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Achievement of genetics in plant reproduction research: the past decade for the coming decade

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In the last decade, a variety of innovations of emerging technologies in science have been accomplished. Advanced research environment in plant science has made it possible to obtain whole genome sequence in plant species. But now we recognize this by itself is not sufficient to understand the overall biological significance. Since Gregor Mendel established a principle of genetics, known as Mendel's Laws of Inheritance, genetics plays a prominent role in life science, and this aspect is indispensable even in modern plant biology. In this review, we focus on achievements of genetics on plant sexual reproduction research in the last decade and discuss the role of genetics for the coming decade. It is our hope that this will shed light on the importance of genetics in plant biology and provide valuable information to plant biologists.

Key words: fertilization, gametophyte, next generation sequencing, reproductive barrier, pollination

INTRODUCTION

In the beginning of the last decade, whole genome sequences in plants were obtained (Arabidopsis Genome Initiative, 2000; International Rice Genome Sequencing Project, 2005). Various kinds of plant research advanced but now it has been recognized that it is not sufficient to understand the overall biological meaning in plants. Almost 150 years have passed since Gregor Mendel established a principle of genetics, known as Mendel's Laws of Inheritance (Mendel, 1866), and a variety of plant species have been applied into plant science with the elements of genetics (Fig. 1). Although technology has been improved at a breakneck speed in the last decade, genetics still plays a prominent role in life science. Thus, understanding the importance of genetics in plant biology is indispensable. In this review, we focus on sexual reproduction as one of the important points in plant genetics. Our hope it is that the importance of genetics will be better understood and provide clues to plant biology, taking full advantage of genetics, in the coming decade.

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Abbreviations: AGPs, arabinogalactan proteins; CMS, cytoplasmic male sterility; LM, Laser microdissection; PMC, pollen mother cell; SI, self-incompatibility

REPRODUCTIVE BARRIERS

Because plants are immovable as we know, pollination is an important process for plants to produce seeds. At the same time, plant needs to reject other or unwanted pollen to protect the species. This is a first step of 'reproductive barriers.' A good example of this is self-incompatibility (SI) in angiosperm. SI is a system which recognizes self/non-self pollen at pollination to promote outcrossing within the same species and is widely spread over 60% of all angiosperm species (summarized in Allen and Hiscock, 2008). Basically, this system is genetically controlled by one locus, called *S* locus, and male and female recognition factors are located tightly within this locus. There are two types of SI systems, gametophytic and sporophytic SI, based on the behavior of SI phenotype in male gametophyte. Over the past quarter-century, there has been great progress to understand the molecular, physiological, and genetic mechanism in these homomorphic SI systems, although heteromorphic SI system in *Primula* is famous as a pioneer work on SI research (Darwin, 1876, 1877) and as one of the Darwin's glorious triumph. We now know the molecular systems of three types of homo-

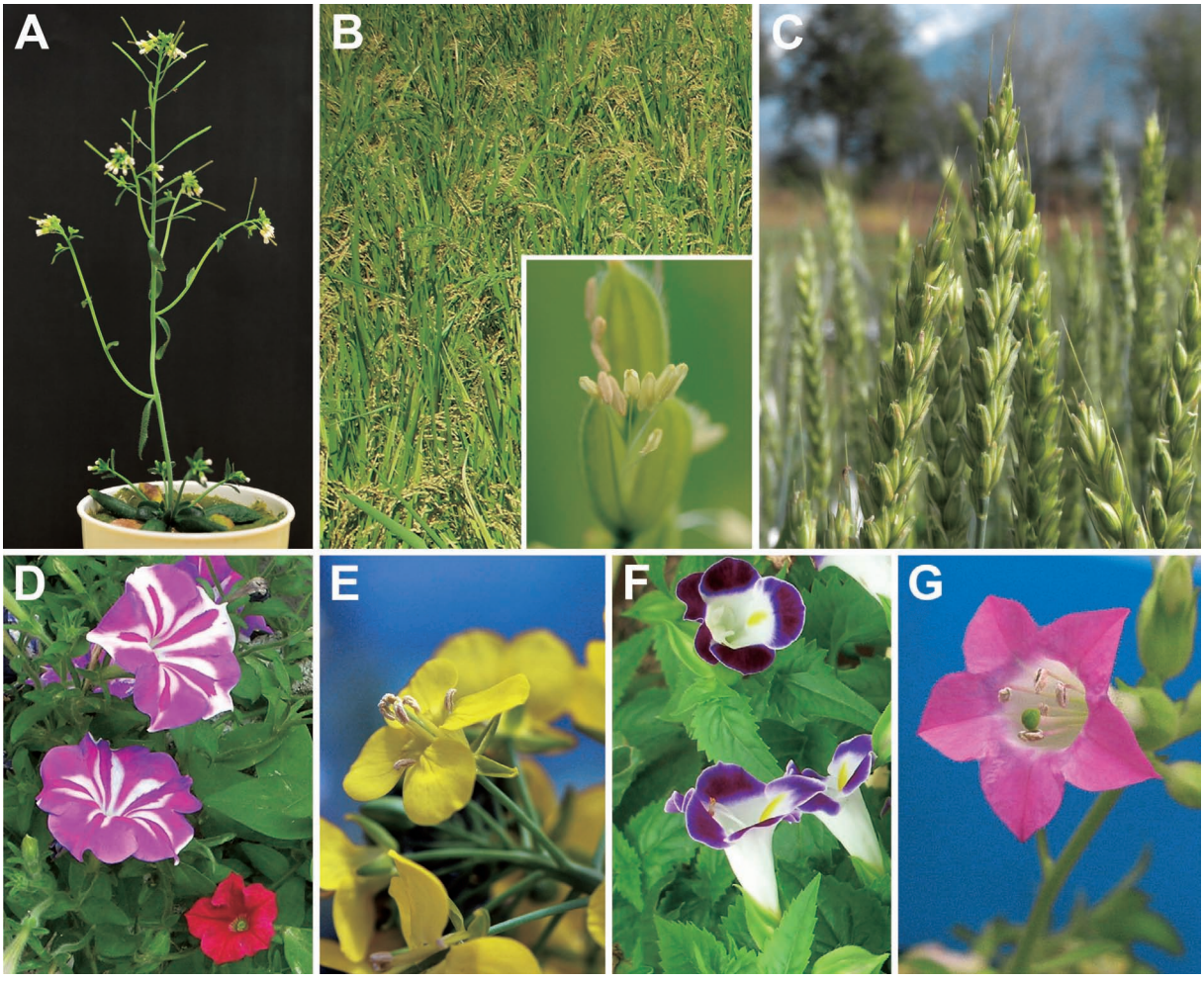

Fig. 1. Plants used in genetic analysis of sexual reproduction. (A) *Arabidopsis*; (B) rice; (C) wheat; (D) *Petunia*; (E) *Brassica*; (F) *Torenia*; (G) tobacco. The wheat photograph is by courtesy of Professor Yasuhiko Mukai, Osaka Kyoiku University.

morphic SI; the *S*-Receptor Kinase (SRK)-based system in Brassicaceae, the *S*-RNase-based system in Solanaceae, Rosaceae and Plantaginaceae, and the *S*-glycoprotein-based system in Papaveraceae. Even though overall the molecular mechanisms underling these systems are still largely unclear, sets of these male and female recognition factors have been identified by genetic and genomic analyses of the *S* locus. Their details are available in recent publications (Franklin-Tong, 2008; Suzuki, 2009). The latest topics in SI research, in addition to the publication of the above articles, are identification of the *trans*-acting small RNA controlling dominance/recessive relationship in the *Brassica* pollen SI factor (Tarutani et al., 2010), the evolution of self-compatibility (SC) in *Arabidopsis* (Tsuchimatsu et al., 2010), and collaborative non-self recognition system in *S*-RNase-based SI (Kubo et al., 2010).

In *Brassica* SI, the dominance/recessive relationships of pollen SI phenotype are often observed between two haplotypes in *S*-heterozygous plants, and the recessive *SP11/SCR*, pollen SI determinant, alleles are suppressed transcriptionally (Shiba et al., 2006). Repression of recessive *SP11* allele is a result of tapetum-specific *de novo* cytosine methylation in its promoter region, and small RNA produced from *SP11*-methylation inducing region (*Smi*), which is located in the flanking region of a dominant *SP11* allele, controls this monoallelic gene silencing (Tarutani et al., 2010).

Arabidopsis thaliana is a self fertile plant belonging to Brassicaceae. From a comparative genetic analysis with *A. lyrata*, a cross relative species having SI, the trace of the *S* locus has been identified in the *A. thaliana* genome, although both male and female SI determinants are inactivated by gene mutations (Kusaba et al., 2001). By ecological and molecular analyses, pseudogenization of pollen determinant gene has been proven to be the first mutation conferring SC of *A. thaliana* and it is nearly fixed in geographically wide samples of European accessions (Tsuchimatsu et al., 2010). A restoration of functional pollen SI determinant, by gene transformation, results in SI on particular *A. thaliana* accession. Thus it

is an experimental reversal of evolution of SI to SC in Brassicaceae (Tsuchimatsu et al., 2010).

Recently, Kubo et al. (2010) showed a newly-found mechanism of *S*-RNase-based SI of petunia, solanaceous plant, in which cross-fertilization is achieved by collaboration among pollen *S* genes. In the *S*-RNase-based SI system, self *S*-RNases entering into the self pollen tube prevent its elongation by cytotoxic effects, whereas non-self *S*-RNases entering into the non-self pollen tube are degraded via the SLF (pollen *S* product)-mediated proteasome pathway, securing the non-self pollen-tube growth. In petunia, the enormous and detailed pollination assays using many transgenic plants with *SLF* transgenes showed that tandemly duplicated *SLF* genes on the *S* locus produce multiple SLFs, which are collaboratively detoxify the non-self *S*-RNases, like animal immune system (Kubo et al., 2010). This finding is definitely an important milestone of the SI research, and provides new insight into the field of plant reproductive genetics.

Genetic reproductive barriers exist at several phases in plant reproduction, including pollination, pollen tube elongation, pre- and post-zygote formation, and embryogenesis. Cytoplasmic male sterility (CMS) is a reproductive barrier in male gamete development. CMS is maternally inherited and is one of the pollen sterility systems in plants. It is regulated by interaction between mitochondria and nuclei, and nuclear-encoded *Rf* genes restore pollen sterility caused by mitochondrial genomic rearrangement (reviewed in Chase, 2007; Fujii and Toriyama, 2008a). Some *Rf* genes encode mitochondria-targeted PentatricoPeptide Repeat (PPR) proteins, and PPR proteins are likely to regulate a post-transcriptional RNA modification of CMS-determining genes in mitochondria (Horn, 2006; Small and Peeters, 2000). Another *Rf* gene encoding a novel protein, RETROGRADE-REGULATED MALE STERILITY (RMS) was also identified in rice CW-type CMS cytoplasm (*Rf17*) (Fujii and Toriyama, 2009). Although each CMS system is unique with respect to the mitochondrial transcript associated with male sterility, it seems that nuclear-mitochondrial gene combinations are common machinery of CMS in many plant species, such as maize (Wise et al., 1999), *Brassica* (Ashutosh et al., 2008), radish (Yasumoto et al., 2009), petunia (Gillman et al., 2007), and wheat (Zhu et al., 2008). Similar to the known examples of retrograde regulation in the yeast RTG signaling, *Drosophila* cell cycle-related signaling, and *Arabidopsis* plastid signaling, the plant CMS might be elaborately regulated by typical retrograde signaling (Fujii and Toriyama, 2008a, 2008b; Fujii et al., 2009). Thus retrograde signals from mitochondria to nuclei are undoubtedly important machinery in plant reproduction.

Genomic imprinting is a postzygotic genetic barrier (Scott and Spielman, 2006; Kinoshita et al., 2008). Genomic imprinting is an epigenetic system that produces unequal expression of maternal and paternal genes. In mammals and plants, the imprinted genes are differentially marked by DNA methylation and/or histone modification (Arnaud and Feil, 2006). The role of maternal and paternal chromosomes differs among each chromosome, and maternal chromosomes usually promote endosperm development while paternal chromosomes repress endosperm development in plants (reviewed in Kinoshita et al., 2008). In the case of plants, *DEMETER* (*DME*) controls DNA methylation in imprinted genes, *FWA* and *FERTILIZATION INDEPENDENT SEED2* (*FIS2*), and it erases DNA methylation of the promoter region in maternally expressed imprinted genes while methylation status is retained in silenced paternal allele (Kinoshita et al., 2004). A paternal allele of another imprinted gene, *MEDEA* (*MEA*), is silenced by the histone methyltransferase activity of its maternal allele, referred to as self-imprinting (Gehring et al., 2006). On the contrary, the paternal allele of imprinted gene, *PHERES 1* (*PHE1*), is expressed by methylation of its 3'-tandem repeat region, while its maternal allele is silenced by de-methylation of the repeat region (Köhler et al., 2005; Makarevich et al., 2008). Although the regulatory mechanism of epigenetic chromosome modifications is different between mammals and plants, polycomb complex PRC2 seems to be a common system for silencing of imprinted genes by DNA methylation both in mammals and plants. Thus, DNA methylation and repression by a polycomb complex are common aspects and play a key role in genomic imprinting in mammals and plants as a reproductive barrier.

Hybrid necrosis (hybrid incompatibility) is an epistatic system of genetic barriers in plant species, and it is also known as a gene flow barrier between species (reviewed in Johnson, 2010). It has epistatic interactions which are observed in many intra- and interspecific plant hybrids, and it contributes to the maintenance of populations or species boundaries. It is caused by interactions between alleles in parental lineages, and a locus in one strain triggers necrosis when combined with a locus in another strain. In *A. thaliana*, among 861 F_1 progenies derived from crosses between 280 parental ecotypes, about 2% of them showed hybrid necrosis (Bomblies et al., 2007). In most cases, epistatic interactions of 2 to 4 loci are involved in this phenotype. Surprisingly, one of the causal genes of hybrid necrosis encodes an NB-LRR type protein, the most common type of disease resistance protein in plants, and activation of gene expression following this involving the plant immune system is also required for the necrosis. Because it is believed to have arisen as a by-product of adaptive evolution, hybrid necrosis may share a common mechanism with a plant autoimmune system in a rapid evolutionary process (Ting et al., 1998; Barbash et al., 2003; Presgraves et al., 2003). Hybrid male sterility in inter-subspecies cross in rice is also

caused by an epistatic interaction at 2 loci, and it is an example of hybrid inviability caused by deleterious epistatic interaction (Kubo et al., 2008). In F_1 hybrid between two major rice subspecies, *japonica* and *indica*, pollen and seed fertilities decrease to approximately 40% and 60% compared to those of parental lines, respectively, although both parental cultivars show normal pollen and seed fertilities. This is caused by epistatic interaction of two genes, *S24* and *S35*, however in this case, it seems not to be a simple epistatic Dobzhansky-Muller model: *S35* locus is dependent on *S24* locus to produce male sterility, but *S24* produces male sterility without a genetic effect of *S35*. A classical Dobzhansky-Muller model proposes a simple scenario to produce hybrid incompatibility between species or isolated populations and depends on the reciprocal genetic interaction between genes that have functionally diverged in the respective species (Brideau et al., 2006). In addition, many hybrid sterility genes have been identified in the inter-subspecies crosses in rice (reviewed in Ouyang et al., 2010). Hybrid lethality would be a severe one of hybrid inviability, leading to embryonic death at early embryogenesis and/or seedling stage. Embryonic death has been found at interspecific crosses in several plant genera such as *Nicotiana* (Zhou et al., 1991), *Gossypium* (Phillips, 1977), and wheat (Zeven, 1981). Although causal genes have not been identified yet, these genes seem to involve programmed cell death, along with chromatin condensation, nuclear fragmentation and vacuolar collapse (Mino et al., 2007; Tezuka et al., 2007). In *Drosophila*, it has been proposed that polygene controls hybrid male sterility (Tao et al., 2003; Moehring et al., 2006). Thus, a variety of epistatic reproductive barriers would exist to maintain and protect a species.

MALE GAMETOPHYTE

Pollen development Pollen is a male reproductive factor to transfer genetic information to the next generation. Many biological events are regulated by complex gene expression in both the gametophytic and sporophytic tissues during pollen development (McCormick, 2004; Scott et al., 2004). After differentiation of the male germline, pollen mother cells (PMCs) undergo meiosis to form tetrad cells of haploid microspores in the anther locule. The microspores then maturate into pollen grains with cell division and formation of the complex pollen wall. Through an asymmetric mitosis, the uninuclear microspore develops into the bicellular pollen with a larger vegetative cell and a smaller generative cell (McCormick, 1993, 2004). The generative cell undergoes a second mitosis to form two sperm cells. In the case of plant species with the tricellular pollen (including *Arabidopsis* and rice), the pollen grain is maturated from the tricellular pollen with the vegetative cell and the two sperm cells. During pollen maturation, the sporophytic tapetum acts as nurse cells for providing nutrition and materials of the pollen wall, and disintegrates in the later stage of the pollen development (Goldberg et al., 1993; Scott et al., 2004). Gene expression in the anther has been intensively studied in important crops and model plants by using conventional cDNA cloning, promoter analysis and microarray (Borg et al., 2009; Honeys and Twell, 2004; Koltunow et al., 1990; Scott et al., 1991; Hihara et al., 1996; Rubinelli et al., 1998; Jeon et al., 1999; Endo et al., 2002, 2004; Amagai et al., 2003; Masuko et al., 2006). In mature pollen grain, pollen-specific transcripts related to cell cytoskeleton, cell-wall re-organization, methionine metabolism, proton pump, and sugar transporter have been detected, whereas as tapetum-specific transcripts related to second metabolisms, fatty acid biosynthesis, protein secretion, and gibberellin signaling cascade have been detected. These different characteristics of gene contents between pollen and tapetum would reflect their different functions in pollen development. In addition, as a recent achievement of a precise transcriptome analysis of pollen and tapetum using laser microdissection-mediated microarray (see below for details), about 38% of anther-expressed genes (10,810/28,141) were synchronized with their expression profiles between pollen and tapetum (Hobo et al., 2008; Suwabe et al., 2008). It is believed that the tapetum acts as a nutritive tissue for pollen development, owing to its high metabolic activity (Scott et al., 2004) and correlation to male sterility (Wilson et al., 2001; Sorensen et al., 2003; Yui et al., 2003; Ariizumi et al., 2004; Yang et al., 2007) and sporophytic self-incompatibility (Takayama et al., 2000; Watanabe et al., 2001). However, this result suggests that tapetum has an identical function with pollen at an earlier stage of pollen development, and after that, it acts as a nurse cell for providing nutrition and materials for pollen wall formation. Thus the tapetum may have dual functions for pollen development and maturation, although this is still speculation.

Because pollen development is carefully orchestrated by the complicated and precise gene networks as mentioned above, it is understandable that pollen development is sensitive to environmental conditions, such as high and low temperatures (Oliver et al., 2005; Endo et al., 2009; Oda et al., 2010), and physiological conditions, such as deficiency of nutrients (Lee and Tegeder, 2004; Kato et al., 2009; Yuan et al., 2009).

Formation of the surface structure (pollen walls) of pollen grains is also an important process for successful pollen development and maturation (Scott et al., 2004). The outermost architecture of pollen walls acts as a kind of protection from the environmental stresses and a pollen-stigma adhesion (Zinkl et al., 1999). The pollen wall consists of inner intine, produced mainly from cellulose and pectin, and outer sporopollenin-based exine. The exine

is further divided into two layers, inner nexine and outer sexine, and provides the species-specific pollen surface structure and cavities storing the pollen coats including lipids and proteins, which are mainly supplied from the tapetum. Through a screening of abnormal exine structure in *Arabidopsis*, 4 types of defective mutants, *kanonashi* (*kns*) series, have been identified (Suzuki et al., 2008). All types of mutant lines have exine-defective phenotypes, such as an abnormality of callose wall and thin exine structure. Along with reports of many other kinds of *Arabidopsis* exine mutants (Ariizumi et al., 2008; Guan et al., 2008; Dobritsa et al., 2009; Morant et al., 2007; Paxon-Sowders et al., 1997, 2001; Zhang and McCormick, 2007), it is obvious that the surface architecture of pollen grain is one of the critical factors for pollen development which leads to a successful reproduction.

Recently, epigenetic gene regulation by small RNA has been suggested in pollen development and function (Fujioka et al., 2008; Takeda et al., 2008; Tarutani et al., 2010). Small RNAs of 18–24 nucleotides are classified into two major classes, microRNA (miRNA) and small interfering RNA (siRNA) (Bartel, 2004; Vazquez, 2006). The miRNAs are processed from single-stranded hairpin-folded precursor RNAs by Dicer ribonuclease, DICER-Like 1 (DCL1). Dicer-generated small RNA then associates with Argonaute (AGO) protein, and the RNA-induced silencing complex (RISC) along with the AGO-small RNA complex regulate their target gene expression accurately by a cleavage of target mRNA, repression of translation, and modification of chromatin structure, in a sequence-specific manner (Brodersen and Voinnet, 2006; Zhang et al., 2006; Tolia and Joshua-Tor, 2007; Kurihara et al., 2009). The list of miRNAs identified to date is available in the miRBase database (http://microrna.sanger.ac.uk/sequences/index.shtml). Meanwhile, siRNAs are derived from longer double-stranded RNAs and are generated through the transcription of inverted repeats or converted from single-stranded RNAs by RNA-dependent RNA polymerases (RDRs). The dsRNAs are then processed into multiple siRNAs through the action of one or more of the DCL proteins. Several distinct classes of siRNAs have been discovered in plants, such as the RDR-dependent siRNA, trans-acting siRNA (ta-siRNA), the repeat associated siRNA (ra-siRNA), and the natural antisense siRNA (nat-siRNA) (Ramachandran and Chen, 2008; Chen, 2010). Large numbers of endogenous siRNAs have been discovered in plants for regulation of gene expression at the transcriptional and post-transcriptional levels, and the regulatory functions of these endogenous siRNAs range from genome stability, environmental stress response, and developmental patterning (Kasschau et al., 2007; Nobuta et al., 2007). Thus, further study will lead to better identification of gene networks regulated by small RNAs in plant reproductive development.

Pollen germination and pollen tube elongation for fertilization In pollination, pollen grain is hydrated, germinates, and elongates the pollen tube into the style toward the ovule for fertilization at the adhesive region between the pollen and papilla cells of the stigma surface (Taylor and Hepler, 1997). In this biological event, many mechanisms such as water and ion channels, protein synthesis, and metabolism are involved. Especially, in most species, an earlier event from hydration to germination occurs very quickly within a couple of minutes when pollen arrives on the stigma surface, thus it has been believed that mature pollen grains store RNAs and proteins required for germination and early tube elongation. Although it is still a matter of debate, many reports support this hypothesis, from the viewpoint of transcriptome, proteome, mutant analysis, and protein/RNA synthesis inhibitor analysis (Becker et al., 2003; Honys and Twell, 2003; Holmes-Davis et al., 2005; Noir et al., 2005; Wang and Okamoto, 2009).

The tip region of the pollen tube has 4 distinct zones: an apical region (clear zone), a subapical organelle-rich zone, a nuclear zone, and a vacuolated zone (Li et al., 1997; Taylor and Hepler, 1997; Hepler et al., 2001; Cheung and Wu, 2008). A striking feature of the pollen tube is an accumulation and fusion of Golgi-derived secretory vesicles at the apical region in the pollen tube apex (Steer and Steer, 1989). The vesicles contain components for cell wall expansion and produce new segments of plasma membrane for tube elongation.

Oscillating growth, typical of fungal hyphae and pollen tubes of lily, is characterized by a periodic oscillatory pattern in which the rate changes in a smooth wave. On the other hand, pulsating growth, typical of petunia and tobacco, is characterized by slower phases of growth followed by growth spurts (pulses) (Picton and Steer, 1982). It seems that the periods of slow growth coincide with the increased thickness by deposition of pectins and arabinogalactan proteins (AGPs) (Li et al., 1992, 1994), and the periods of pulse correspond to thinner cell wall formation (Li et al., 1995, 1997; Pierson et al., 1995), although lily pollen tubes show a uniform deposition of these wall components in the tube, even though they show a marked oscillation in growth rate. The mechanisms underlying the oscillating, or pulsating, growth are not completely understood yet, but it is clear that many important factors contribute to this process. For example, Ca^{2+} and H^{+} in the cell wall space play a crucial regulatory role in controlling the yield properties of the wall and in elevating Ca^{2+} flux involved in the regulation of self-incompatibility in *Papaver rhoeas* (Franklin-Tong et al., 1993). At the tube tip, an influx of K^{+} ions has also been observed. Thus the tip-focused ion gradients would regulate the internal structural zonation for cytoplasmic streaming as well as the vesicle fusion. Actin microfilaments accumulate in the apical region of the growing pollen tube and

seem to be involved in the transport of secretory vesicles essential for cell elongation (Derksen et al., 1995). Actin-binding proteins such as myosin, spectrin, profilin, and Rho GTPases have been identified from many plant species, suggesting that they participate in regulation of actin microfilaments and membrane-associated signal transduction.

After pollination and germination, the emerging pollen tube makes contact with the extracellular matrix of the pistil and grows into the transmitting tract of the style to deliver the male gametes to the ovary. The transmitting tract plays an important role in the extension or movement of the pollen tube to the ovary, and stylar components may serve several functions during pollen tube growth including guidance, nutrition, and structural integrity (Cheung et al., 1995; Wu et al., 1995; Jauh and Lord, 1996). A good example of this is a gametophytic self-incompatibility system in Solanaceae, Rosaceae, and Plantaginaceae. In Solanaceae and Rosaceae, as we mentioned above, it is triggered by interaction between stylar and pollen SI factors, *S*-RNase and SLF/SFB, and tube growth of self-incompatible pollen is arrested by the following ubiquitin-mediated protein degradation system, 26S proteasomal pathway (Hua et al., 2008; Sassa et al., 2010). In the case of Plantaginaceae, in a SI specific manner, the Ca^{2+}-dependent signaling network triggers programmed cell death, resulting in the inhibition of incompatible pollen tube growth (de Graaf et al., 2006; Wheeler et al., 2009). As another example, HT-B, 120K, and transmitting tissue-specific (TTS) proteins, a variety of AGPs, have been isolated from stylar tissues (Du et al., 1994, 1996; Lind et al., 1996). HT-B and 120K proteins are suggested to be necessary for functioning of the *S*-RNase (McClure et al., 1999; O'Brien et al., 2002; Schultz et al., 1997; Hancock et al., 2005). TTS proteins are incorporated into the pollen tube surface and wall, and the glycosylated TTS protein promotes pollen tube growth. It has also been reported that *SKP1* (*S-phase kinase-associated protein 1*)-like genes, one of the major components of SCF complex, are essential for normal lily pollen tube growth (Chang et al., 2009). In all cases, stylar proteins, together with basic biological machineries, are essential for pollen tube growth and may contribute to the guidance of the pollen tubes to the ovary.

When the pollen tube arrives at the embryo sac of the ovule, it enters into the embryo sac through the micropylar end, the entrance of tube for fertilization, and subsequently two sperm cells are dispersed for embryogenesis with the egg cell and endosperm development with the central cell, the so called double fertilization. It has long been suggested that the pollen tube is guided by diffusible attractants produced by the ovule, and recently two attractant proteins were identified from *Torenia fourneri* and maize (Okuda et al., 2009; Márton et al., 2005). Attractant molecule LUREs (LURE1 and LURE2) in *T. fourneri* are expressed in the synergid cell and are secreted to the micropylar end of the filiform apparatus of the embryo sac. These are small cystein-rich peptides of ~9.8 kDa, belonging to a subgroup of defensin-like proteins, and recombinant proteins of LUREs attract pollen tube within a ~50 μm range. Similarly, *Zea mays* EGG APPARATUS1 (ZmEA1) has been identified as a micropylar guidance factor. It is a small plasma membrane protein and belongs to the EA1-like gene family (Gray-Mitsumune and Matton, 2006). It is expressed in synergid cell and the egg cell, and it can attract the pollen tube at the micropyle of the ovule. In *Arabidopsis*, synergid cell-factors, *MYB98* (Kasahara et al., 2005; Punwani et al., 2007) and *FERONIA*/*SIRENE* (Escobar-Restrepo et al., 2007), have also been identified for their micropylar pollen tube guidance and pollen tube reception. In all cases, the synergid cell is likely to be a key player for successful guidance of the pollen tube into the female gametophyte for successful fertilization. In addition, other guidance factors, such as *magatama* (Shimizu and Okada, 2000; Shimizu et al., 2008), *central cell guidance* (Chen et al., 2007), and *gex3* (Alandete-Saez et al., 2008), have been suggested, thus a variety of factors would be involved in a pollen tube guidance.

FEMALE GAMETOPHYTE

In angiosperms, the female gametophyte, a.k.a. megagametophyte, develops within the ovule. In the most common form, called *Polygonum* type, megaspore mother cell undergoes production of seven cells belonging to four identical cell types: an egg cell, two synergids, a central cell, and three antipodals, through megasporogenesis and megagametogenesis. The cellular structure and organization of the female gametophyte have been well studied since the mid 20th century (Maheshwari, 1950), however, few studies have focused on the molecular and genetic mechanisms underlying this biological event because of the inaccessibility and small size of the female gametophyte. Therefore it was referred to as "the forgotten generation" (Brukhin et al., 2005). Although a technical difficulty, it is obvious that the *Polygonum* type female gametophyte contains essential reproductive functions such as pollen tube guidance, reception of pollen tube, fertilization, and embryogenesis, with only seven cells. Thus, it is important to understand its function at the molecular level. For this, molecular genetic analysis of the female gametophyte is one of the choices to dissect these functions and their underlying molecular mechanisms. In a screening of gametophytic mutants, simple Mendelian rules cannot be applied in the genetic analysis of the progeny, but female gamete-defective mutants can be identified by a combination of characteristic features, reduced seed set and a segregation distortion in F_2 progeny on reciprocal crosses with the wild type

plants (Christensen et al., 1998; Howden et al., 1998; Page and Grossniklaus, 2002). By this screening strategy, a larger number of female gamete-defective mutants have been identified (summarized in Yadegaria and Drews, 2004). A similar strategy can be applied to a screening of defective mutants in the later stage of female gametophyte development, such as embryogenesis and the early stage of seed development (Shimizu et al., 2008; Hakozaki et al., 2008). Development of a female gametophyte and embryo occurs within the ovule, with a collaboration of gametophytic and sporophytic tissues, and moreover, reproductive specific mechanisms such as meiosis and unequal cell division are involved. In the recent mutant analysis, T-DNA insertion of *AGO9* (*ARGONAUTE 9*) gene in *Arabidopsis* resulted in abnormal ovule development, indicating that sporophytically-expressed AGO9 protein plays an important role in female gametophyte development via a small RNA pathway (Olmedo-Monfil et al., 2010). Thus the isolation of gametophytic female mutants can be a resource to pave the way for dissecting the molecular mechanisms involved in gametophyte development and basic biological machineries underlying embryogenesis and seed development.

On the other hand, genetic analysis of apomixis is not easy. Apomixis is a well-known plant reproductive system of asexually-reproduced seed formation (Bicknell and Koltunow, 2004). From the point of view of plant breeding, molecular understanding of apomixis is no doubt important for future introduction of apomixis into sexual crops to produce "clone seeds" maintaining heterosis (van Dijk, 2008). Needless to say, apomixis research is closely related to female gametophyte development. Apomixis consists of apomeiosis (avoidance of meiosis), parthenogenesis, and endosperm development with or without fertilization. Although many researchers have extensively studied apomixis in *Boechera*, *Hieracium*, *Hypericum*, *Pennisetum*, and *Taraxacum* plants (Catanach et al., 2006; Huo et al., 2009: Schallau et al., 2010; Sharbel et al., 2010; Vijverberg et al., 2010), a precise understanding of genetic regulation of apomixis still remains veiled. In addition to the difficulty of dissection of the phenomena in ovules, a genetic approach (such as map-based cloning and a transgenic experiment) is difficult or time-consuming because most of these apomicts are not model plants and have a heteromorphic or hemizygous chromosome with the apomixis locus, whose recombination is frequently suppressed. It is noteworthy that mutation of *DYAD* gene in *Arabidopsis* causes apomeiosis (Ravi et al., 2008). Although the apomixis phenotype of *DYAD* is recessive, and natural apomixis is fundamentally regulated by dominant loci, genetic regulation of apomeiosis in *Arabidopsis* has made an impact in plant reproductive science (Noyes, 2008). For a tool of future understanding of genetic regulation of apomixis, deletion mutants for apomeiosis and parthenogenesis in *Hieracium* are expected to be used in efficient map-based cloning of apomixis-related genes (Catanach et al., 2006).

ADVANCED TECHNOLOGY AND INFORMATION FOR GENETICS

In the last decade, full-genome nucleotide sequencing of the model plants *A. thaliana* and rice was completed in 2000 and 2005, respectively (Arabidopsis Genome Initiative, 2000; International Rice Genome Sequencing Project, 2005). Soon after, the innovation of the next generation sequencing platform (the second generation sequencer), such as Genome Analyzer (Illumina), 454 Genome Sequencer FLX (Roche), and SOLiD (Life Technologies), has extensively improved the research environment for nucleotide sequencing. Such recent advanced technology makes it possible to obtain a large number of nucleotide sequences and various kinds of fundamental data sets, such as genes, cDNAs, ESTs, DNA markers and gene expression profiles. This is referred to as omics technologies.

As we mentioned above, plant male and female reproductive organs are embedded and surrounded by sporophytic organs. Thus, a precise isolation of targeted cells is a critical challenge in plant reproduction research. To overcome this difficulty, a laser microdissection (LM) technology is a case in point. LM is a powerful tool for isolating specific cell types from sectioned specimens of heterogeneous tissues (Asano et al., 2002; Kerk et al., 2003; Nakazono et al., 2003; Day et al., 2005; Nelson et al., 2006; Ohtsu et al., 2007a). A tissue section that contains the cell type of interest is placed on a microscope stage, and a laser beam separates the target cells from the rest of the tissue. A current LM system, such as Veritas Laser Microdissection System (Molecular Devices), is capable in collecting the target cells/tissue with high precision and efficiency, without low contamination rate of other unwanted cells/tissues. Recently, many microarray analyses using cell types isolated by LM have been conducted in plant biology. Targeted plant cell types of the LM-microarray include embryos (Casson et al., 2005; Spencer et al., 2007), coleoptile epidermis and vascular tissues (Nakazono et al., 2003), shoot apical meristems (Ohtsu et al., 2007b; Zhang et al., 2007), root pericycles (Woll et al., 2005; Dembinsky et al., 2007), silique replums (Cai and Lashbrook, 2006), and stamen abscission zones (Cai and Lashbrook, 2008). In reproductive organs, such analysis has been conducted in pollen and tapetum cells of rice (Hobo et al., 2008; Suwabe et al., 2008; Watanabe, 2008; Fujita et al., 2010). Using this transcriptome data, gene expression profiles in pollen and tapetum, along with developmental stages, have been elucidated independently, and phytohormone biosynthesis and *cis*-regulatory elements underlying male gametophyte development have been investigated (Hirano et al.,

2008; Mihara et al., 2008). Such achievement can be accomplished only by precise cell type-specific transcriptome. More recently, the LM-microarray was also applied to the cell-type-specific transcriptome of female gametophyte cells; synergids, egg and central cells, showing similar transcriptomes between *Arabidopsis* and human gametes (Wuest et al., 2010).

A combination of LM and the second generation sequencer is a better way for a whole transcriptome analysis, but now the third generation sequencer is becoming more available. The concept of the third generation is different from that of the second generation, and the most striking feature of the third generation is single molecule sequencing at high accuracy. The second generation sequencer focuses on the quantity and rapidity of nucleotide sequencing, and on the other hand, the third one focuses on the quality and preciseness of the data. This means that a very small number of DNAs from a tiny cell is sufficient for sequencing, and not much artificial amplification of DNA samples is necessary. Thus native whole transcriptome information of the tissue/cell of interest can be obtained by a combination of LM and the third generation sequencer technologies. At moment, three platforms, Genetic Analysis System (Helicos), Single Molecule Real Time technology (Pacific Biosciences), and nanopore sequencing (Oxford Nanopore technologies), comprise this third generation sequencer, and other types of platforms will also be launched in the near future.

With an increasing number of comprehensive genomic, transcriptomic and proteomic analyses, large-scale text-based omics data are rapidly accumulating. In addition, numerical-based and phenotype-based databases are becoming more established (Kuromori et al., 2009; Kojima et al., 2009; Mano et al., 2009; Ogata et al., 2009). This situation indicates the necessity of an integrated database among various types of omics data (Swarbreck et al., 2008; Rice Annotation Project, 2008; also reviewed in Shinozaki and Sakakibara, 2009). This wealth of comprehensive resources and web databases allow us to extract essential new biological information beyond a dataset obtained from an individual study. For effective and efficient handling of such large data sets, various kinds of bioinformatics tools are being developed for plant biosciences and systems biology (Obayashi et al., 2007; reviewed in Suwabe and Yano, 2008).

In addition to a light microscope and electron microscope, the bio-imaging system is also critical to grasp appropriate subcellular structure and cytological characteristics of complex plant reproductive tissues. In living pollen, visualization of mitochondria and plastids has been established (Matsushima et al., 2008; Tang et al., 2009), and precise monitoring of actin dynamics in papilla cells and Ca^{2+} dynamics in pollen tubes is also successful in pollination studies (Iwano et al., 2007, 2009). Thermal and fluorescence imaging of chlorophyll is also a well established technique as a non-destructive method in vegetative tissues (reviewed in Chaerle et al., 2007). These kinds of visual imaging technologies in living cells have a particular advantage of examining spatial and temporal information dynamism of the cell, along with growth and development of plants.

FUTURE PERSPECTIVE OF GENETICS

As mentioned above, innovation of emerging technologies and accumulation of omics data are a treasure-trove for biologists, but at the same time, this by itself tells us nothing about biology. To take full advantage of them, a collaboration of a variety of research studies, such as molecular biology, physiology, biochemistry, epigenetics, and bioinformatics, is one of the keys for successful achievement in plant science. A cooperative relationship among every research area of expertise will make it possible to understand complex biological phenomena from all angles, and such endless scientific trials will lead to the next advance in plant reproduction research. Of course, needless to say, genetics is one of the most important collaborators of the integrated research system.

Importance of genetics never changes since Mendel found that each character is governed by specific factor, which is known as ‘gene’ today. Using the Mendelian genetics with a theoretical concept of biological functions regulated by genes, we have effectively studied various plant phenomena as described above. Although it is impossible to achieve comprehensive analysis of the entire gene networks by classical genetics, the emerging new technologies will continuously help our understanding of complex genetic phenomena as in the last decade. The mysteries of yet-to-be-defined mechanisms of plant reproductive phenomena, such as apomixis, polyploidy, heteromorphic incompatibility, unilateral incompatibility, inter-species incompatibility, and incongruity, are expected to be solved by genetics together with unborn technologies in the coming decade.

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