focusing electrophoresis, the *S*-specific proteins SLGs were identified (Hinata *et al.*, 1982), leading to the subsequent isolation of the stigma *S* determinant SRK (Stein *et al.*, 1991). The pollen determinant SP11/SCR was identified with two genetic approaches, the cloning and sequencing of the *S*-locus region (Suzuki *et al.*, 1999; Schopfer *et al.*, 1999) and an allelic gene search using a fluorescent differential display technique (Takayama *et al.*, 2000). In the case of S-RNase GSI system, the same stigma determinant was identified in the Solanaceae, Rosaceae, and Plantaginaceae, while their relative pollen determinants have been reported recently. The pistil S proteins were first identified in *Nicotiana alata* as abundant glycoproteins that cosegregate with the pistil *S* haplotype (Anderson *et al.*, 1986). The search for the pollen S determinant took considerably longer and was only recently resolved by the identification of the SLF through genomic analysis of the *S*-locus region (Lai *et al.*, 2002) and the confirmation through transformation analysis (Sijacic *et al.*, 2004). The stigma determinant for the *Papaver* GSI system was identified using iso-electric focusing of stigma exatracts to identify S-linked proteins and confirmed using an *in vitro* bioassay system where pollen growth is inhibited at the presence of the stigma S proteins of the same *S*-haplotype (Foote *et al.*, 1994). The pollen determinant, PrpS (*Papaver rhoeas* pollen S), has recently been identified to be a single-copy gene linked to the stigma S determinant (Wheeler *et al.*, 2009).

In the case of the *S*-*Z* two loci grass SI system, neither determinant has so far been identified. In the current study, three different approaches- comparative genetics, differential gene

expression analysis and genetic mapping, were applied to investigate the grass SI system and make progress towards the identification of the stigma *S* determinant and components involved in regulating the SI response.

The rice genome was used as a template for the identification of new molecular markers for the two major SI loci, *S* and *Z*. Comparative mapping approaches have been previously applied in *Lolium perenne*, e.g. for heading-date evaluation (Armstead *et al*., 2004) and the mapping of crown rust resistance genes (Armstead *et al*., 2006b). A requirement for such an approach to be effective is a high degree of genetic synteny between model species and target species. For *L. perenne*, conserved synteny has been established between regions of its genome with that of the rice and the Triticeae (Jones *et al*., 2002b; Alm *et al*., 2003; Armstead *et al*., 2004; Sim *et al*., 2005). Thorogood *et al*. (2002) reported the locations of the *L. perenne* SI controlling loci, *S* and *Z*, on LG1 and LG2, respectively. Based on syntenic relationships between the *L. perenne* and the rice genome, regions on rice chromosome R5 and R4 were identified with a useful degree of conservation with regions on *L. perenne* LG1 and LG2, which contain the *S* and *Z* loci, respectively. This identification provides the rational for marker development for the *S* and *Z* loci through a comparative mapping strategy. It is also the basis for the identification of the SI candidate genes and genes potentially involved in SI responses through the second approach of this study using differential gene expression analysis.

A second approach used in this work was to generate *L. perenne* SI cDNA libraries via subtracted suppression hybridization, in order to enrich differentially expressed genes between pollinated stigma and unpollinated mature/immature stigma. Through this

differential expression analysis, transcripts involved in various pollen-stigma interactions (compatible and incompatible) as well as those related with stigma development were identified in the SI cDNA libraries. In all the well studied SI systems the stigmatic SI genes are expressed before the pollen-stigma contact (Anderson *et al*., 1986; Nasrallah *et al*., 1988), thus the stigmatic genes involved in the *L. perenne* incompatibility response should have been theoretically present in the SI cDNA libraries developed by subtracting pollinated stigmas with unpollinated immature stigmas. As a result of *in-vitro* pollination, it was apparent that the SI cDNA libraries contained non-stigma specific genes generated by pollen. Therefore, the pollen specific cDNA library was developed with the intention to eliminate the possible inclusion of pollen specific genes in the identified stigmatic SI candidates. However, the pollen specific library might not be comprehensive for all pollen specific genes and some pollen expressed genes would still be present in the SI cDNA libraries after library comparison.

Novertheless, the genes identified in the current study provide a useful starting point for future studies. Protein kinases (Can3, Can4 and Can94) were identified. There have been evidences for the involvement of protein kinases in various SI responses, implying the role of protein kinases in the *L. perenne* SI response and the possible relationship between different SI systems. Putative pathogenesis-related (PR) protein (Can130) was identified, indicating the parallels between incompatible host-pathogen and incompatible pollen-stigma interactions. In the SI response, PRs might play the same role as in plant innate immunity in regulating pollen tube growth through a Ca^{2+}-dependent programmed cell death response. Calcium related proteins (Can3 and Can94) were also identified. They might function through regulating Ca^{2+} influxes or actin reorganization in the mediation of pollen tube growth.

Additional pollen expression analysis of the identified ten SI candidate genes would clarify their tissue specific identity (this experiment was not included in this study because of the restriction of flowering time for pollen collection). If the ten SI candidates were expressed in pollen, the possibility of them being involved in the SI response is still valid. Determination of their genetic map positions in relation to the *S* or *Z* loci would lead to a better understanding of their involvement in controlling the SI specificity in *L. perenne*. There are similarities between the grass GSI, *Papaver* GSI and SSI systems, implying the mechanistic links between these SI systems (reviewed by Yang et al. 2008). Therefore biochemical studies such as in *Papaver* (Franklin-Tong and Franklin, 2003; de Graaf *et al*., 2006) via *in-vitro* germination of pollen might offer an alternative approach to study the involvements of the identified candidate genes in the Poaceae SI response through protein functions. Through evaluating the reactions of pollen tube upon treatments with the identified candidates, whether they play a role in regulating pollen germination or tube growth could be revealed. Functional genomic approaches such as RNA interference (RNAi) (Small, 2007) could also be applied for the validation of the functions of the identified candidate genes. If they are involved in the SI response, silencing their functions might lead to the disruption of SI and hence the pollen tube growth. Furthermore, because of the *S*-*Z* two loci complementary system in *L. perenne*, both the *S* and *Z* alleles of the pollen need to match with an *S* and *Z* allele pair combination in the stigma to exhibit SI reaction. Through knocking out either one of the *S* or *Z* alleles, the SI would be lost, leading to the self-compatibility. T-DNA insertional mutagenesis has been applied successfully in *Arabidopsis*, *Medicago truncatula* and rice for functional genomics researches (Tadege *et al*., 2005). However, this approach requires an efficient transformation and regeneration system, which is not so well established in *Lolium*

thus limiting the application of T-DNA tagging in studying gene functions in *Lolium*. Furthermore, it should also be noted that T-DNA insertion might induce deletions and rearrangements in the target gene and complicates the analysis.

Polymorphic markers for mapping candidate genes were developed for *Can10*, *Can130*, *Can136* and the *Lolium* G10-protein gene (identified with comparative bioinformatics). *Can10* and *Can130* did not show genetic relationships with the *S* or *Z* loci and *Can136* was located on LG1, but too far away (~38 cM) from *S* to be involved in determining the *S* locus specificity. However, they might function in the downstream reactions that mediate the incompatibility response. *Lolium* G10-protein gene was mapped at a distance of about 20 cM away from the *S* locus, therefore it is not likely to be a SI component of *L. perenne*. The finding of the four similar G10-proteins on rice R1, R4, R5 and R12 in the syntenic regions of the *L. perenne* SI loci is still an interesting point for further investigation from the evolutionary aspect of SI systems. It has been suggested that SI systems may have evolved independently at least 21 times during the evolution of flowering plants (Steinbachs and Holsinger, 2002) and there was a widely accepted hypothesis addressing the origin of complex multi-locus SI systems and the derivative status of the one-locus SI systems (Lundqvist 1975; Østerbye, 1975; Lundqvist, 1990). It was also assumed that gene duplication is involved in the evolution of multi-locus SI systems (Lundqvist, 1954; Pandey, 1980). Elucidation of the relationship between the G10 proteins and the *L. perenne* SI system might provide some indication for the evolutionary property of SI systems.

As mentioned before, identification of the SI candidate genes from the SI cDNA libraries was based on the synteny between the *L. perenne S* and *Z* loci regions and the rice genome regions.

Transcripts selected as potential SI candidates were homologous to rice genes within the syntenic regions of the *S* and *Z* loci. One problem possibly involved in this strategy is that rice is a self-compatible grass species. Therefore no homologs of the *S* or *Z* gene may be present in the rice genome. However, it is also possible that the *S* or *Z* genes are present in rice but simply inactivated similarly to the situation of the *S* genes in self-fertile *Arabidopsis thaliana* (Kusaba *et al*., 2001). A further consideration is if the *S* and *Z* genes are present in the rice genome, they might not be at the syntenic locations compared to the SI grass species. The scenario was observed between *Brassica* and *Arabidopsis* where the *S* locus is translated as a unit between two distant chromosomal locations (Kusaba *et al*., 2001). If a similar scenario would apply to the Poaceae, then the syntenic regions of *Lolium S* and *Z* loci might be at different locations on rice R5 and R4. Consequently, the identified ten SI candidate genes might not be the *L. perenne* SI components. However, their involvement in the incompatibility response still makes them a valuable resource for exploring the under characterised downstream reactions and signal transduction pathways triggered by the SI response in the Poaceae species.

An alternative approach to enable the identification of the *S* and *Z* genes is through map-based cloning. The tightly linked markers developed in the fine mapping populations in this study provide useful information towards an effective map-based cloning strategy and the development of functional markers (Andersen and Lübberstedt, 2003) for the *S* and *Z* loci, which will assist in practical plant breeding programmes. Work is ongoing at IBERS to develop fine-scale maps for the *S* and *Z* loci towards the identification of allelic markers. The maintenance of heterogeneity at the *S* and *Z* loci by means of allele specific marker selection can circumvent SI induced seed yield loss and facilitate the exploration of heterosis.

If either the pollen or stigma SI genes in the Poaceae can be identified, it would be possible to find the partner determinant genes through sequencing of neighbouring genomic sequences as previously demonstrated in *Brassica*, the RNase and *papaver* SI systems. However, caution is required here, for the reason that the orders, orientations and distances between *SRK*, *SCR/SP11* and other *S*-locus genes have been shown to vary considerably among species and even haplotypes (Fukai *et al*., 2003). For instance, insertion of retrotransposons into the *S*-locus region has recently been reported as the reason for the length difference in the *S*-locus haplotype between *Brassica oleracea* and *B*. *rapa* (Fujimoto *et al*., 2006).

Up to date, a majority of the studies on the grass SI system were based on genetic approaches such as differential gene expression and genetic mapping. Under the circumstance that the SI components are identified, there would still be a lack of understanding on the mechanism of the grass SI response. Therefore, a parallel strategy to investigate protein function should be considered and might also facilitate the search of the SI determinants. An example is the successful application of isoelectric focusing technique in the finding of the *S*-specific proteins in *Brassica*, Solanaceae, Rosaceae and Papaveraceae (Nishio and Hinata, 1977; Singh and Kao, 1992; Nakanishi *et al.*, 1992; Sassa *et al.*, 1993). In *L. perenne*, the populations with segregating genotypes for SI loci have been generated (Dr Danny Thorogood, personal communication). Hence, the isoelectric focusing technique can be used to detect stigmatic *S*-specific proteins by correlating allelic differences with genotypes.

An *in vitro* bioassay system was established for *Papaver* and led to the identification of the female *S* determinant (Foote *et al.*, 1994) as well as the subsequent analysis of the mechanism

of pollen inhibition in *Papaver* SI. The basic requirement for developing such a bioassay is a robust method for *in vitro* pollen germination and growth. In *Lolium*, a successful *in vitro* pollen germination system has also been reported (Ahloowalia, 1973). Therefore, the establishment of an *in vitro* bioassay system for *Lolium* is potentially feasible and would provide another path towards the understanding of the mechanism in *Lolium* SI.

Appendix:

Appendix A. Sequences of Lolium BAC clones identified with primers designed from the four G10 candidate gene homologs in rice.

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>Loilum_BAC703
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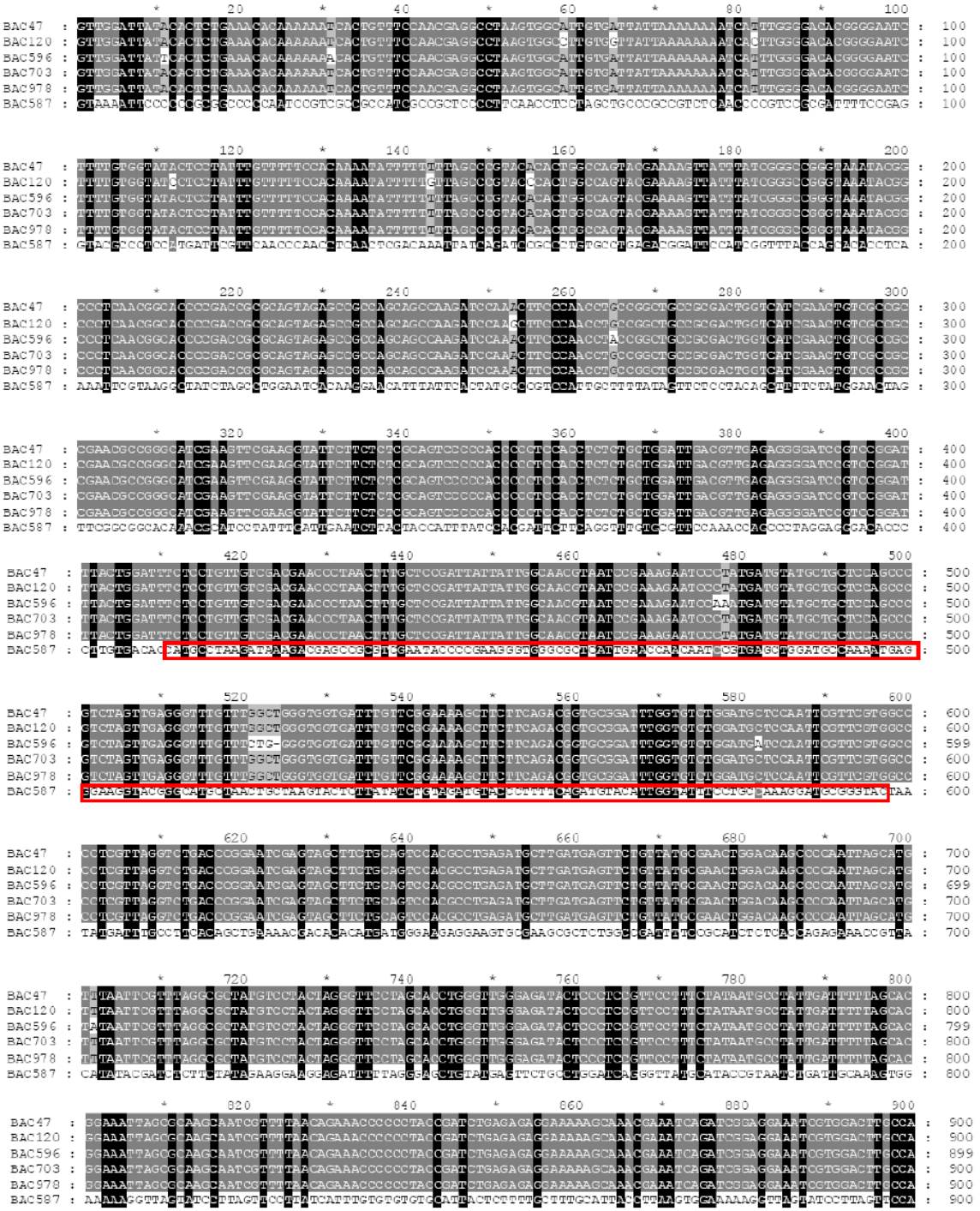
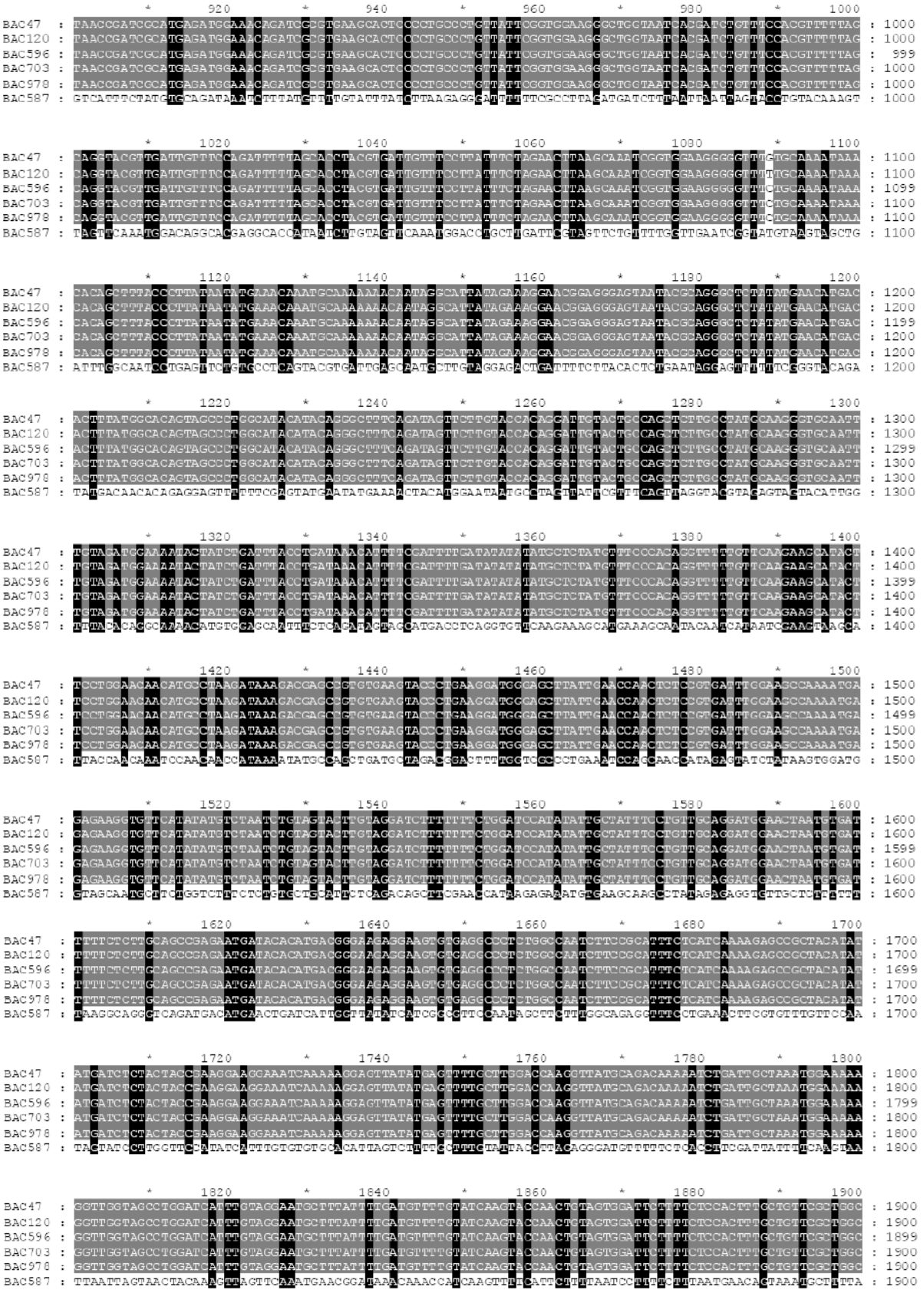
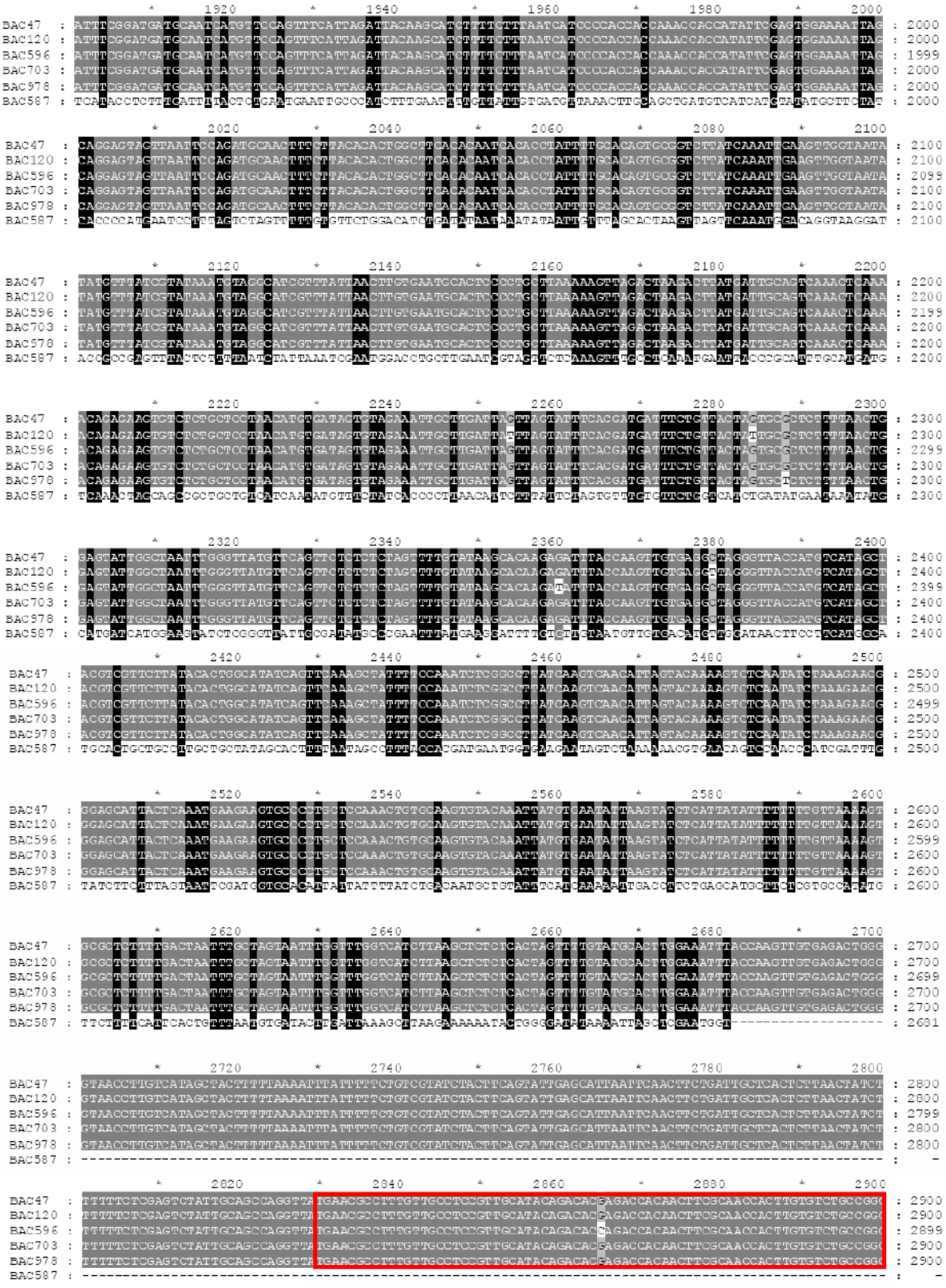
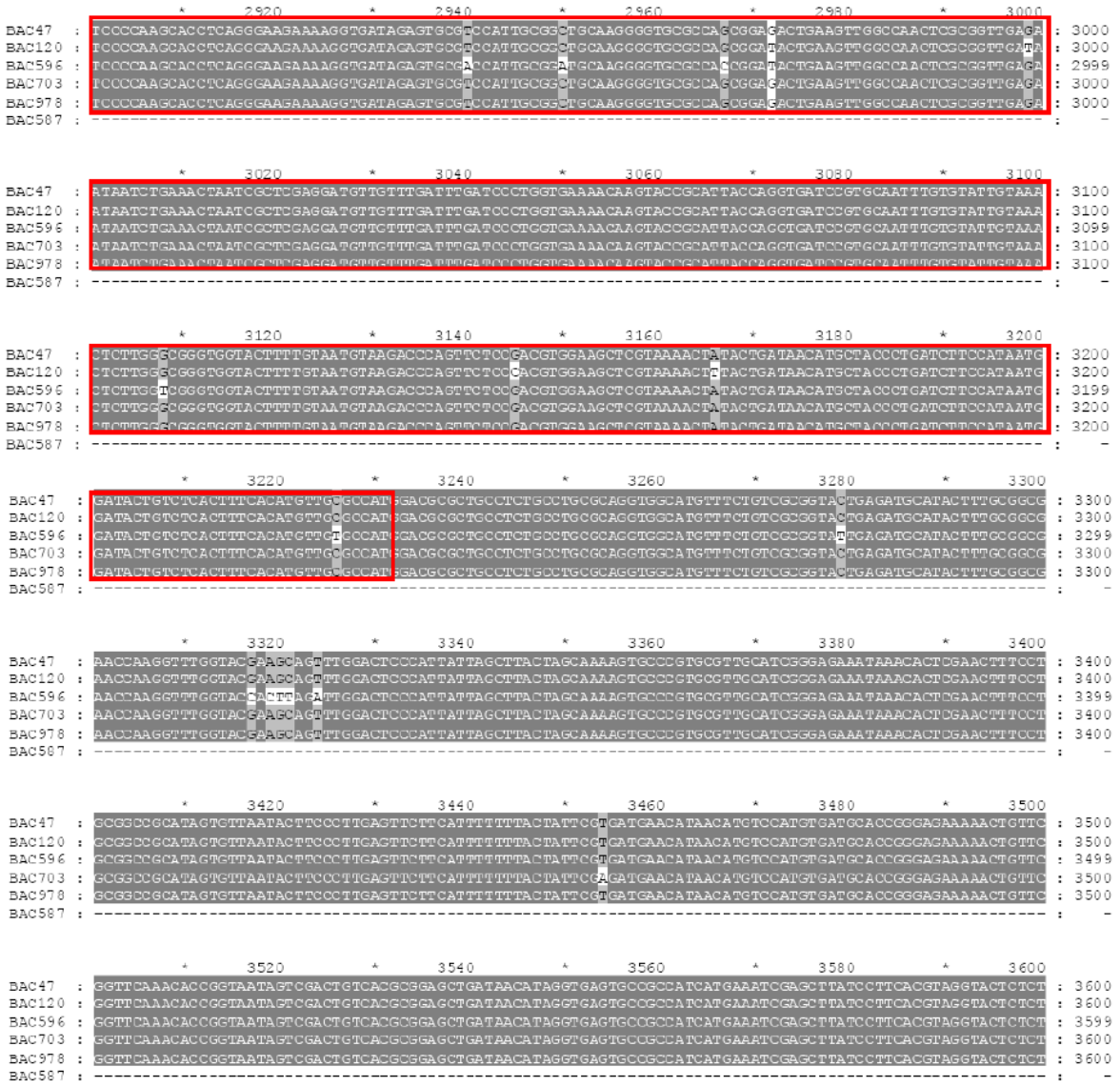
*Appendix B. Sequence alignments of the six identified Lolium BAC clones homologous to the rice G10-protein. The predicted coding sequences are highlighted in red boxes. The alignment was performed using GeneDoc (**http://www.psc.edu/biomed/genedoc**). Predictions of coding sequences were carried out using BESTORF (**http://linux1.softberry.com/berry.phtml**).*









Appendix C. Rice homologies of the transcripts identified in the pollen specific cDNA library. They were listed in the order of the physical appearance on the rice genome: according rice sequence and rice gene annotation information. BLAST searches for rice homology were carried out through the BLASTN search function in the Rice Genome Annotation Project with the Osa1 rice genome coding sequences (CDS) database.

Rice Sequence	p	rice annotation
LOC_Os01g04170	2.50E-05	expressed protein
LOC_Os01g05430	1.00E-11	membrane protein, putative, expressed
LOC_Os01g15220	4.00E-10	expressed protein
LOC_Os01g46850	4.40E-23	AGP16, putative, expressed
LOC_Os01g52214	0.31	NADH-ubiquinone oxidoreductase 20 kDa subunit, mitochondrial precursor, putative, expressed
LOC_Os01g57960	2.60E-43	unspliced-genomic retrotransposon protein, putative, unclassified, expressed
LOC_Os01g57974	1.50E-13	unspliced-genomic retrotransposon protein, putative, unclassified

Rice Sequence	p	rice annotation
LOC_Os01g58010	1.60E-24	ATP synthase a chain, putative
LOC_Os01g58060	4.30E-10	retrotransposon protein, putative
LOC_Os02g12060	0.0013	low-molecular-weight cysteine-rich protein LCR66 precursor, putative
LOC_Os02g12950	0.2	hypothetical protein
LOC_Os02g13850	6.70E-01	protein kinase, putative, expressed
LOC_Os02g28600	2.80E-02	F-box domain containing protein
LOC_Os02g35660	3.10E-10	helix-loop-helix DNA-binding domain containing protein, expressed
LOC_Os02g48690	7.00E-08	hypothetical protein
LOC_Os02g58250	7.60E-06	expressed protein
LOC_Os03g01610	1.50E-11	major pollen allergen Ory s 1 precursor
LOC_Os03g02240	1.60E-05	AT-GTL1, putative, expressed
LOC_Os03g18170	7.90E-05	mitogen-activated protein kinase kinase kinase 1, putative, expressed
LOC_Os03g42530	4.70E-32	unspliced-genomic retrotransposon, putative, centromere-specific
LOC_Os03g58764	2.50E-01	F-box domain containing protein, expressed
LOC_Os03g60470	2.60E-16	glycine-rich protein A3, putative, expressed
LOC_Os04g03660	0.95	conserved hypothetical protein
LOC_Os04g12120	5.20E-01	transposon protein, putative, unclassified
LOC_Os04g29210	3.10E-03	FAD binding domain containing protein
LOC_Os04g32680	3.00E-14	pollen-specific protein C13 precursor
LOC_Os04g47900	9.50E-01	ATTIM23-2, putative, expressed
LOC_Os04g48010	2.50E-04	signal transducer, putative, expressed
LOC_Os04g52950	5.30E-19	nitrate-induced NOI protein, expressed
LOC_Os05g02780	2.20E-01	glycine-rich protein A3, putative, expressed
LOC_Os05g02870	4.10E-06	ATPase, coupled to transmembrane movement of substances, putative
LOC_Os05g02890	6.20E-06	stigma/style ABC transporter, putative
LOC_Os05g04584	3.30E-02	3-N-debenzoyl-2-deoxytaxol N-benzoyltransferase, putative, expressed
LOC_Os05g08410	0.9998	ATP10 protein, expressed
LOC_Os05g16100	0.042	hypothetical protein
LOC_Os05g22770	9.99E-01	retrotransposon protein, putative
LOC_Os05g22840	1.00E+00	conserved hypothetical protein
LOC_Os05g23880	0.087	lipoxygenase A, putative, expressed
LOC_Os05g51900	5.40E-06	expressed protein
LOC_Os06g04070	0.093	arginine decarboxylase, putative, expressed
LOC_Os06g05880	7.60E-18	profilin-2, putative, expressed
LOC_Os06g12820	8.70E-01	expressed protein
LOC_Os06g21410	3.20E-05	pollen-specific arabinogalacta protein BAN102, putative, expressed
LOC_Os06g22820	2.40E-05	CAK1AT, putative, expressed
LOC_Os06g33210	0.15	UDP-N-acetylglucosamine transporter
LOC_Os06g42320	4.50E-01	hypothetical protein
LOC_Os06g43340	7.70E-01	retrotransposon protein, putative
LOC_Os07g05280	0.46	retrotransposon protein, putative
LOC_Os07g13810	0.38	cytokinin-N-glucosyltransferase 1, putative
LOC_Os07g44744	2.60E-29	expressed protein
LOC_Os08g08760	1.70E-38	ubiquitin family protein, expressed

Rice Sequence	p	rice annotation
LOC_Os08g10940	0.99	retrotransposon protein, putative
LOC_Os09g25040	2.10E-02	BHLH transcription factor, putative
LOC_Os09g25330	4.40E-01	signal transducer, putative, expressed
LOC_Os09g30040	0.62	expressed protein
LOC_Os10g02520	9.00E-01	transposon protein, putative, Mutator sub-class
LOC_Os10g17680	7.30E-66	profilin A, putative, expressed
LOC_Os10g21280	3.70E-60	ribulose bisphosphate carboxylase large chain precursor, putative, expressed
LOC_Os10g28020	7.60E-01	unspliced-genomic acylamino-acid-releasing enzyme, putative, expressed
LOC_Os10g40090	1.50E-12	beta-expansin 1a precursor, putative
LOC_Os10g41980	0.999	expressed protein
LOC_Os11g01270	0.15	expressed protein
LOC_Os11g11340	3.20E-01	unspliced-genomic CIP111, putative, expressed
LOC_Os11g17954	0.91	transposon protein, putative, Pong sub-class, expressed
LOC_Os11g19880	0.9996	O-methyltransferase ZRP4, putative
LOC_Os11g39490	0.992	jacalin-like lectin domain containing protein
LOC_Os12g07760	0.093	hypothetical protein
LOC_Os12g23610	1.80E-09	unspliced-genomic ATP synthase, A subunit family protein
LOC_Os12g42650	3.10E-07	pollen preferential protein, putative

Appendix D. Rice homologies of the transcripts identified specifically in SI cDNA library 1. They were listed in the order of the physical appearance on the rice genome: according rice sequence and rice gene annotation information. BLAST searches for rice homology were carried out through the BLASTN search function in the Rice Genome Annotation Project with the Osa1 rice genome coding sequences (CDS) database.

Rice Sequence	p	rice annotation
LOC_Os01g15800	3.50E-01	conserved hypothetical protein
LOC_Os01g33420	1.30E-06	alpha-galactosidase/ hydrolase, hydrolyzing O-glycosyl compounds
LOC_Os01g49370	1.60E-10	expressed protein
LOC_Os01g57960.1	3.00E-19	retrotransposon protein, putative, unclassified
LOC_Os01g58060.1	1.00E-30	hypothetical protein
LOC_Os01g58220	5.10E-18	protein translation factor SUI1 homolog 2
LOC_Os02g26170	8.80E-17	conserved hypothetical protein
LOC_Os02g43470	9.50E-15	glutamate dehydrogenase A
LOC_Os03g17870	6.60E-09	metallothionein-like protein 1
LOC_Os03g42530.1	5.00E-21	retrotransposon protein, putative, unclassified
LOC_Os03g55730	9.70E-01	seven transmembrane domain protein
LOC_Os04g12000	0.46	retrotransposon protein
LOC_Os04g25454	1.00E-01	retrotransposon protein
LOC_Os04g26400	9.50E-01	expressed protein
LOC_Os04g39560	6.20E-01	expressed protein
LOC_Os04g45970	6.00E-18	glutamate dehydrogenase 2
LOC_Os04g53450	6.00E-12	expressed protein
LOC_Os05g07050	8.20E-01	pre-mRNA-processing-splicing factor 8
LOC_Os05g22840	6.70E-05	conserved hypothetical protein
LOC_Os05g39930	5.60E-01	spotted leaf protein 11

Rice Sequence	p	rice annotation
LOC_Os05g41900	1.80E-16	protein translation factor SUI1
LOC_Os05g49200	2.00E-47	Aspartic proteinase oryzasin 1 precursor
LOC_Os06g16380.1	7.00E-57	hypothetical protein
LOC_Os06g36240	5.30E-13	pollen allergen Phl p 11
LOC_Os06g42280	1.30E-01	transposon protein, putative, CACTA, En/Spm sub-class
LOC_Os06g49530	9.98E-01	F-box domain containing protein
LOC_Os08g08980	6.80E-40	germin-like protein subfamily 1 member 7 precursor
LOC_Os08g13440	1.70E-89	germin-like protein subfamily 1 member 17 precursor
LOC_Os08g28560	9.90E-01	expressed protein
LOC_Os08g39230	9.50E-01	hypothetical protein
LOC_Os09g00998	9.80E-68	expressed protein
LOC_Os09g39930	1.10E-08	serine/threonine-protein kinase NAK
LOC_Os10g33830	9.00E-01	MATH domain containing protein
LOC_Os11g03780.1	7.00E-45	Alpha-L-arabinofuranosidase C-terminus family protein
LOC_Os11g31450	9.98E-01	expressed protein
LOC_Os11g47809	1.50E-12	metallothionein-like protein 1
LOC_Os12g03530	4.20E-42	arabinoxylan arabinofuranohydrolase isoenzyme AXAH-I
LOC_Os12g03470.1	9.20E-52	Alpha-L-arabinofuranosidase C-terminus family protein, expressed
LOC_Os12g05860	3.90E-25	Germin-like protein subfamily 1 member 8 precursor
LOC_Os12g29434	1.00E+00	wall-associated kinase-like 1
LOC_Os12g38064	1.40E-05	Metallothionein-like protein 1, putative, expressed

Appendix E. Rice homologies of the transcripts identified specifically in SI cDNA library 4 and 5. They were listed in the order of the physical appearance on the rice genome: according rice sequence and rice gene annotation information. BLAST searches for rice homology were carried out through the BLASTN search function in the Rice Genome Annotation Project with the Osa1 rice genome coding sequences (CDS) database.

Rice Sequence	p	rice annotation
LOC_Os01g08340	2.80E-71	zinc finger family protein, putative, expressed
LOC_Os01g10890	5.00E-69	CBL-interacting serine/threonine-protein kinase 15, putative, expressed
LOC_Os01g17390	4.10E-08	cyclin-like F-box
LOC_Os01g42010	3.80E-01	expressed protein
LOC_Os01g43620	9.99E-01	hypothetical protein
LOC_Os01g48410	6.30E-76	pollen-specific kinase partner protein, putative, expressed
LOC_Os01g56230	4.40E-56	expressed protein
LOC_Os01g56790	9.10E-24	expressed protein
LOC_Os01g59180	8.00E-86	cyclin-like F-box
LOC_Os01g70780	4.80E-02	SVP1-like protein 2, putative, expressed
LOC_Os02g02360	2.70E-01	expressed protein
LOC_Os02g02410	3.10E-07	luminal binding protein precursor 3, putative, expressed
LOC_Os02g02560	1.90E-43	UTP-glucose-1-phosphate uridylytransferase, putative, expressed
LOC_Os02g07250	4.80E-01	expressed protein
LOC_Os02g07900	8.20E-72	Clathrin assembly protein, putative, expressed
LOC_Os02g14290	0.9995	Scramblase family protein, expressed
LOC_os02g16000	2.60E-35	GAMYB-binding protein, putative, expressed

Rice Sequence	p	rice annotation
LOC_Os02g16040	6.50E-62	Ubiquitin-conjugating enzyme E2-17 kDa, putative, expressed
LOC_Os02g25900	5.70E-01	pollen signalling protein with adenylyl cylase activity, putative, expressed
LOC_Os02g31290	6.70E-03	Mei2, putative, expressed
LOC_Os02g32740	6.70E-59	SNAP25 homologous protein SNAP30,putative,expressed
LOC_Os02g35300	4.60E-06	transposon protein
LOC_Os02g39790	7.80E-49	S-adenosylmethionine decarboxylase proenzyme, putative, expressed
LOC_Os02g42290	9.96E-01	ATP-dependent Clp protease proteolytic subunit, putative, expressed
LOC_Os02g44470	9.10E-52	Actin-deploymerizing factor, putative, expressed
LOC_Os02g46480	1.50E-24	expressed protein
LOC_Os02g47900	0.23	SET domain protein SDG117, putative, expressed
LOC_Os02g55910	1.60E-02	Monogalactosyldiacylglycerol synthase family protein, expressed
LOC_Os03g06890	1.50E-21	expressed protein
LOC_Os03g11410	5.40E-51	Mitochondrial processing peptidase beta subunit, mitochondrial precursor, putative, expressed
LOC_Os03g11734	9.90E-16	antiporter/drug transporter
LOC_Os03g16140	9.70E-17	digalactosyldiacylglycerol synthase 2,putative,expressed
LOC_Os03g20020	4.50E-86	beta-fructofuranosidase, putative, expressed
LOC_Os03g24940	0.98	proline-rich family, putative, expressed
LOC_Os03g27110	1.20E-25	hydrolase,alpha/beta fold family protein, putative, expressed
LOC_Os03g29350	1.00E-01	von Willebrand factor type A domain containing protein, expressed
LOC_Os03g44890	2.00E-02	C2 domain-containing protein, putative, expressed
LOC_Os03g46090	1.00E+00	Endonuclease/Exonuclease/phosphatase family protein, expressed
LOC_Os03g58764	7.00E-01	F-box domain containing protein
LOC_Os03g59300	9.80E-01	expressed protein
LOC_Os03g60470	1.60E-15	expressed protein
LOC_Os03g61530	2.20E-07	pectinaesterase inhibitor domain containing protein, expressed
LOC_Os03g62140	1.60E-02	DnaJ domain containing protein
LOC_Os04g01150	6.70E-95	phagocytosis and cell motility protein ELM01, putative, expressed
LOC_Os04g22230	2.60E-05	pathogenesis-related protein PRB1-3 precursor
LOC_Os04g37780	1.00E+00	histone H3
LOC_Os04g42090	1.40E-53	S-adenosylmethionine decarboxylase proenzyme, putative, expressed
LOC_Os04g46630	1.60E-11	beta-expansin 2 precursor
LOC_Os04g47170	1.10E-08	pollen-specific kinase partner protein
LOC_Os04g49954	4.90E-07	expressed protein
LOC_Os04g50216	1.50E-80	gtk16 protein
LOC_Os04g56450	6.80E-38	Protein phosphatase 2C containing protein, expressed
LOC_Os05g15690	2.50E-12	beta-expansin 2 precursor
LOC_Os05g33820	1.00E-46	lipase
LOC_Os05g34110	5.70E-05	myb-like DNA-binding domain, SHAQKYF class family protein
LOC_Os05g35860	9.20E-01	retrotransposon protein, putative, unclassified
LOC_Os05g38290	3.60E-10	Protein phosphatase 2C containing protein, expressed

Rice Sequence	p	rice annotation
LOC_Os05g39870	3.60E-17	CBL-interacting serine/threonine-protein kinase 15, putative, expressed
LOC_Os05g40700	1.80E-22	expressed protein
LOC_Os05g41270	6.80E-51	Calcium-dependent protein kinase, isoform 2, putative, expressed
LOC_Os05g43540	2.60E-40	expressed protein
LOC_Os05g47940	1.90E-06	transposon protein
LOC_Os05g48640	2.10E-36	pollen-specific kinase partner protein
LOC_Os05g51680	1.20E-06	SCP-like extracellular protein
LOC_Os05g51790	1.90E-02	ATP binding protein
LOC_Os06g33210	1.10E-01	UDP-galactose transporter family protein, expressed
LOC_Os07g33850	1.90E-51	small GTP-binding protein domain containing protein, expressed
LOC_Os07g37900	0.99995	retrotransposon protein, putative, unclassified
LOC_Os08g32170	2.20E-01	oxidoreductase
LOC_Os08g44660	4.60E-02	EF hand family protein, expressed
LOC_Os09g15320	g4.8E-20	Ubiquitin-conjugating enzyme E2M, putative, expressed
LOC_Os10g13940	2.70E-44	antiporter/drug transporter
LOC_Os10g35930	5.00E-44	LIM domain containing protein, expressed
LOC_Os11g06130	9.96E-01	PHD-finger family protein, expressed
LOC_Os11g13860	1.30E-49	Protein kinase domain containing protein, expressed
LOC_Os11g44880	2.00E-10	kinesin motor protein, putative, expressed
LOC_Os12g03490	0.993	hypothetical protein
LOC_Os12g04180	1.30E-03	RNA recognition motif family protein, expressed
LOC_Os12g08810	3.60E-82	VTC2, putative, expressed
LOC_Os12g12860	3.00E-115	Calcium-dependent protein kinase, isoform AK1, putative, expressed
LOC_Os12g35030	0.98	Plus-3 domain containing protein, expressed
LOC_Os12g35710	8.10E-16	extensin-like domain containing protein, putative, expressed

Appendix F Publication

"How far are we from unraveling self-incompatibility in grasses?" is a review article on gametophytic self-incompatibility (SI) in grasses. The grass SI system is still poorly understood and the review article summarizes current knowledge as well generate insights into the studies in grass SI system. It covers topics in the introduction, evolution and known genetic controls of the grass SI system. It compares the grass SI system with other single- and multi- locus gametophytic SI (GSI) systems, also discusses the relation of the grass GSI system with the sporophytic SI system. It makes a generalization that the grass SI system exhibits features from both known GSI systems and SSI systems, supported by some of the recent findings and observations through the studies carried out by the authors via a comparative genetics approach. To understand the grass GSI system would lead to a better understanding of plant evolution, plant development and taxonomy. Furthermore an understanding could lead to exploitation in plant breeding programmes involving self-incompatible grass species.

Review

New Phytologist

Research review

How far are we from unravelling self-incompatibility in grasses?

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Key words: comparative mapping, evolution, gametophytic, grasses, self-incompatibility, sporophytic.

Summary

The genetic and physiological mechanisms involved in limiting self-fertilization in angiosperms, referred to as self-incompatibility (SI), have significant effects on population structure and have potential diversification and evolutionary consequences. Up to now, details of the underlying genetic control and physiological basis of SI have been elucidated in two different gametophytic SI (GSI) systems, the S-RNase SI and the *Papaver* SI systems, and the sporophytic SI (SSI) system (*Brassica*). In the grass family (Poaceae), which contains all the cereal and major forage crops, SI has been known for half a century to be controlled gametophytically by two multiallelic and independent loci, *S* and *Z*. But still none of the gene products for *S* and *Z* is known and only limited information on related biochemical responses is available. Here we compare current knowledge of grass SI with that of other well-characterized SI systems and speculate about the relationship between SSI and grass SI. Additionally, we discuss comparative mapping as a tool for the further investigation of grass SI.

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Self-incompatibility in grass species

The grass family (Poaceae) is the fourth largest family of flowering plants, comprising > 10 000 species of economic and ecological importance (Watson & Dallwitz, 1992). Grasses include all the cereal crops and 75% of the cultivated forage crops (Nelson & Moser, 1995). They are more extensively adapted to all temperature ranges and rainfall habitats than any other family of flowering plants (Watson & Dallwitz, 1992). Self-incompatibility (SI) has been known in flowering plants for over a century since Darwin's description in 1876 (Darwin, 1876). SI was defined by deNettancourt (1977) as 'the inability of a fertile hermaphrodite seed plant to produce zygotes after self-pollination'. The pistil is able to distinguish self and same-species nonself pollen with common *S* alleles, and prevent fertilization, thus circumventing the tendency towards self-fertilization and consequent inbreeding. The promotion of outbreeding through SI has played a significant role in the evolution and diversification of grass species (Pandey, 1977). The effectiveness of SI in grasses limits to a certain extent the efficient production of inbred lines and hybrids in plant breeding but also ensures the maintenance of heterozygosity in wild populations and, potentially, could contribute to adaptive success. SI is widely distributed in

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Appendix Table 1 Sequences selected for alignment to design primers of STS markers. 'Rice loci' is according to the definition of MSU Rice Genome Annotation (Osa1) Release 5. 'TA' refers to Plant Transcript Assemblies (http://plantta.tigr.org/index.shtml).

Marker	**Rice loci**	**Species**	**TA accession**	**Species**	**TA accession**	**Species**	**TA accession**
05g25850	LOC_Os05g25850	*Triticum turgidum*	AJ612359	*Triticum aestivum*	CK213770	*Zea mays*	CO447632
05g30800	LOC_Os05g30800	*Triticum aestivum*	BT009132	*Hordeum vulgare*	BE421023	*Zea mays*	TA105465_4577
05g33100	LOC_Os05g33100	*Triticum aestivum*	TA65531_4565	*Hordeum vulgare*	BF621527	*Festuca arundinacea*	TA3236_4606
05g43480	LOC_Os05g43480	*Festuca arundinacea*	DT708671	*Hordeum vulgare*	TA57722_4513	*Zea mays*	EE033636
05g46550	LOC_Os05g46550	*Triticum aestivum*	TA78791_4565	*Hordeum vulgare*	TA28577_4513	*Zea mays*	TA113168_4577
04g54940	LOC_Os04g54940	*Triticum aestivum*	TA51686_4565	*Hordeum vulgare*	BU973147	*Zea mays*	DR803119
04g55260	LOC_Os04g55260	*Triticum aestivum*	TA91070_4565	*Zea mays*	AI783268		
04g55570	LOC_Os04g55570	*Triticum aestivum*	CA729291	*Hordeum vulgare*	TA50985_4513		

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List of definitions and abbreviations

°C	centigrade
µg	microgram
µl	microlitre
µM	micromolar concentration
ABP	actin-binding protein
ADF	actin deploymerizaing factor
AFLP	amplified fragment length polymorphism
ARC	armadillo repeat-containing
AT	annealing temperature
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
BC_1	backcross
BLAST	Basic Local Alignment Search Tool
bp	base pair
BP	biological process
BRET	bioluminescence resonance energy transfer
BSA	bovine serum albumin
Ca^{2+}	calcium
$[Ca^{2+}]_i$	cytosolic free Ca^{2+}
CAPS	cleaved amplified polymorphism sequence
CBL	calcineurin B-like calcium sensor protein
CC	cellular component
CDK	cyclin-dependent kinase
cDNA	complementary DNA
cDNA-AFLP	cDNA-amplified fragment length polymorphism
CDPK	calcium-dependent protein kinase
CDS	coding sequence
CK	casein kinase
cM	centiMorgan
CO	*CONSTANS*
CRres	crown rust resistance
Ct	threshold cycle
CTAB	cetyltrimethyl ammonium bromide
CV	coefficient of variation
cyt c	cytochrome c
ddH_2O	double distil H_2O
DAG	diacylglycerol
DH	double haploid
DNA	deoxyribonucleic acid

DNase	deoxyribonuclease
dNTP	deoxyribonucleioside triphosphate
ds	double-strand
DTT	dithiothreitol
E	amplification efficiency
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis(*β*-aminoethyl ether)-N,N'-tetraacetic acid
eSRK	soluble form of *S*-locus receptor protein kinase
EST	expressed sequence tag
EtBr	ethidium bromide
F_2	second filial generation
FRET	fluorescence resonance energy transfer
g	acceleration of gravity
GAP	GTPase activator protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GEF	guanine nucleotide exchange factor
GO	Gene Ontology
GOT/3	isozyme glutamate oxalacetatetransaminase
GSI	gametophytic self-incompatibility
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
het	heterozygous
hom	homozygous
HV	hypervariable
Hz	hertz
IBERS	Institute of Biological, Environmental and Rural Sciences
ILGI	International Lolium Genome Initiative
IP3	inositol 1,4,5-triphosphate
IPTG	isopropyl β-D-1-thiogalactopyranoside
IRGSP	International Rice Genome Sequencing Project
ISSR	inter simple sequence repeat
La	lanthanum
LG	linkage group
LOD	log of odds
M	expression stability parameter
MAPK	mitogen-activated protein kinase
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
MAS	marker assisted selection
MF	molecular function

MLPK	M locus protein kinase
mJ	millijoule
ml	millilitre
mM	millimolar concentration
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
NIL	near isogenic line
nm	nanometer
NTC	no template control
Osa1	MSU Rice genome annotation
PA	phosphatidic acid
PCD	programmed cell death
PCR	polymerase chain reaction
PhSBP1	*Petunia hybrida* S-RNase binding protein 1
PGI-2	isozyme phosphoglycoisomerase
PIP2	phosphatidylinositol 4,5-bisphosphate
PP	pollen factor
ppm	parts per million
PR	pathogenesis-related
QTL	quantitative trait loci
RACE	rapid amplification of cDNA ends
RAP-DB	Rice annotation project database
RAPD	randomly amplified polymorphic DNA
RDA	representational difference analysis
REC	recombination threshold value
Rf	recombination frequencies
RFLP	restriction fragment length polymorphism
RIL	recombinant inbred lines
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	reverse transcription
SAGE	serial analysis of gene expression
SBP	S protein binding protein
SC	self-compatibility
SCP	sperm-coating glycoprotein
SCR	*S*-locus cysteine-rich protein

SD	standard deviation
SDS	sodium dodecyl sulfate
Se	standard deviation of the residuals
SFBB	*S* locus F-box brother
SI	self-incompatibility
SLF/SFB	*S*-locus F-box
SLFL	*S*-locus F-box genes with low allelic sequence polymorphism
SLG	*S*-locus glycoprotein
SNP	single nucleotide polymorphism
SNRK	SNF1-related protein kinase
SP11	*S*-locus pollen protein 11
sPPases	soluble inorganic pyrophosphatases
SRK	*S*-locus receptor protein kinase
ss	single-strand
SSH	suppression subtractive hybridization
SSI	sporophytic self-incompatibility
SSR	simple sequence repeat
StdDev	standard deviation
STS	sequence tagged site
TAS	telomeric repeat associated sequence
TBE	Tris/Borate/EDTA
TDF	transcribed derived fragment
THL	thioredoxin-H-like
TLC	TRAM, LAG1 and CLN8
Tris-Cl	trishydroxymethylaminomethane chloride
U	unit
UBP	ubiquitin-specific protease
v/v	volume/volume
w/v	weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside