Cytoplasmic contribution in interspecific crosses of Solanum tuberosum L. (2n=4x=48) with a Mexican wild species S. demissum Lindl. (2n=6x=72) and subsequent backcrosses

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Cytoplasmic contribution in interspecific crosses of *Solanum tuberosum* L. (2*n*=4*x*=48) with a Mexican wild species *S. demissum* Lindl. (2*n*=6*x*=72) and subsequent backcrosses

Solanum tuberosum L. (2*n*=4*x*=48)とメキシコ原産野生種 *S. demissum* Lindl. (2*n*=6*x*=72)の種間交雑ならびに戻し交雑後代における細胞質の寄与

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General introduction

When sources of variation for a character of interest (ex. disease or pest resistance) cannot be found within existing genotypes in a species, it seems sensible to look at related species or genera and examine the possibility to introgress traits from them into the one of interest. A high proportion (over 80%) of genes introduced to our crop species through interspecific or inter-generic hybridization relates to pest and disease resistances (Brown and Caligari 2008). This trend continues today whereby wild related species to our crop species are continually being screened and evaluated to identify new genes for resistance to crop diseases.

One of examples of this procedure is found in introgression of tomato mosaic virus (ToMV) resistances into the cultivated tomato (*Lycopersicon esculentum* Mill.**)**. ToMV causes serious loss of yield and fruit quality in commercial tomato crops. Any form of resistance to ToMV in a wild species of tomato was first mentioned by Porte et al. (1939), who found that plants of *L. hirsutum* Dunal, grown from seeds collected by Blood and Tremelling in the years 1937-38, were symptomless even though virus was present in their tissues. Later, Watson and Heinrich (1951) studied the inheritance of ToMV-tolerance in hybrids of *L. esculentum* × *L. hirsutum*. Further studies by Clayberg (1960) and Pecaut (1962, 1964) finally identified a dominant gene *Tm-1* (reviewed by Pelham 1966, 1972). Another single dominant allelic gene *Tm-2* was isolated from *L. peruvianum* (L.) Mill., which conferred a higher level of ToMV resistance than *Tm-1* (Soost 1958, 1959, 1963). Unfortunately, however, *Tm-2* was tightly linked to an undesirable recessive allele *netted-virescent* (*nv*), which caused stunting and yellowing in the homozygous condition (Clayberg et al. 1960).

Alexander (1963) found an additional gene from a cross with *L. peruvianum*. Pecaut (1965, 1966) and Schroeder et al. (1967) studied the allelic relationship of the gene *Tm-2* and Alexander (1963)'s new resistance gene, and found both genes located on the same locus, or extremely closely linked to each other. Because of the apparent allelism with *Tm-2*, the gene symbol *Tm-2*^{2} was assigned (Anonymous 1970).

Another example was found in *Sinapis arvensis* L. of the family *Brassicaceae*. *S. arvensis* is a useful and valuable source of blackleg (*Leptosphaeria maculans*) resistance for oilseed rape (*Brassica napus* L.), because this species contains resistance to a specific isolate of *L. maculans* which has been found to overcome resistance originating from B genome species of *Brassica.* The inter-generic hybrid from *B. napus* × *S. arvensis* has been successfully backcrossed to *B. napus* (Plümper 1995), which is until now the major source of blackleg resistance (Snowdon et al. 2000).

Successful interspecific crossing depends on two factors: obtaining viable seeds from plants in the F_1 and subsequent generations and eliminating undesirable characters from the donor species. More often, however, interspecific hybrids suffer a loss in reproductive capacity with both F_1 and later generations, showing a greater or lesser degree of hybrid abortion or sterility. Therefore, it is required to elucidate the mechanisms for obtaining mature hybrid seeds and subsequent hybrid's fertility.

Normal seed formation after fertilization depends on the endosperm growth, which is controlled by a balance between male and female gametes. In *Arabidopsis*, imprinting genes play important roles for seed development in interspecific crosses (reviewed in Kinoshita et al. 2008; Köhler et al. 2010). The imprinted genes *MEDEA* (*MEA*) and *FERTILIZATION-INDEPENDENT-SEED2* (*FIS2*) show maternal-specific expression and repress endosperm proliferation (Kiyosue et al. 1999; Ingouff et al.

2005), while *PHERES1* (*PHE1*) shows paternal-specific expression and promotes endosperm proliferation (Köhler et al. 2003, 2005). *MEA* and *FIS2* along with non-imprinted genes *FERTILIZATION-INDEPENDENT-ENDOSPERM* (*FIE*) and *MULTICOPYSUPPRESSOR-OF-IRA1* (*MSI1*) form Polycomb repressive complex 2 (PRC2) proteins (Guitton and Berger 2005; Baroux et al. 2007; Huh et al. 2008), which suppress endosperm cellularization (Kang et al. 2008). A dosage-dependent regulation model of these imprinted genes has been proposed in the interspecific and inter-ploidy crosses of *Arabidopsis* (Dilkes and Comai 2004; Josefsson et al. 2006). Recent findings suggest more specifically that dosage-sensitive loss of PRC2 proteins results in dysregulation of AGAMOUS-LIKE Type-1 MADS domain transcription factors (AGL) such as PHE1, AGL62 and AGL90. These AGLs are co-regulated and interacting as heterodimers consisted with combinations of at least six member proteins in *Arabidopsis* (Walia et al. 2009). Endosperm cellularization in inter-ploidy *Arabidopsis* hybrid seeds with paternal genome excess is delayed or fails completely (Scott et al. 1998; Dilkes et al. 2008), which is consistent with increased expression of *PHE1* and its proposed interaction partner *AGL62* (de Folter et al. 2005) and impaired PRC2 proteins (Erilova et al. 2009). Thus, maternal PRC2 protein has a role serving as a dosage sensor for increasing paternal AGL contributions, establishing the molecular basis for dosage sensitivity (Erilova et al. 2009). As the result, increased paternal genome contribution as well as lack of PRC2 function predominantly affects endosperm development. Such endosperm growth controlled by a balance between maternally contributed PRC2 protein and paternally contributed AGLs might be a conserved mechanism (Köhler et al. 2010). In potato, however, little is known on the molecular basis for seed development in interspecific crosses with its relatives (the tuber-bearing *Solanum* species).

A conceptual explanation, known as the Endosperm Balance Number (EBN) hypothesis (Johnston et al. 1980), has been proposed for endosperm development in interspecific crosses in potato (Ehlenfeldt and Ortiz 1995). According to this hypothesis, a balance of 2:1 maternal to paternal EBN dosage in the endosperm, independent of ploidy, is required for normal endosperm development. EBN values for various potato species have been determined based on the ease of crossability between standard testers as pollen parents and the species in question, and 2x(1EBN), 2x(2EBN), 4x(2EBN), 4x(4EBN) and 6x(4EBN) species have been identified (Hanneman 1994). The same biological concept, the polar-nuclei activation (PNA) hypothesis has been proposed by Nishiyama and Yabuno (1978) to explain the diverse interspecific crosses in the genus *Avena* (Katsiotis et al. 1995). The degree of the polar nuclei activation is expressed by the 'activation index' (AI), which is the ratio of the 'activating value' (AV) of the male gamete to the 'response value' (RV) of the female gamete. In a self-pollinated plant AV=RV, and the AI = (AV/2RV) × 100 = 50%, which is the most balanced condition resulting in normal endosperm development. Depending on the AI of the polar nuclei, the kernel type becomes different: AI < 20% - small inviable kernels, 20% < AI < 30% - small viable kernels, 30% < AI < 80% - normal viable kernels, and 80% < AI - large shriveled-empty inviable kernels (Nishiyama and Yabuno 1978). Although for judging the seed viability, plumpness, germinability and/or size were considered (Johnston and Hanneman 1980), seed size itself was not a criterion in determining EBN values. In contrast, kernel size was an important criterion to determine the species' AV or RV.

Analyses of hybrids between Mexican species of 4x(2EBN) and a South American, colchicine-doubled 4x(2EBN) *S. commersonii* Dunal, or hybrids between a South American 4x(2EBN) *S. acaule* Bitt. and a colchicine-doubled 4x(2EBN) *S. commersonii*,

disclosed lack of recombination and segregation for EBN in these hybrids, suggesting that these 4x(2EBN) species carry EBN in a genetically similar way (Bamberg and Hanneman 1990; Bamberg 1994). Ehlenfeldt and Hanneman (1988) obtained exceptional inter-EBN hybrids (1.5 EBN) from the cross between 2x(1EBN) *S. commersonii* and 2x(2EBN) *S. chacoense* Bitt., and conducted a complete diallele cross including the exceptional hybrids and their parents. They observed that a slight excess of maternal dosage produced viable seeds of reduced size, while a slight excess of paternal dosage produced large seeds or aborted seeds. Based on the observation they proposed a genetic model for EBN, controlled by three unlinked, additive loci in a threshold-like system (Ehlenfeldt and Hanneman 1988). Alternatively, Camadro and Masuelli (1995) proposed a model that the EBN is controlled by two independent loci with two alleles in homozygosity per genome; that is, 4x(2EBN) *S. acaule* carrying in homozygosity the alleles "0.5" and "0", 2x(1EBN) *S. commersonii* carrying the alleles "0.5" and "0" and 2x(2EBN) *S. gourlayi* Hawkes carrying the alleles "0.5" and "0.5". Therefore, although the EBN is practically useful to predict success or failure of a given interspecific cross in potatoes (Ortiz and Ehlenfeldt 1992), genetic understanding of EBN has still been controversial. The previous studies were conducted using exceptional hybrids from inter-EBN crosses, so that seemingly, these materials never generated fertile progenies enabled to assess their EBNs. Consequently, studies on genetic and molecular bases of EBN have been greatly limited due to lack of genetic materials.

In potato breeding, *S. demissum* Lindl. is one of the oldest wild species used as a resistance source to late blight in the history of modern breeding (Rudorf 1950; Ross 1986; Plaisted and Hoopes 1989). Until now, 11 hypersensitive-type resistance genes (*R1-R11*) were identified and incorporated into cultivars (Ross 1986). *S. demissum* can

be easily crossed with the common potato (*S. tuberosum* L.) only when used as a female parent and produces pentaploid hybrids. The resultant pentaploid hybrids are non-functional as males, and only crossable as female parents (Black 1943a; Dionne 1961; Irikura 1968). The male sterility of the backcrossed progeny persists even after ten or more successive generations of backcrossing. Thus, the *S. demissum* cytoplasm was preferentially transmitted to the bred varieties. Although Dionne (1961) suggested that this male sterility is attributed to the interaction of a cytoplasmic factor of *S. demissum* and nuclear factors contributed by the male parents, the underlying molecular mechanism is still unknown.

To elucidate as a goal the genetic and molecular basis for the seed formation mechanism in interspecific crosses, I investigated genetic factors causing unilateral incompatibility between *S. tuberosum* and *S. demissum* in this study. In Chapter I, crossing experiments were conducted between *S. tuberosum* and *S. demissum* and for the subsequent progenies. As I found a difference in crossability between interspecific F_1 hybrids, pollen DNA and mRNA were compared between the reciprocal F_1 hybrids in Chapter II and III, respectively. In Chapter IV, the *S. demissum* cytoplasm-specific DNA marker was developed. Based on these findings, the effect of *S. demissum* cytoplasm on crossability and the usefulness of *S. demissum* cytoplasm-specific marker in potato breeding are discussed in Conclusion.

Chapter I

Factors affecting crossability in *Solanum tuberosum* × *S. demissum* and the progenies

Introduction

Interspecific or inter-generic cross-incompatibility has been a hindrance in all of plant breeding programs. One of cross-incompatibilities previously observed is unilateral incompatibility (UI). UI is defined by the one-way success of crossing between self-compatible (SC) and self-incompatible (SI) plants, which is widely known in various plant genera. SC × SI interspecific cross usually succeeds, whereas the reciprocal cross SI × SC fails due to pollen-tube inhibition (Lewis and Crowe 1958). The UI has also been observed in SC × SC, SC × SI, and SI × SI combinations (Abdalla and Hermsen 1972; de Nettancourt 1977). Among gametophytic SI species, UI has been observed in *Nicotiana* (Pandey 1964), *Petunia* (Mather 1943), *Lycopersicon* (McGuire and Rick 1954; Martin 1961, 1964), *Antirrhinum* (Harrison and Darby 1955), and *Solanum* (Malheiros-Gardé 1959; Grun and Radlow 1961; Pandey 1962a, b; Grun and Aubertin 1966; Abdalla and Hermsen 1972). In potato and its relatives (the tuber-bearing *Solanum* species), almost all diploids are SI, controlled by the gametophytic SI locus (Pushkarnath 1942; Pandey 1962a; Cipar et al. 1964; Thompson and Kirch 1992). And UI in the tuber-bearing *Solanum* species has also been reported between several species combinations (Marks 1965; Camadro et al. 2004). Three major hypotheses to explain UI has been proposed (Hermsen and Sawicka 1979): 1) self-incompatibility alleles which have dual function (Lewis and Crowe 1958; Pandy

1964), 2) specific genes independent of S locus (Grun and Radlow 1961; Martin 1963; Grun and Aubertin 1966), and 3) both S alleles and specific gene's power competition (Abdalla and Hermsen 1972).

In the family Solanaceae, SI specificity is determined by an S locus, encoding S-ribonucleases (S-RNases) expressed in the pistil (McClure et al. 1989) and S-locus F-box (SLF) proteins expressed in pollen (Sijacic et al. 2004). Li et al. (2010) demonstrated that in *Solanum*, a pollen-expressed *Cullin1* gene with high similarity to *Petunia* SI factors interacts genetically with a gene near the S locus to control UI. And cultivated tomato and related red- or orange-fruited species (all SC) exhibit the same loss-of-function mutation in this gene, whereas the green-fruited species (mostly SI) contain a functional allele; hence, similar biochemical mechanisms underlie the rejection of both self and interspecific pollen. However, the differential timing of rejection of self versus interspecific pollen tubes demonstrates that there are also differences between SI and UI (Hardon 1967; Hogenboom 1973; Liedl et al.1996; Covey et al. 2010).

One of wild potato species, *S. demissum* is highly self-fertile, yet it shows UI with the common potato (*S. tuberosum*). The genomic structure of *S. demissum* may be complicated. From the cytological observation of chromosome pairing behaviors at metaphase I, it has been thought that *S. demissum* shares A genome in common with the common potato (AAA^tA^t) and many other South American species, and the second and third genomes are partially homologous to each other but different from A genome (Marks 1955; Matsubayashi 1962; Irikura 1976). Thus, the genome formula has been proposed for *S. demissum* as $AADDD^dD^d$ (Matsubayashi 1991). However, the nuclear and chloroplast or mitochondrial DNA analyses indicated very close genetic similarity between *S. demissum* and A genome species (Hosaka et al. 1984; Debener et al. 1990;

Spooner at al. 1991; Kardolus et al. 1998; Lössl et al. 1999; Nakagawa and Hosaka 2002). Thus, the degree of genomic differentiation between D and A genomes is obscure and the putative ancestral species of D genome is unknown (Hawkes 1990). Despite such ambiguity and polyploidy, we can easily obtain the pentaploid hybrid from *S. demissum* × *S. tuberosum* when *S. demissum* was used as a female parent (Black 1943a; Cooper and Howard 1952; Irikura 1968). For example, Irikura (1968) reported that 314 pollinations on *S. demissum* with *S. tuberosum* pollen produced 175 berries with 5,171 seeds resulting in the 56% berry-setting rate and 29.5 seeds/berry, while the reciprocal cross on 621 *S. tuberosum* flowers with S. *demissum* pollen produced 6 berries with 980 seeds, resulting in the 1% berry-setting rate and 163.3 seeds/berry. The resultant pentaploid F_1 hybrids produce abundant normal-looking pollen grains, but are non-functional as males, and usually produce seeds only if backcrossed with the pollen of *S. tuberosum* (Dionne 1961).

In this chapter, to elucidate the genetic mechanism of UI between *S. tuberosum* and *S. demissum* and in the backcross progenies, thousands of crosses with various combinations were made and berry-setting rates, seeds/berry, seed size and pollen tube growth were examined. From the observation, I found significant differences in seed size and berry-setting rates between reciprocal interspecific hybrids. At least three genetic factors were involved in normal seed development, 1) a cytoplasmic factor, and nuclear genome-encoded factors functioned 2) in female gametophyte and 3) in pollen. Among them, the cytoplasmic factor, or maternally inherited factor from *S. demissum* showed the most prominent effect on the crossability: *S. demissum* cytoplasm reduced berry-setting rates in all cross combinations. Underlying molecular basis of EBN is also discussed.

Materials and Methods

Plant materials

Seeds of 25 accessions of *S. demissum* with different PI numbers were obtained from the Potato Introduction Station (NRSP-6), Sturgeon Bay, Wisconsin, USA. An advanced breeding line Saikai 35 was always used as a *S. tuberosum* material except for inter-variety crosses where Pike and Desiree were also used. Most of *S. tuberosum* cultivars have T-type chloroplast DNA, as defined by Hosaka (1986), and β-type mitochondrial DNA, as defined by Lössl et al. (1999), (Lössl et al. 2000). However, Saikai 35, descended maternally from *S. phureja* Juz. & Buk., so that it has S-type chloroplast DNA and ε-type mitochondrial DNA. For this reason, Saikai 35 does not display cytoplasmic male sterility as compared to other cultivars of *S. tuberosum* (Grun 1979). F_1 and BC_1 progenies analyzed in this study were all derived from 5H109-5 (*S. demissum* PI 186551) and Saikai 35. The parental *S. demissum* clone 5H109-5 has W-type chloroplast DNA and α-type mitochondrial DNA. Since *S. demissum* is highly self-fertile and homogeneous within family as evidenced by random amplified polymorphic DNA analysis (Hosaka, unpublished), 5H109-5 and the selfed progeny were all assumed to be genetically identical and collectively referred to as D. And Saikai 35 was referred to as T hereinafter. The cross between T as female and D as male generated TD family (6H38) and the reciprocal cross DT family (6H37). Three TD (6H38-2, -8 and -19) and three DT (6H37-5, -6 and -15) hybrids were counted for somatic chromosome numbers by Ono (2010), all of which had 60 chromosomes (Fig. 1c). Four plants of TD family (6H38-7, -8, -19 and -23) and four plants of DT family (6H37-2, -6, -13 and -23) were crossed as female with T, deriving BC_1 families (TD)T and (DT)T, respectively. Four plants of TD family (6H38-43, -58, -84 and -73) and

one plant of DT family (6H37-23) were crossed as female with D, deriving BC_1 families (TD)D and (DT)D, respectively. Somatic chromosome numbers were determined by Ono (2010) for 100 plants of (TD)T (derived from 6H38-19 × T) and 105 plants of (DT)T (derived from 6H37-6 × T), the former ranging from 49 to 60 with an average of 53.8 (SD=1.93) and the latter ranging from 49 to 59 with an average of 53.5 (SD=1.98). There was no significant difference between the two BC_1 families in the chromosome number ($t = 1.15$, $P > 0.25$).

Hybridity test

Total DNA was extracted from fresh leaves by the method of Hosaka and Hanneman (1998). Amplified fragment length polymorphism (AFLP) analysis (Vos et al. 1995) was performed to compare parents and the reciprocal hybrids. Total DNA was double-digested by *Msp*I and *Eco*RI, ligated to adapters, pre-amplified and selectively amplified by the method essentially described in Vos et al. (1995). Adapter and primer sequences were described in Xiong et al. (1999). For pre- and selective amplification, polymerase chain reaction (PCR) was set up in volume of 10 µl consisting of 0.3 µM primer, 5 µl of Ampdirect® Plus (Shimadzu Co., Japan) and 0.25 units *Taq* DNA polymerase (Nova *Taq*™ Hot Start DNA polymerase, Novagen®, USA). The amplification products were electrophoresed on 4% denaturing polyacrylamide gels, and silver-stained (Bassam et al. 1991).

Crossing

All crosses were made in an ordinary manner. Berries were collected one month after pollination and seeds were extracted after another one month. When seeds were extracted, matured berries were squeezed in water and the debris was flushed out to

collect only plump seeds. The seeds were dried naturally and then in a desiccator, followed by counting number of seeds and measuring the weight.

Observation of pollen tube growth

Flowers were emasculated a day before flowering and pollinated. Styles and ovaries were collected 48 h later after pollination, fixed in FAA (3 part ethanol : 1 part glacial acetic acid) at 4°C for 24 h, and after rinsing in water for 30 min, stored in 70% ethanol at 4°C. The pollen tube growth was observed using the aniline blue method modified from Sitch and Snape (1987). The samples were washed and rehydrated in distilled water for 30 min, and then softened in 70% lactic acid in a boiling water bath for 10 min. After cooling to room temperature, samples were washed in distilled water for 1 h and left in 0.1 M K_3PO_4 buffer at 4°C overnight. They were then stained in decolorized aniline blue solution (0.2% w/v in 0.1 M K_2HPO_4 buffer, pH 11.0) for 3 h. The stained samples were cut with a pair of tweezers, mounted under a cover slip and examined using a fluorescence microscope (Nikon HB-10101AF). The yellow florescence emitted by the stained callose plugs and the linings of pollen tubes were visualized under ultra-violet light.

Results

Reciprocal crosses between S. tuberosum *and* S. demissum

One hundred and twenty-two plants raised from 25 *S. demissum* accessions with different PI numbers (3-10 seedlings each) were selfed. The mean berry-setting rate among 122 plants was very high (92.8%) compared with those of *S. tuberosum* (Saikai

35) self- or inter-variety crosses (Table 1). The number of seeds per berry (166.8) and the mean seed weight (0.41 mg) were not much different from those of Saikai 35 self- and inter-variety crosses.

Crosses between *S. tuberosum* (T) and *S. demissum* (D) differed reciprocally (Table 1). When *S. demissum* was female (D × T), all 110 plants (2-5 plants each of 25 accessions) set berries with the mean berry-setting rate of 81.2%. The reciprocal cross (T × D) with the pollen from 17 of 28 *S. demissum* plants (1-6 plants each of 9 accessions) was also successful, although the mean berry-setting rate was low (18.7%). The D × T cross produced the significantly lower number of seeds per berry (34.0) and the heavier mean seed weight (0.94 mg) than the T × D cross (113.2 and 0.39 mg, respectively) (Fig. 1a), D self or T self ($P < 0.001$). The seed sizes obtained from these reciprocal crosses were considerably uniform within a berry.

Hybridity test of reciprocal interspecific hybrids

DNA samples from 7 TD hybrids and those from 6 DT hybrids were bulked, separately. Using 126 AFLP primer pairs, over 12,500 DNA fragments were compared between the two bulked DNA samples, which always showed identical and the sum of the parental AFLP banding patterns (Fig. 1b), except for a few bands reported in the next chapter. These results supported their hybrid status.

Crossing behavior of TD and DT reciprocal hybrids

Selfing and sib-crossing of DT hybrids as male were all unsuccessful (Table 2), whereas 4 of 17 TD hybrids set 1-3 berries by selfing and 6 of 53 sib-mating combinations using TD hybrids as male set 1-3 berries, resulting in relatively low mean berry-setting rates (1.7-5.6%) and small numbers of seeds/berry (3.7-10.1).

Table 1 Crossing behavior in *S. tuberosum*, *S. demissum* and their reciprocal crosses

Cross combination[a]	Flowers	Berries	Successful crosses			Seeds/berry		Seed weight (mg)	
			No.[b]	Mean (%)[c]	SD	Mean[d]	SD	Mean[d]	SD
Saikai 35 self	69	47	1	68.1	-	219.5	-	0.49	-
Saikai 35 × Pike	77	33	1	42.9	-	143.4	-	0.60	-
Desiree × Saikai 35	25	15	1	60.0	-	ND	-	0.73	-
S. demissum (122) self	449	418	119	92.8	19.62	166.8	47.22	0.41	0.063
Saikai 35 × *S. demissum* (28)	232	45	17	18.7	20.93	113.2	61.71	0.39	0.032
S. demissum (110) × Saikai 35	488	395	110	81.2	22.77	34.0	19.27	0.94	0.201

[a] Combinations shown in order of female × male. The number of genotypes involved in the cross combination given in parenthesis

[b] No. of successful cross combinations

[c] Mean of the berry-setting rates (percent berries/flowers) over all cross combinations

[d] Mean among successful cross combinations

ND no data, SD standard deviation

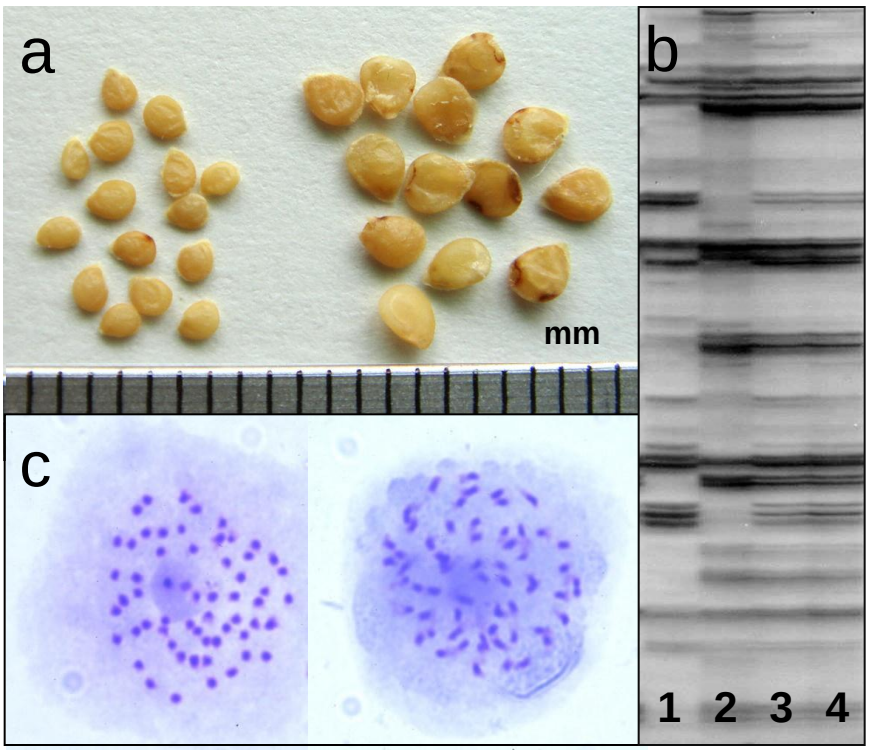

Fig. 1 Differences of reciprocal interspecific hybrids between *S. tuberosum* cv. Saikai 35 (T) and *S. demissum* (D). **a** Seed size in TD (left) and DT (right). **b** AFLP banding patterns (1 T, 2 D, 3 TD bulk, 4 DT bulk). **c** Somatic chromosomes in the root tip cells of TD (left, 6H38-19, 2*n*=60) and DT (right, 6H37-6, 2*n*=60)

Table 2 Crossing behavior of reciprocal F_1 hybrids TD (Saikai 35 × *S. demissum* 5H109-5) and DT (reciprocal)

Cross combination[a]	Flowers	Berries	Successful crosses			Seeds/berry		Seed weight (mg)	
			No.[b]	Mean (%)[c]	SD	Mean[d]	SD	Mean[d]	SD
TD (17) self	161	7	4	4.2	8.42	10.1	2.25	0.73	0.081
DT (55) self	223	0	0	0	-	-	-	-	-
TD (14) × DT (24)	118	0	0/41	0	-	-	-	-	-
DT (13) × DT (11)	66	0	0/23	0	-	-	-	-	-
TD (11) × TD (10)	120	7	5/33	5.6	16.09	3.7	1.76	1.03	0.254
DT (10) × TD (10)	79	1	1/20	1.7	7.45	7	-	0.69	-
TD (71) × T	426	141	52	30.8	28.17	33.2	14.09	0.97	0.107
DT (21) × T	183	45	14	24.5	26.23	32.3	13.61	0.93	0.162
T× TD (31)	103	0	0	0	-	-	-	-	-
T × DT (25)	103	0	0	0	-	-	-	-	-
TD (28) × D	171	34	14	25.3	31.84	84.3	34.70	0.59	0.036
DT (40) × D	150	30	16	21.7	31.03	62.2	26.09	0.53	0.080
D × TD (16)	123	78	16	64.9	22.11	46.2	17.55	0.63	0.078
D × DT (9)	94	19	7	24.2	25.63	30.0	17.74	0.62	0.082

[a] Combinations shown in order of female × male. The number of genotypes involved in the cross combination given in parenthesis. T denotes Saikai 35. D denotes plants derived by selfing *S. demissum* 5H109-5

[b] The number of successful cross combinations. Sib-crosses are indicated by the numbers of successful cross combinations/total combinations

[c] Mean of the berry-setting rates (percent berries/flowers) over all cross combinations

[d] Mean among successful cross combinations

SD standard deviation

TD × T showed the almost similar crossing behavior to DT × T. 73.2% of TD hybrids and 66.7% of DT hybrids set berries with the mean berry-setting rates of 30.8% and 24.5% and the mean seeds/berry of 33.2 and 32.3, respectively. DT (grown from large seeds) × T produced large seeds of 0.93 mg, and interestingly, TD (grown from small seeds) × T produced also large seeds of 0.97 mg. When TD and DT were used as male onto T, no berry set.

Likewise, TD × D showed the almost similar crossing behavior to DT × D: 50.0% of TD and 40.0% of DT hybrids set berries with the mean berry-setting rates of 25.3% and 21.7%, respectively. The mean seeds/berry was 62.2-84.3 and the mean seed weight of 0.53-0.59 mg. In the reciprocal crosses, although all 16 TD and 7 of 9 DT hybrids set berries onto D, TD and DT hybrids showed significantly different performances. TD hybrids set berries with the significantly higher berry-setting rate (64.9%) and larger number of seeds/berry (46.2) than DT hybrids (24.2% and 30.0, respectively) ($P < 0.001$ and $P = 0.023$, respectively). Their mean seed weights were almost similar (0.62-0.63 mg). In these reciprocal crosses with D, however, it was noticed that berries contained many large empty shriveled, or aborted seeds (uncounted), and even the size of plump seeds remarkably varied in a berry, although the standard deviation among mean seed weights for each cross combination was not high (Table 2).

Crossing behavior of BC_1 plants

In any cross combinations involving BC_1 plants (TD)T, (DT)T, (TD)D and (DT)D, successfulness of crossing largely differed between individual plants, or likely segregated. Moreover, successful crosses produced berries containing many large empty shriveled, aborted seeds, and a wide range of seed size variation was observed (Fig. 2). In a few cross combinations, seed size within a berry was relatively uniform.

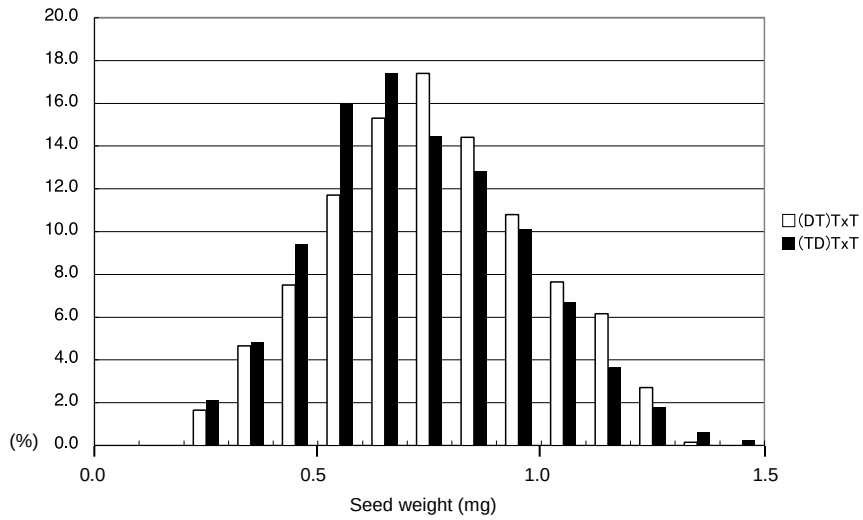

Fig. 2 Variation in seed weight observed among BC_2 seeds obtained by combined data from 10 randomly chosen cross combinations for each of (TD)T × T and (DT)T × T

43 of 112 (TD)T and 10 of 83 (DT)T plants set berries by selfing. Thus, (TD)T was more successful (the berry-setting rate of 25.1%) and produced a higher number of mean seeds/berry (55.9) than (DT)T (8.4% and 19.3, respectively) (Table 3). In the sib-crosses, again, (TD)T plants were more successful as males: the pollen from (TD)T plants set more berries (13.7-14.8%) and produced the higher number of seeds/berry (35.3-48.7) than that from (DT)T plants (3.3-11.0% and 14.8-27.2 seeds/berry, respectively). In the crosses with T, (TD)T × T and (DT)T × T showed higher berry-setting rates (28.6-49.2%) and heavier mean seed weight (0.80-0.84 mg) than their reciprocal crosses (1.5-14.7% and 0.57-0.67 mg, respectively). In addition, (TD)T × T showed higher berry-setting rates than (DT)T × T (49.2% vs. 28.6%), and T × (TD)T higher than T × (DT)T (14.7% vs. 1.5%). In the reciprocal crosses with D, the berry-setting rates were relatively high except for the cross D × (DT)T (8.4%). (TD)T × D and (DT)T × D produced the larger number of mean seeds/berry (66.3-74.3) and smaller seed weight (0.45-0.48 mg) than their reciprocal crosses (14.4-31.5 and 0.80-0.85 mg, respectively).

Selfing of (TD)D and (DT)D plants was almost unsuccessful. One (DT)D plant set one seedless berry, and another one set two selfed berries, of which only one contained one seed weighing 0.50 mg. Thus, the mean seeds/berry for (DT)D family became 0.50. Likewise, five of 111 (TD)D plants set 14 berries by selfing, but only six berries from one plant contained a total of 11 seeds. (TD)D × T and (DT)D × T set berries (the mean berry-setting rates of 22.7% and 14.5%, respectively), but the number of seeds/berry was low (5.3 and 5.2, respectively). T × (TD)D and T × (DT)D set no berry, although the number of pollinations might not be sufficient. In the reciprocal crosses of (TD)D and (DT)D with D, only (TD)D × D showed the moderate berry-setting rate (13.4%). In the cross D × (TD)D, only one of seven plants set one

berry containing the relatively high number of seeds (85) with uniformly small size (the mean seed weight of 0.30 mg, ranging from 0.2 to 0.4 mg). (DT)D × D and the reciprocal crosses failed to set berry.

Observation of pollen tube growth

Pollen tubes in reciprocal crosses between T and D penetrated styles normally, reached the bottom of styles (Fig. 3b) and entered gaps between ovules (Fig. 3c), which strongly indicated normal fertilization occurred. Even in unsuccessful crosses such as selfing TD and DT, or T × TD and T × DT, pollen tubes were penetrating through styles towards ovaries (Fig. 3a) and reached ovaries (Fig. 3d). In the crosses D × TD and D × DT, a differential crossing ability was found as described above. Yet, pollen tubes of both DT (less successful parent, Fig. 3e) and TD (successful parent, Fig. 3f) apparently reached ovaries.

Parental and cytoplasmic effects

Relevant mean values of the berry-setting rate, seeds/berry and seed weight were extracted from Tables 1-3 and arranged to display parental (as female or male) and cytoplasmic (D or T cytoplasm) differences in Table 4.

There was a tendency that the percentage berries/flower was positively correlated with mean seeds/berry (r = 0.503), but the mean seeds/berry was negatively correlated with seed weight (r = -0.460). Thus, the berry-setting rate, seeds/berry and seed weight were likely associated among them, indicating that if a certain cross easily set berries, more number of seeds with small sizes were expected.

In addition to this general association, I found three tendencies. First, the cytoplasmic difference was prominent. Irrespective of being crossed as male or female,

Table 3 Crossing behavior of BC_1 hybrid families (TD)T, (DT)T, (TD)D and (DT)D

Cross combination[a]	Flowers	Berries	Successful crosses			Seeds/berry		Seed weight (mg)	
			No.[b]	Mean (%)[c]	SD	Mean[d]	SD	Mean[d]	SD
(TD)T (112) self	442	102	43	25.1	37.32	55.9	42.63	0.62	0.101
(DT)T (83) self	289	27	10	8.4	24.44	19.3	18.00	0.68	0.076
(TD)T (31) × (DT)T (19)	85	9	6/35	11.0	27.72	27.2	19.54	0.82	0.206
(DT)T (33) × (DT)T (30)	121	4	4/48	3.3	11.50	14.8	17.73	0.59	0.238
(TD)T (68) × (TD)T (41)	206	31	23/88	13.7	25.84	48.7	28.57	0.65	0.100
(DT)T (21) × (TD)T (18)	61	10	6/27	14.8	29.63	35.3	11.59	0.62	0.076
(TD)T (87) × T	493	212	70	49.2	34.22	53.3	26.22	0.80	0.130
(DT)T (36) × T	190	41	21	28.6	32.82	50.6	22.98	0.84	0.108
T × (TD)T (33)	118	18	12	14.7	22.62	53.8	19.82	0.67	0.131
T × (DT)T (43)	128	3	3	1.5	5.97	31.3	26.35	0.57	0.068
(TD)T (38) × D	142	51	24	41.8	39.31	66.3	43.60	0.45	0.097
(DT)T (33) × D	119	41	21	37.8	38.31	74.3	39.64	0.48	0.094
D × (TD)T (40)	247	109	33	42.2	29.62	31.5	23.55	0.85	0.227
D × (DT)T (46)	258	22	14	8.4	16.01	14.4	13.37	0.80	0.230

(TD)D (111) self	772	14	1 (+4)	2.5	13.12	1.8	-	0.58	-
(DT)D (29) self	201	3	1 (+1)	2.3	9.68	0.5	-	0.50	-
(TD)D (75) × T	376	85	27 (+5)	22.7	32.29	5.3	3.92	0.88	0.253
(DT)D (16) × T	87	9	5 (+1)	14.5	21.91	5.2	2.17	0.76	0.131
T × (TD)D (6)	9	0	0	0.0	-	-	-	-	-
T × (DT)D (8)	11	0	0	0.0	-	-	-	-	-
(TD)D (53) × D	215	36	16	13.4	23.01	30.9	19.20	0.50	0.093
(DT)D (7) × D	27	0	0	0.0	-	-	-	-	-
D × (TD)D (7)	11	1	1	14.3	37.80	85	-	0.30	-
D × (DT)D (7)	9	0	0	0.0	-	-	-	-	-

[a] Combinations shown in order of female × male. The number of genotypes involved in the cross combination given in parenthesis. T denotes Saikai 35. D denotes plants derived by selfing *S. demissum* 5H109-5

[b] The number of successful cross combinations. Sib-crosses are indicated by the numbers of successful cross combinations/total combinations. Plus numbers in parentheses indicate the number of plants that set only seedless berries

[c] Mean of the berry-setting rates (percent berries/flowers) over all cross combinations

[d] Mean among successful cross combinations

SD standard deviation

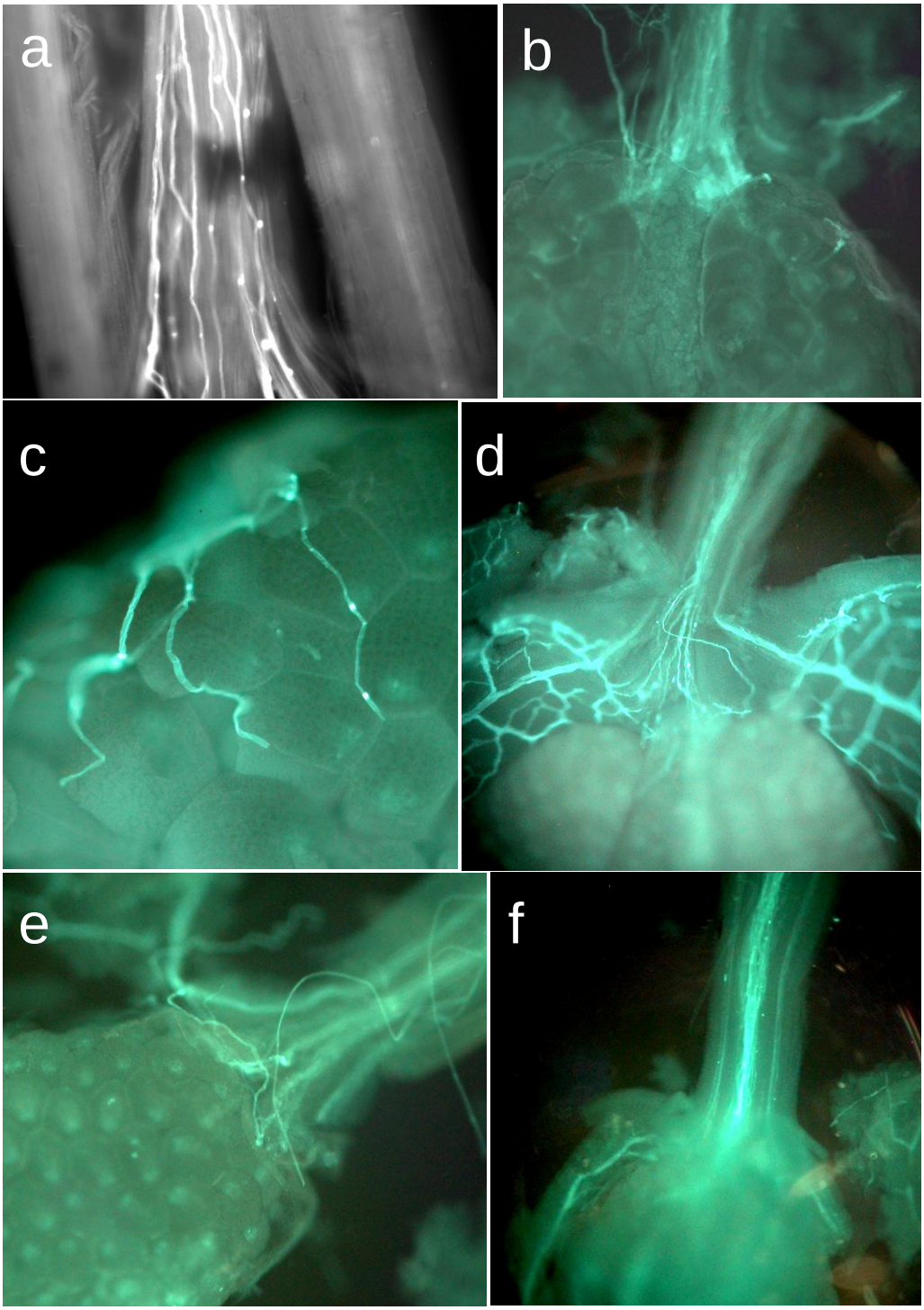

Fig. 3 Florescence microscopic observations of pollen tube growth, penetrating style (**a**) and reaching ovaries (**b-f**). **a** Selfing TD (6H38-10). **b** D (7H16-16) × T. **c** T × D (7H16-8). **d** T × DT (6H37-6). **e** D (7H16-17) × DT (6H37-15). **f** D (7H16-8) × TD (6H38-8)

F_1 and BC_1 progenies with T cytoplasm always showed higher berry-setting rates (the average of 2.04 times) than those with the D cytoplasm. Second, irrespective of cytoplasm or male parent, when F_1 and BC_1 progenies were crossed as females, the berry-setting rates decreased with increasing D-derived germplasm in the female; that is, (TD)D < TD < (TD)T or (DT)D < DT < (DT)T (theoretically consisted of the average of 82%, 60% and 33% D germplasm, respectively). The third finding was a little complicated. When the F_1 and BC_1 progenies were used as males, berry-setting rates were optimized under a certain balance: onto T, only (TD)T or (DT)T hybrids with 33% D germplasm set berries, whereas onto D, TD or DT hybrids with 60% D germplasm showed the highest berry-setting rates. In the latter case, (TD)T or (DT)T plants with less D germplasm (33%) in pollen produced heavier seeds, while (TD)D or (DT)D plants with larger D germplasm (82%) in pollen produced smaller seeds.

Discussion

It is generally known that *S. demissum* easily sets berries with the pollen of *S. tuberosum*, while the reciprocal cross is unsuccessful. The resultant pentaploid F_1 hybrids are non-functional as males, and produce seeds only if backcrossed with the pollen of *S. tuberosum* (Black 1943a; Cooper and Howard 1952; Irikura 1968). The present study reconfirmed this UI in the cross between a large number of *S. demissum* accessions (D) and *S. tuberosum* (T, a breeding line Saikai 35). However, the degree of UI was not complete, but rather quantitative as observed in the berry-setting rates; 81.2% in D × T vs. 18.7% in T × D. Dionne (1961) suggested that the non-functional pollen of DT hybrids was attributed to the interaction of a cytoplasmic factor or factors

Table 4 Summary of parental and cytoplasmic effects on crossability

D cytoplasm	Berries/flower (%)	Seeds/berry	Seed weight (mg)	T cytoplasm	Berries/flower (%)	Seeds/berry	Seed weight (mg)
DT self	0.0	-	-	TD self	4.2	10.1	0.73
DT sib	0.8	7	0.69	TD sib	2.5	3.7	1.03
(DT)T self	8.4	19.3	0.68	(TD)T self	25.1	55.9	0.62
(DT)T sib	7.4	27.1	0.61	(TD)T sib	12.9	44.2	0.69
(DT)D self	2.3	0.5	0.50	(TD)D self	2.5	1.8	0.58
As female							
(DT)T × T	28.6	50.6	0.84	(TD)T × T	49.2	53.3	0.80
DT × T	24.5	32.3	0.93	TD × T	30.8	33.2	0.97
(DT)D × T	14.5	5.2	0.76	(TD)D × T	22.7	5.3	0.88
(DT)T × D	37.8	74.3	0.48	(TD)T × D	41.8	66.3	0.45
DT × D	21.7	62.2	0.53	TD × D	25.3	84.3	0.59
(DT)D × D	0.0	-	-	(TD)D × D	13.4	30.9	0.50
As male							
T × (DT)T	1.5	31.3	0.57	T × (TD)T	14.7	53.8	0.67

T × DT	0.0	-	-	T × TD	0.0	-	-
T × (DT)D	0.0	-	-	T × (TD)D	0.0	-	-
D × (DT)T	8.4	14.4	0.80	D × (TD)T	42.2	31.5	0.85
D × DT	24.2	30.0	0.62	D × TD	64.9	46.2	0.63
D × (DT)D	0.0	-	-	D × (TD)D	14.3	85	0.30

in *S. demissum* with nuclear factors contributed by the male parents. However, the pollen from TD hybrids that had T cytoplasm was also non-functional on T, and in fact, the pollen from both TD and DT hybrids were functional on D (Table 2). These facts make it implausible to attribute the non-functional pollen on *S. tuberosum* to male sterility caused by interaction with the *S. demissum* cytoplasm. Although UI is usually explained by differential pollen tube growth between reciprocal crosses, no apparent inhibition of pollen tube growth was observed in this study in any cross combinations that failed seed formation. Thus, the UI between *S. tuberosum* and *S. demissum* and the subsequent incompatibilities occurred in backcrossing were likely caused by a post-zygotic failure of seed formation.

Both *S. tuberosum* and *S. demissum* readily produced plump seeds when crossed with 4x(4EBN) testers, thus having been assigned 4EBN (Johnston and Hanneman 1980; Hanneman 1994). Although for judging the seed viability, plumpness, germinability and/or size were considered (Johnston and Hanneman 1980), seed size itself was not a criterion in determining EBN values. In this study an apparent size difference between TD and DT seeds was observed, the latter being significantly larger than the former.

In diallele crosses among *S. commersonii* (1EBN), *S. chacoense* (2EBN) and their exceptional hybrids (1.5EBN), average to small sized seeds with 1 viable : 1 aborted seed ratio were observed when the female parent had a higher EBN value than the male, while average to large sized seeds with 1 viable : 7 aborted seed ratio were observed when the female parent had a lower EBN value than the male (Ehlenfeldt and Hanneman 1988). If the present results are explained by the PNA hypothesis (Nishiyama and Yabuno 1978), the AI in the cross D × T could be around or over 80%, thus the AV of *S. tuberosum* being slightly larger than the RV of *S. demissum*, because

DT hybrid seeds were large viable to aborted resulting in lower number of seeds per berry (Table 1) and a lower germination rate of DT hybrid seeds compared with TD hybrid seeds (data not shown). Assuming the AV of *S. tuberosum* is 4 (derived from 4EBN), the AI of >80% could be obtained if the RV of *S. demissum* is <2.5. Consequently, the AI of the reciprocal cross becomes less than 31.3% (= [2.5 / (2 × 4)] × 100), predicting the formation of relatively smaller viable or inviable seeds, which fit the present result regarding the seed size. Both EBN and PNA hypotheses predict that a slight excess of maternal dosage will produce small seeds, while a slight excess of paternal dosage will produce large seeds (Nishiyama and Yabuno 1978; Ehlenfeldt and Hanneman 1988; Ehlenfeldt and Ortiz 1995). Early seed abortion would likely cause berry-dropping, lowering the berry-setting rate. Therefore, it could be concluded that that the UI occurred between *S. demissum* and *S. tuberosum* was caused by the imbalance of EBN or AV, *S. demissum* having a slightly lower EBN than *S. tuberosum*.

It was disclosed that three genetic factors involved in normal seed development: 1) a cytoplasmic factor, and nuclear genome-encoded factors functioned 2) in female gametophyte and 3) in pollen. Among the three factors, a cytoplasmic, or maternally inherited factor showed the most prominent effect on crossability. Irrespective of being crossed as male or female, F_1 and BC_1 progenies with D cytoplasm always showed lower berry-setting rates (the average of 2.04 times) than those with the T cytoplasm. Thus, the D cytoplasm has likely a suppressor effect on crossability.

Reducing D germplasm in female gametophyte increased or recovered berry-setting rates (Table 4), indicating nuclear genome-encoded factor(s) of D functioned negatively as a suppressor for endosperm development. In pollen, the proportion of D germplasm for optimized berry-setting rates depended upon whether the female was D or T (Table 4), implying that the EBN-like balance system functioned.

Three additive loci in a threshold-like system (Ehlendfeldt and Hanneman 1988) or two independent loci with two alleles in homozygosity (Camadro and Masuelli 1995) have been proposed for EBN-controlling genes, and the EBN-controlling genes would segregate and are randomly distributed into gametes. Apparently, these nuclear genome-encoded factor(s) of D were segregating in the BC_1 populations due to aneuploidy and recombination. BC_1 plants as aneuploids showed wide variation in berry-setting rates due to segregation in berry-setting/non-berry-setting plants and the degree of berry-setting rate in each of berry-setting plants (Table 3). In addition, the number of seeds/berry and seed weight also varied between plants (Table 3, Fig. 2). Since the postulated EBN-controlling genes have additive effect (Ehlenfeldt and Hanneman 1988), both TD and DT hybrids would have the same EBN value consisted of a half of T and a half of D. Nevertheless, TD and DT hybrids were apparently different in the crossing behaviors, particularly in crosses onto D (Table 2). Thus, other factors than EBN was likely involved.

Selfing of TD or DT hybrids was extremely difficult (Table 2). As summarized in General introduction, endosperm growth in *Arabidopsis* is controlled by a balance between maternally contributed PRC2 proteins and paternally contributed AGLs (Erilova et al. 2009; Köhler et al. 2010). If the same system is functioning in potato, it could be expected that the amount and kind of active proteins in the gametes produced from TD and DT hybrids are highly variable among gametes due to random association of subunit gene products genetically segregated from a half of T and a half of D genomes. Consequently, effective PRC2 proteins are rarely balanced in selfing with targeted AGL transcription factors, resulting in seed formation failure. Therefore, it is suggested that the molecular mechanism of EBN must be understood quantitatively, or dosage-dependently, and also qualitatively, because appropriate recognition of PRC2

proteins with target AGL genes could be important for AGL proteins to promote normal endosperm proliferation.

Furthermore, since genes functioning as maternal factors and those as paternal factors are essentially different as shown in *Arabidopsis*, it might be too audacious to assign an EBN value peculiar to the species. EBN value for a species was determined by pollinating with the pollen of EBN standard tester clones, which, strictly to say, determined an EBN value of polar nuclei of the species, not necessarily identical with the EBN value of sperm nucleus. In fact, *S. demissum* set berries with pollen of 4x(4EBN) tester clones, whereas it as pollen parent can set berries on 3x(1.5EBN) species 3x *S. commersonii*, 2x(2EBN) species *S. phureja*, *S. stenotomum* Juz. & Buk., *S. verrucosum* Schltdl. and F_1 hybrid of *S. chacoense* × *S. phureja*, and 4x(2EBN) species *S. acaule*, *S. oxycarpum* Schiede, *S. polytrichon* Moric. and *S. stoloniferum* Schlechtd. et Bché. (Black 1943b; Hawkes 1958; Dionne 1961; Marks 1965). These indicate that the D pollen may have a slightly lower EBN than its polar nuclei and this level of imbalance is still tolerable in nature, producing many small viable seeds on its selfing (Table 1).

Even such recent knowledge is considered to understand the present data, it remains unknown why T germplasm always improve berry-setting rates, or inversely, D germplasm reduced berry-setting rates (Table 4). Evolutionarily, *S. demissum* is undoubtedly a successful polyploid in nature, featured by high chromosome number, self-fertility and homozygosity, while *S. tuberosum* is of recent origin by autotetraploidy (Hawkes 1979). Considering highly homozygous allohexaploid nature of *S. demissum*, duplicated loci may suffer suppression and silencing by chromatin modification or histone activation changes (Jones and Hegarty 2009) in the long history of polyploidy stabilization process, resulting in reorganization of EBN-controlling genes. Namely,

among three pairs of genomes in *S. demissum*, one or two pairs of genomes may have zero EBN value.

In conclusion, the UI between *S. demissum* and *S. tuberosum* can be explained by slightly lower EBN of *S. demissum* than that of *S. tuberosum*. However, EBN is not as simple as being denoted by single digits. Imbalance of EBN in the interspecific hybrids must be considered as quantitative and qualitative mismatches between maternal and paternal factors, because it is likely a balance between maternal protein complex composed with subunit proteins encoded by different genes and paternal gene products.

Chapter II

Reciprocal differences in DNA sequence and methylation status of the pollen DNA between F_1 hybrids of *Solanum tuberosum* × *S. demissum*

Introduction

Reciprocal difference refers to differential phenotypic expression observed between reciprocal F_1 hybrids, and the phenomenon is widely observed for various traits in various plant species (Burke et al. 1998; Campbell and Waser 2001; Tiffin et al. 2001; Rhode and Cruzan 2005; Gonzalo et al. 2007). In a perennial wild flower *Penstemon davidsonii* Greene, reciprocal F_1 hybrids differed significantly in fruit set, seed number, seed weight, number of days to fruit maturity, vegetative growth and performance in field gardens (Kimball et al. 2008). In maize (*Zea mays* L.), inter-varietal F_1 hybrids showed reciprocal differences in germination and traits such as whole-kernel growth rates (Groszmann and Sprague 1948) and embryo and endosperm dry weights (Bagnara and Daynard 1983). These differences occur mainly by the maternal effect as cytoplasmic factors of mitochondrial and chloroplast genomes, and xenia (refers to the effect of pollen on the endosperm phenotype in the same generation) (Roach and Wulff 1987).

In the previous chapter (Chapter I), the unilateral incompatibility between *Solanum tuberosum* (T) and *S. demissum* (D) was reconfirmed. Furthermore, I found that the obtained hybrid seeds were reciprocally very different in size: the average seed weights were 0.39 mg from T × D and 0.94 mg from D × T. The further crossing experiments in various combinations revealed reciprocal differences in crossability

between T (female) × D (male) hybrids (TD hybrids) and the reciprocal ones (DT hybrids). Based on these crossing results, I suggested at least three factors likely involved in hybrid seed development in these specific crosses: 1) a cytoplasmic, or maternally inherited factor, 2) a nuclear-encoded factor functioning in female gametophyte and 3) a nuclear-encoded factor functioning in pollen and affected by a balance between male and female gametes. Increasing the *S. tuberosum* nuclear germplasm and possessing *S. tuberosum* cytoplasm by backcrossing in both male and female gametophytes always resulted in superior berry-setting rates (Chapter I). Especially when TD and DT hybrids were crossed as pollen parents onto *S. demissum,* a significantly higher berry-setting rate was obtained in TD (64.9%) compared with DT (24.2%). Due to the maternal inheritance of chloroplast and mitochondrial DNA, the genetic information was slightly different between TD and DT. However, most of genetic information was encoded in the nuclear DNA, which was composed of a half of *S. demissum* and a half of *S. tuberosum* genomes, and should be identical between TD and DT hybrids. Nevertheless, the pollen from TD and DT hybrids functioned differently.

It is widely recognized that DNA methylation affects gene activity mainly by repressing gene expression or transcription (reviewed in Bird and Wolffe 1999), whereas its demethylation promotes gene expression and activates silent genes (Cervera et al. 2002; Rutherford and Henikoff 2003). In plants, this mechanism likely occurs because methylation affects the accessibility of several plant proteins to their target DNA sequences (Gierl et al. 1988). Proteins with affinity for methylated sequences also have been isolated (Ehrlich 1993). Therefore, DNA methylation is one of most important epigenetic mechanisms for plant development and the regulation of fertilization in fungi and plants (Martienssen and Colot 2001). The DNA cytosine

methylation changes were observed among several plant interspecific hybrids, allopolyploids and introgression lines (Comai et al. 2000; Madlung et al. 2002; Levy and Feldman 2004). The role of DNA methylation is well documented on seed development in *Arabidopsis* (reviewed in Kinoshita 2007). The endosperm was specifically hypomethylated (demethylated), lower than other tissues (Adams et al. 2000; Vinkenoog et al. 2000). And imprinted genes of female origin are activated and interacted with those of male origin (reviewed in Baroux et al. 2002). Such tissue-specific methylation differences between endosperm and leaf or other organ tissues has also been reported in maize (Lauria et al. 2004; Lu et al. 2008), rice (Xiong et al. 1999) and sorghum (Zhang et al. 2007). However, the methylation rate of male gametophyte, or pollen has been studied poorly.

In this chapter, I investigated a global view of the DNA differences and cytosine methylated DNA differences between pollen DNA from reciprocal F_1 hybrids TD and DT, and between pollen and leaf DNA using methylation-sensitive amplified polymorphism (MSAP) analysis (Reyna-López et al. 1997). As expected, I found DNA level differences originated from chloroplast and mitochondrial DNA. In addition, I found other DNA sequence and DNA methylation level differences, which is to my knowledge the first report detecting difference at such levels between reciprocal hybrids.

Materials and methods

Plant materials

In this study, a *S. tuberosum* breeding line Saikai 35 (referred to as T) and

seedlings of 6H36 and 7H16 families (collectively referred to as D) derived by selfing from one of *S. demissum* PI 186551 plants were used as parents. Saikai 35 has S-type chloroplast DNA and ε-type mitochondrial DNA descended from *S. phureja*, while the parental *S. demissum* clone has W-type chloroplast DNA and α-type mitochondrial DNA. The interspecific hybrid family 6H38 was obtained by the cross between *S. tuberosum* as female and *S. demissum* as male (TD), while 6H37 family was obtained by the reciprocal cross (DT).

DNA extraction

Mature pollen grains were collected from many T individuals and seedlings of D. For interspecific hybrids, 16 TD and 9 DT genotypes were clonally propagated and grown in the field to obtain sufficient amount of pollen grains to extract DNA. Collected pollen grains were stored at -30 °C until DNA extraction. Approximately 50-120 mg of pollen grains per genotype were used to extract DNA by the method of Hosaka and Hanneman (1998). Simultaneously, DNA from fresh leaves was extracted from T, D, 7 TD and 6 DT genotypes.

Methylation-sensitive amplified polymorphism (MSAP) analysis

The MSAP analysis was performed to detect differences in DNA sequences and DNA methylation status as well. The MSAP analysis is an adaptation of the amplified fragment length polymorphism (AFLP) technique (Vos et al. 1995), in which the isoschizomers *Hpa*II and *Msp*I were used instead of a usually used frequent cutter enzyme *Mse*I (Reyna-López et al. 1997). *Hpa*II and *Msp*I recognize the same four-base sequence (CCGG) and cut with differential sensitivity to DNA methylation of internal or external cytosine. *Hpa*II does not cut the recognition site if the internal

cytosine is full-methylated, whereas *Msp*I is insensitive and cuts it. If the external cytosine is hemi-methylated, *Msp*I does not cut, whereas *Hpa*II cuts the recognition site. Thus, if the presence/absence of an AFLP band was different between *Eco*RI+*Hpa*II double-digest (E/H digest) and *Eco*RI+*Msp*I double-digest (E/M digest) of the same DNA sample, it was regarded as a methylation-sensitive band caused by a difference of DNA methylation status.

Genomic DNA (500 ng) was digested with 12.5 units of *Msp*I (Takara Bio, Japan) or *Hpa*II (TOYOBO, Japan) separately by overnight incubation at 37 ºC. After the digestion reaction was stopped by incubating at 70°C for 10 min, digested DNA was precipitated by ethanol, dried, and re-suspended in 10 μl of distilled water. The second digestion was performed with 12.5 units of *Eco*RI (Takara Bio, Japan) in volumes of 25 μl by overnight incubation at 37°C. The double-digested DNA fragments were ligated to the adapters by adding 24 μl of ligation mixture [2× ligation buffer (Invitrogen), 0.1 pmol *Eco*RI adapter and 1 pmol *Hpa*II/*Msp*I adapter] and 1 μl of T4 DNA ligase (1 unit/μl, Invitrogen), and incubated overnight at 20°C. The adapter and pre-amplification primer sequences were the same as those described by Xiong et al. (1999). Pre-amplification was performed with 2.5 μl of the above DNA in volumes of 25 μl consisting of 0.3 μM pre-amplification primers, 12.5 μl of Ampdirect® Plus (Shimadzu, Japan) and 0.25 units of *Taq* DNA polymerase (Nova *Taq*™ Hot Start DNA Polymerase, Novagen®, USA). Pre-amplified products were adjusted to the concentration of 5 ng/μl. Selective amplification was performed with the same components in volumes of 10 μl with 2 μl of 5 ng/μl pre-amplified DNA. For selective amplification seven *Eco*RI primers and two sets of nine *Msp*I/*Hpa*II primers were used. *Eco*RI primers consisted of the core sequences of 5'-GACTGCGTACCAATTC and three selective nucleotides ANN (NN denotes CC,

AG, CA, CG, CT, AC or GC). Two *Msp*I/*Hpa*II primer sets were used, both consisted of the same core sequences of 5'-ATCATGAGTCCTGCTCGG. The first primer set had three selective nucleotides ANN and the second one TNN (NN denotes TA, CG, GC, TG, CT, CC, TC, AC or GT). Thermal profiles for pre- and selective amplifications were those described in the original AFLP protocol (Vos et al. 1995). The amplification products were electrophoresed on 4% denaturing polyacrylamide gels and visualized by silver-staining (Bassam et al. 1991).

Bulked segregant analysis

Because potato is a highly heterozygous tetraploid crop, interspecific hybrids would become heterogeneous. Thus, bulked segregant analysis (Michelmore et al. 1991) was employed to compare TD and DT. Pollen DNA samples of 16 TD and 9 DT genotypes were pre-amplified separately, bulked with equal amounts as TD and DT bulked samples, and then, subjected to selective amplification. If different bands were found between TD and DT bulked pollen DNA samples, these bands were examined from individually pre-amplified samples. For bulked leaf DNA samples, 7 TD and 6 DT leaf DNA samples were respectively mixed prior to double-digestion.

Isolation and characterization of amplified bands

The target bands were cut out from polyacrylamide gels dried on the glass plate using a razor blade. The peeled gel fragment containing a target band was rehydrated in 10 µl of sterile water overnight, and centrifuged at 12,000 g for 10 min. The target DNA was amplified by PCR from 2 µl of the supernatant using the same primer pairs as those for the selective amplification. The thermal condition was 10 min at 95°C, then, 25 cycles of 1 min at 95°C, 1 min at 65°C and 1 min at 72°C, followed by final

extension of 5 min at 72°C. After PCR amplification, unincorporated primers and deoxynucleotide triphosphates were removed by ethanol precipitation prior to sequencing. The PCR products were sequenced from both directions with forward and reverse primers separately using BigDye terminator version 3.1 on ABI 3100 (Applied Biosystems). The homology search of the obtained sequences was performed using the BLASTN program.

Results

Detection of methylation-sensitive bands

For MSAP analysis two sets of *Hpa*II/*Msp*I selective amplification primers were used. Primer sequences of the first and second sets consisted of the same core sequences plus three selective nucleotides ANN and TNN, respectively. Nine primers of each set had the same set of additional two selective nucleotides, and all combinations with the same seven *Eco*RI primers were used. Using a total of 126 pairs of selective amplification primers, bulked pollen and leaf DNA of interspecific reciprocal F_1 hybrids TD and DT were examined (Table 5, Fig. 4). The total numbers of AFLP bands from bulked pollen DNA samples of TD and DT were 23,527 and 23,525, while those from bulked leaf DNA samples were 23,530 and 23,533, respectively. The total numbers of methylation-sensitive bands from bulked pollen DNA of TD and DT were 2,090 and 2,082, while those from bulked leaf DNA were 2,078 and 2,087, respectively. Thus, there was no significant difference in the total band numbers among four DNA samples, and in the methylation-sensitive band numbers as well. However, the numbers of methylation-sensitive bands detected from

Table 5 The number of AFLP bands detected in *Eco*RI/*Hpa*II (E/H)- and *Eco*RI/*Msp*I (E/M)-digested bulked pollen and leaf DNA samples of TD and DT, amplified using the first 63 primer pairs and the second 63 primer pairs, and the methylation rates

Primer pairs	Sample	No. of total bands						No. of methylation-sensitive bands						Methylation rate (%)	
		E/H-digested		E/M-digested		Combined		E/H-digested		E/M-digested		Combined			
		TD	DT	TD	DT	TD	DT	TD	DT	TD	DT	TD	DT	TD	DT
First 63 primer pairs	Pollen	5,334	5,330	5,956	5,958	11,290	11,288	121	114	743	742	864	856		
	Leaf	5,334	5,339	5,962	5,962	11,296	11,301	114	119	742	742	856	861		
Second 63 primer pairs	Pollen	5,677	5,677	6,560	6,560	12,237	12,237	189	189	1,037	1,037	1,226	1,226		
	Leaf	5,677	5,674	6,557	6,558	12,234	12,232	188	189	1,034	1,037	1,222	1,226		
Total															
126 primer pairs	Pollen	11,011	11,007	12,516	12,518	23,527	23,525	310	303	1,780	1,779	2,090	2,082	8.88	8.85
	Leaf	11,011	11,013	12,519	12,520	23,530	23,533	302	308	1,776	1,779	2,078	2,087	8.83	8.87

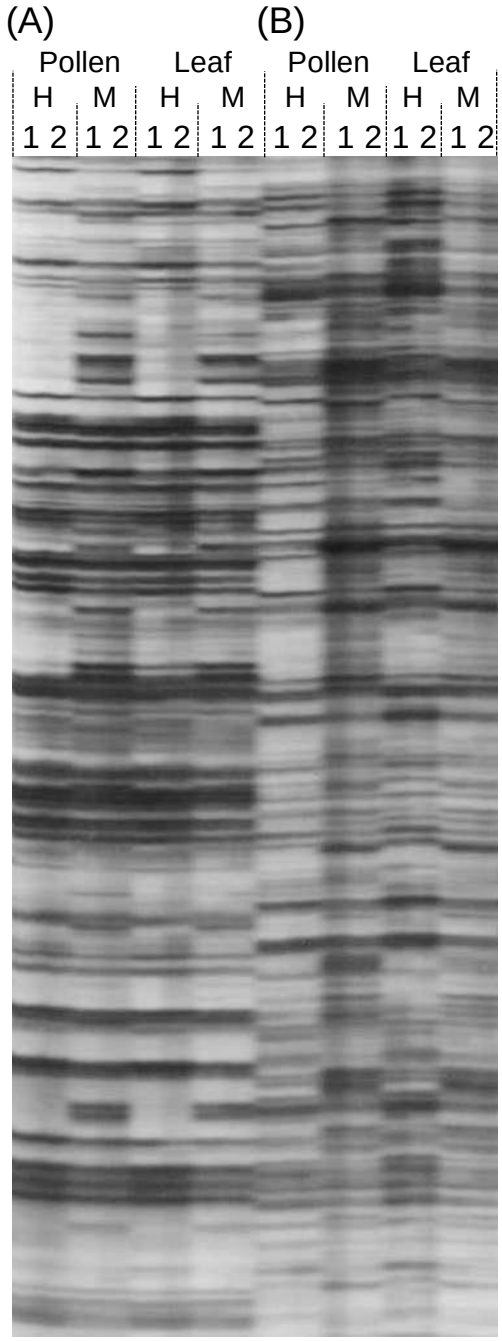

Fig. 4 AFLP banding patterns of *Eco*RI/*Hpa*II-digested (H) and *Eco*RI/*Msp*I-digested (M) bulked pollen or leaf DNA of interspecific reciprocal F_1 hybrids TD and DT (labeled 1 and 2, respectively), amplified using primer pairs E-AGC and M/H-AGT (A), and E-ACA and H/M-AGT (B)

E/M-digested samples (1,776-1,780 bands) were much higher than those from E/H-digested samples (302-310 bands), the mean proportion being 85.3% and 14.7%, respectively, which also reflected on the total band numbers of E/M digests higher than those of E/H digests. When two primer sets were compared, the first set of primers generated a total of 45,175 bands from four samples (11,288-11,301 bands) and the second set of primers generated 48,940 bands (12,232-12,237 bands per sample), respectively, in which 3,437 and 4,900 bands were methylation-sensitive, respectively. Thus, compared with the first set primers, the second set primers increased total band numbers to 1.1 times and methylation-sensitive bands to 1.4 times, which increased the detection frequency of methylation-sensitive bands from 7.6% to 10.0%.

Rates of methylation-sensitive bands

When the DNA methylation rate was calculated as the percentage of the total number of methylation-sensitive bands over total band numbers detected in both E/H and E/M digests (Table 5), those for pollen DNA samples of TD and DT were 8.88% and 8.85%, and those for leaf DNA samples of TD and DT were 8.83% and 8.87%, respectively. Thus, average DNA methylation rates of pollen and leaf DNA samples were 8.87% and 8.85%, respectively.

Different banding types in TD and DT

Only for the first set primers, pollen and leaf DNA samples of TD and DT were electrophoresed together on the same gels. Thus, the presence/absence of each band among four samples for each of TD and DT could be compared (Table 6). A total of 6,085 bands were amplified from TD and the same number of bands from DT, which were categorized into 13 types. 5,197 bands (85.4%) of type 1 were present in all

Table 6 Banding types different among *Eco*RI/*Hpa*II (E/H) and *Eco*RI/*Msp*I (E/M) digests of bulked pollen and leaf DNA samples using the first primer set, and the number of bands in each type in TD and DT, respectively

Banding pattern					No. of bands	
Type	Pollen		Leaf			
	E/H	E/M	E/H	E/M	TD	DT
1	+	+	+	+	5,197	5,197
2	-	+	-	+	727	727
3	+	-	+	-	111	112
Tissue-specific						
4	-	-	+	+	6	9
5	+	+	-	-	0	1
6	-	+	+	+	12	12
7	+	-	+	+	5	2
8	+	+	-	+	14	15
9	+	+	+	-	2	3
10	+	-	-	-	5	0
11	-	+	-	-	4	3
12	-	-	+	-	1	4
13	-	-	-	+	1	0
Total					6,085	6,085

samples of TD, and similarly in DT. The bands of types 2 and 3 were methylation-sensitive bands, observed in E/M digests of pollen and leaf DNA, but not in the E/H digests (type 2), or vice versa (type 3). Types 4-13, counting 50 bands (0.82%) in TD and 49 bands (0.81%) in DT, were differentially amplified between leaf and pollen DNA. Among these tissue-specific bands, those of types 4 and 5 (12.0% in TD and 20.4% in DT) were detected as DNA sequence differences, while those of types 6-13 were regarded as DNA methylation differences (88.0% in TD and 79.6% in DT).

Differences in pollen DNA between TD and DT

TD and DT bulked DNA samples showed mostly the same AFLP banding patterns (Fig. 4). Yet, 57 bands from 43 primer pairs were different between TD and DT, of which 35 bands were specifically found in pollen DNA (Table 7).

For these 57 bands, the pollen DNA samples of 16 TD and 9 DT plants were individually analyzed. Forty-nine bands segregated for presence/absence within each population. The presence or absence of eight bands (Band 1 to 8) was uniform within each of TD and DT populations and consistently different between TD and DT plants (Fig. 5, Table 8). Irrespective of methylation-sensitive restriction enzymes, Band 1 to 3 were detected in all DT but not in any TD plants, while Band 4 and 5 were detected in all TD but not in any DT plants. Thus, these bands were regarded as the differences in DNA sequences. Band 6 to 8 were detected in all E/M digests of TD and DT plants, whereas in E/H digests, Band 6 was present in all TD but absent in DT plants, and Band 7 and 8 present in all DT but absent in TD plants. Presence or absence of these bands in the parental T and D was also analyzed (Table 8). Band 1 to 5 of F_1 hybrids were only present in the female parent, that is, Band 1 to 3 of DT were shared with D, while Band 4 and 5 of TD shared with T. Band 6 was present in D and absent in T in both

Table 7 Differences between reciprocal F_1 hybrids TD and DT detected in *Eco*RI/*Hpa*II (E/H)- and *Eco*RI/*Msp*I (E/M)-digested bulked DNA samples

E/H-digested		E/M-digested		No. of bands
TD	DT	TD	DT	(pollen-specific)
+	-	+	-	5 (0)
-	+	-	+	6 (0)
+	-	+	+	19 (14)
+	-	-	-	6 (5)
-	+	+	+	16 (14)
-	+	-	-	4 (1)
+	+	-	+	1 (1)

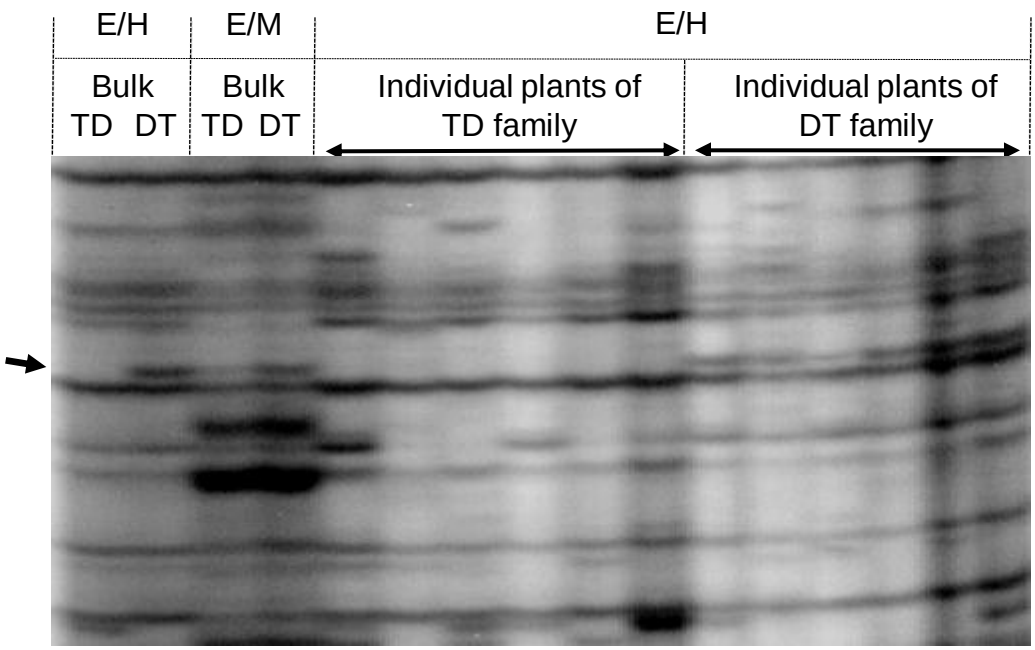

Fig. 5 Comparison between *Eco*RI/*Hpa*II-digested (E/H) and *Eco*RI/*Msp*I-digested (E/M) bulked pollen DNA samples revealed a methylation sensitive AFLP band different between interspecific reciprocal F_1 hybrids TD and DT (arrowed, Band 7), whose presence or absence in individual DNA samples was consistent within and between populations

Table 8 Presence (+) or absence (-) of the different AFLP bands detected in the *Eco*RI/*Hpa*II (E/H)- and *Eco*RI/*Msp*I (E/M)-digested pollen DNA of individual genotypes of TD (16 genotypes of 6H38 family) and DT (9 genotypes of 6H37 family) and their parents T and D

Band	Selective primer[a]	E/H-digested				E/M-digested			
		T	TD	DT	D	T	TD	DT	D
1	AGC/ACC	-	All -	All +	+	-	All -	All +	+
2	AGC/ATC	-	All -	All +	+	-	All -	All +	+
3	AGC/TTC	-	All -	All +	+	-	All -	All +	+
4	AAC/ACG	+	All +	All -	-	+	All +	All -	-
5	AAC/ATG	+	All +	All -	-	+	All +	All -	-
6	AGC/TGC	-	All +	All -	+	-	All +	All +	+
7	AGC/TCT	-	All -	All +	+	+	All +	All +	+
8	AAG/TTA	-	All -	All +	-	+	All +	All +	+

[a] *Eco*RI primer and *Msp*I/*Hpa*II primer

E/H and E/M digests. Band 7 and 8 were present in E/M digests of both parents, whereas in E/H digests, Band 7 was present only in D and Band 8 absent in both parents. These eight bands detected in pollen DNA were also examined in leaf DNA. Only the difference of Band 8 was pollen-specific, while the others were shown similarly in leaf DNA.

Sequencing analysis

Bands 1-8 were eluted from the polyacrylamide gels, re-amplified and sequenced (Table 9). The determined sequences are available from the National Center for Biotechnology Information (accession numbers given in Table 9). Band 1 and 2 showed the same sequences with the size of 170 bp. Since each band was sequenced from both directions using the *Eco*RI and *Hpa*II/*Msp*I primers used for the selective amplification, it was found that Band 2 was amplified by a mismatch of one base in the region corresponding to the selective nucleotides of the *Hpa*II/*Msp*I primer. Similarly, Band 4 and 5 had the same sequences with the size of 334 bp, the latter band having been amplified by a mismatch. BLAST-search indicated that Band 1, 6 and 7 showed no or less than 40% homology with any known sequences (Table 9). Band 3 (205 bp) shared 97% homology with a part of *S. tuberosum* cv. Desiree chloroplast DNA. Band 4 shared 97% homology with a part of *Nicotiana tabacum* L. mitochondrial DNA. Band 8 shared 87% homology with a part of non-coding region of *Vitis vinifera* L. mitochondrial DNA, but not with any Solanaceous mitochondrial DNA. Instead, it shared 42% homology with a part of *S. lycoperisicum* chromosome 2 or 38% homology with a part of *S. demissum* chromosome 5.

Table 9 Sequence homology of the six bands by BLAST-searching

Band	Source (E/H-digested DNA)	Size (bp)	Accession No.	Sequence with the highest homology	(%)
1 and 2	6H37-16 and 6H37-19	170	HR505437	Unknown	
3	6H37-16	205	HR505438	*pet D* (cytochrome b6/f complex subunit IV) coding region in chloroplast DNA of *Solanum tuberosum* cv. Desiree (DQ386163)	97
4 and 5	6H38-8 and 6H38-45	334	HR505439	orf 175 (hypothetical protein) in mitochondrial DNA of *Nicotiana tabacum* (BA000042)	97
6	6H38-3	185	HR505440	Unknown	-
7	6H37-19	331	HR505441	Unknown	-
8	6H37-16	287	HR505442	Non-coding region in mitochondrial DNA of *Vitis vinifera* (GQ220323)	87

Discussion

Using 126 primer pairs, pollen DNA generated a total of 23,527 AFLP bands from the *S. tuberosum* × *S. demissum* (TD) hybrid and 23,525 bands from the reciprocal one (DT). With unknown reason the second set primers amplified 1.1 times more number of bands than the first set primers, although both primer sets had the same GC content with only difference of one nucleotide substitution from A to T in the first position of three selective nucleotides. Even comparing DNA samples digested with methylation-sensitive isoscizomers *Hpa*II and *Msp*I, TD and DT hybrids showed almost identical AFLP banding patterns (Fig. 4) and the same methylation rates (8.88% in TD and 8.85% in DT, respectively). Yet, I found at least six differences between TD and DT. As expected, one difference possibly occurred in chloroplast DNA (Band 3) and another one in mitochondrial DNA (Band 4). In addition, I found Band 1, which occurred by a DNA sequence level difference or a difference of DNA methylation that did not cause differential sensitivity between the two enzymes, and Bands 6-8, which occurred as DNA methylation level differences. To my knowledge, this is the first report detecting reciprocal differences in DNA sequence or DNA methylation other than those in cytoplasmic DNA.

Band 1 was detected in all DT hybrids and the *S. demissum* parent. This indicated that the Band 1 was transmitted maternally and of chloroplast or mitochondrial DNA origin. Potato chloroplast DNA was completely sequenced (Chung et al. 2006). Mitochondrial DNA in the Solanaceous model plant tobacco (*Nicotiana tabacum*) has also been completely sequenced (Sugiyama et al. 2005). Nevertheless, Band 1 shared no homology with any known sequences. Band 1 will be addressed further in Chapter IV.

Band 3 and 4 were apparently originated in chloroplast and mitochondrial DNA, respectively, because of the high sequence homology and specificity to maternal parents. It has been known that the parental *S. demisssum* and *S. tuberosum* genotypes have different chloroplast DNA (W and S types, respectively) and mitochondrial DNA (α and ε types, respectively), which might be associated with Band 3 and 4. Previously, in Chapter I, I found that the cytoplasmic difference was a major contributory factor to the reciprocal difference in crossability between TD and DT. However, it remains unknown whether Band 3 or 4 is related to contributory factors to crossability difference.

In plant, apparent non-Mendelian inheritance or re-modeling of parental methylation patterns has been observed to occur in certain situations, like in several plant interspecific hybrids, allopolyploids and introgression lines (Madlung et al. 2002; Liu and Wendel 2003; Levy and Feldman 2004; Liu et al. 2004; Salmon et al. 2005; Lukens et al. 2006; Marfil et al. 2006). Unlike such epigenetic methylation, the presence or absence of Band 6 to 8 were consistent within all of 16 plants of TD or all 9 plants of DT. Thus, these methylated DNA regions should have some functional meanings. Imprinted genes or parent-of-origin dependent genes are regulated by differential DNA methylation between parental alleles (Kiyosue et al. 1999; Ingouff et al. 2005; Kinoshita 2007; Köhler et al. 2010). Imprinting was extensively studied for endosperm genes in maize (Gutiérrez-Marcos et al. 2004) and *Arabidopsis* (Gehring et al. 2009; Hsieh et al. 2009). By comparison of mRNA and protein profiling screened for cold germination and desiccation tolerance, Kollipara et al. (2002) identified 336 of 32,496 and 656 of 32,940 cDNA fragments, or 117 of 2,641 and 205 of 1,876 detected proteins to be differentially expressed between reciprocal maize hybrids. They hypothesized that these differentially expressed genes were either directly or indirectly

associated with the imprinting phenomenon. Although the functional roles of Bands 6-8 remain unknown, these might be related to one of regulating factors, especially for the differential crossability or endosperm development. Particularly, Band 8 would be interesting because its difference between TD and DT was specifically found in pollen.

The methylation rates of pollen and leaf DNA were almost similar to each other (8.85-8.88% and 8.83-8.87%, respectively). Since *Hpa*II and *Msp*I do not show differential sensitivity to DNA methylation of full-methylated external cytosine and hemi-methylated internal cytosine, the present DNA methylation rates were apparently underestimated. Yet, I can suggest that the sporophyte and the male gametophyte DNA are methylated to the same degree at least in a genome-wide view. On the other hand, hypomethylation in endosperm is well documented in *Arabidopsis* (Adams et al. 2000; Vinkenoog et al. 2000; Gehring et al. 2009; Hsieh et al. 2009). In maize, the endosperm exhibited 13% reduction in total cytosine methylation level compared with leaf and embryo (Lauria et al. 2004), and in sorghum, endosperm exhibited 11% reduced methylation rate than leaf in inbred and hybrid lines (Zhang et al. 2007). In addition, Zhang et al. (2007) revealed higher expression of the endosperm-specific hypomethylated six genes by RT-PCR analysis. Thus, hypomethylation of the endosperm is generally recognized to induce expression of many genes, which plays an important role for seed development or seed formation (Lund et al. 1995a, b; Gutiérrez-Marcos et al. 2004). By genome-wide high-throughput bisulfate sequencing, Hsieh et al. (2009) demonstrated that virtually the entire endosperm genome was hypomethylated in *Arabidopsis*. Further, they found endosperm hypomethylation was accompanied by hypermethylation of embryo transposable elements (TEs). Likewise in the *Arabidopsis* pollen, Slotkin et al. (2009) found that sperm-derived DNA was hypermethylated and vegetative nucleus-derived DNA was hypomethylated. TEs,

triggered by hypomethylation and reactivated in endosperm or vegetative cell in pollen, might generate short interfering (si)RNAs that move to embryo or sperm cell and enhance silencing of TEs in the embryo or sperm cell to secure the progeny (Hsieh et al. 2009; Slotkin et al. 2009; Mosher and Melnyk 2010). Therefore, what I observed in this study for pollen DNA might be the total or average methylation rate of vegetative and sperm cell DNA, resulting in the almost similar methylation rate to that of leaf DNA.

Although the comparison was made using bulked DNA samples, 0.81-0.82% of bands were differently methylated between leaf and pollen (Table 6). By the whole genome transcriptome analysis of the sperm cell in *Arabidopsis*, Borges et al. (2008) suggested that distinct mechanisms might be involved in regulating the epigenetic state of the paternal genome, and identified numerous candidate genes involved in sperm cell development and fertilization pathways. Differentially methylated DNA fragments observed in this study might be associated with such tissue-specific expression as known in various tissues in various plant species (Adams et al 2003; Ingouff et al. 2005; Wang et al. 2006; Chaudhary et al. 2009).

Chapter III

Transcript profiling of pollen from interspecific reciprocal F_1 hybrids of *Solanum tuberosum* × *S. demissum*

Introduction

Previously in Chapter I, a significantly higher crossing rate was obtained in interspecific F_1 hybrids of *Solanum tuberosum* as female and *S. demissum* as male (TD hybrids) than in reciprocal DT hybrids when they were used as pollen parents on to D. In Chapter II, to reveal this reciprocal differences, DNA sequences and methylation status of pollen DNA were compared between TD and DT using methylation-sensitive amplified polymorphism (MSAP) analysis. Six distinct DNA bands were found as reciprocal differences between TD and DT, in which three bands were detected as DNA methylation level differences. DNA methylation is generally recognized to suppress gene expression as regulatory factors (Jacobsen and Meyerowitz 1997; Jones and Takai 2001). Thus, reciprocal differences are also speculated to occur at a transcription level.

Recent advances in high-throughput microarray and sequencing technologies have facilitated to conduct a genome-wide transcription analysis. Wang et al. (2008) showed for the time the changes in the transcriptome from desiccated mature pollen grains to hydrated pollen grains and then to pollen tubes of *Arabidopsis*. They found that the overall transcription of genes, both in the number of expressed genes and in the levels of transcription, was increased. Borges et al. (2008) used fluorescence-activated cell sorting to isolate sperm cells from *Arabidopsis* and GeneChip analysis of their

transcriptome at a genome-wide level was conducted. Comparative analysis of the sperm cell transcriptome with those of representative sporophytic tissues and of pollen showed that sperm has a distinct and diverse transcriptional profile (Borges et al. 2008). Transcription levels of reciprocal hybrids were investigated by a genome-wide survey in *Arabidopsis thaliana* (Andorf et al. 2010), rice (He et al. 2010; Luo et al. 2011) and maize (Guo et al. 2004).

In this study, a whole-genome transcript analysis was performed to the pollen mRNA of T, D, TD and DT using a high-throughput sequencer in order to disclose interspecific reciprocal differences at a transcription level.

Materials and Methods

Plant materials

In this study, we used as parents a *S. tuberosum* breeding line Saikai 35 (referred to as T), and seedlings of 10H3 families derived by selfing from one of *S. demissum* PI 186551 plants (referred to as D). The interspecific hybrid family 6H38 was obtained by the cross between *S. tuberosum* as female and *S. demissum* as male (TD), while 6H37 family was obtained by the reciprocal cross (DT). T, 14 TD and 11 DT plants were clonally propagated and grown in the same field. D was grown in greenhouse at the same time.

RNA extraction

Mature pollen grains were collected, quick-frozen in liquid nitrogen, and ground to powder with a mortar and a pestle. Total RNA was extracted using the RNeasy®

Plant Mini Kit (QIAGEN, ME, USA). To eliminate possible contaminated genomic DNA, all RNA samples were treated for 20 min at 37°C with DNase (TURBO DNA-*free*™, Ambion, TX, USA). These RNA samples were quantified using ND-1000 NanoDrop spectrophotometer (Thermo Scientific, DE, USA) and the quality was checked by Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). Equal amounts of RNA from 14 TD and 11 DT samples were bulked as TD and DT, respectively. Four cDNA libraries from T, D, TD and DT were generated using the mRNA-Seq Sample Prep Kit (Illumina, CA, USA).

Illumina sequencing

Four cDNA libraries were sequenced by a commercial service provider (Hokkaido System Science Co., Ltd., Sapporo, Japan). A 75 base single-end run was performed on Illumina Genome Analyzer IIx platform with one sample per lane of a flow cell. All 75 bases of each read were filtered for sequence quality and complexity and assembled using the Velvet program (Zerbino and Birney 2008) for *de novo* assembly. A hash length in base pairs (*k*-mer length) was determined with a series of *k*-mers to optimize the Velvet assembly toward higher transcript contiguity (longer transcript length) and specificity (less spurious overlaps). Overlapping contigs were grouped and regarded as a transcript using the program Oases (version 0.1.8, http://www.ebi.ac.uk/~zerbino/oases/). Sequence reads of each sample were mapped with a maximum of two nucleotide mismatches on assembled transcripts to quantify the abundance of the transcripts using the program Bowtie (Langmead et al. 2009). The abundance, or the coverage of each transcript was determined by read counts and normalized as reads per kilobase of exon model per million mapped reads (RPKM). The RPKM value of read density reflects the molar concentration of a transcript in the

starting sample by normalizing for RNA length and for the total read number in the measurement. This facilitates transparent comparison of transcript levels both within and between samples.

Single nucleotide polymorphism (SNP) genotyping

Based on visual inspection of mapped reads to a transcript using the graphical viewer program Tablet (Milne et al. 2010), SNPs were searched, which enabled to separate one transcript into multiple transcript variants. Parental origins of the transcript variants were determined by comparison with parental transcripts.

Homology search and functional annotation

To deduce the putative function, transcript sequences were subjected to BLASTX analysis. The results of only the best hit were extracted, and their functional roles were deduced by annotating to GO Slim terms in categories of molecular function and biological process. The GO Slim term was searched using public databases of the Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org/tools/bulk/go/index.jsp), Sol Genomics Network (http://solgenomics.net/search/template) and The European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/).

Results

Data summary

After the low-quality sequence data were filtered, a total of 3,035,230 - 3,204,206 kilo bases (72.5 - 80.3 %) sequences per sample were generated by a single run of 75

cycles using Illumina Genome Analyzer IIx (Table 10). The trimmed high-quality sequence reads were assembled using Velvet program at different *k*-mer length of 51, 61, 69 and 73. The best assembly was found by $k = 69$. Using a hash length of 69-mers, all reads were assembled, resulting in a total of 37,238 contigs of at least 100 bp length. These contigs were grouped and constructed into transcripts using Oases program with default parameters. This resulted in a total number of 13,020 transcripts of 9,366 loci. These transcripts were mapped to the complete chloroplast genome and mitochondrial mRNAs of *S. tuberosum*, which disclosed 47 (36 loci) chloroplast transcripts and 21 (21 loci) mitochondrial transcripts. High-quality reads were mapped with a maximum of two nucleotide mismatches on transcripts of the respective samples. As no read was mapped to some transcripts of a sample, the number of mapped transcripts varied from 12,595 in T to 13,018 in TD. The number of mapped reads to each transcript was normalized as RPKM values. Although RPKM values varied largely among loci from 0.0 to 13547.2, 70.3-77.3% of transcripts showed < 1,000 RPKM values (Table 10, Figs. 6 and 7). The mean RPKM value in each sample ranged from 151.2 in D to 156.5 in T, and the difference among the four samples was not significant as tested by ANOVA (F= 2.61, $P < 0.001$).

Pairwise comparison among RPKM values of T, D, TD and DT

RPKM values were compared using x^2 test against 1 : 1 ($P < 0.05$) for each transcript by pairwise comparisons among T, D, TD and DT (Table 11). The largest number of transcripts significantly different between given two samples was obtained from a comparison between parents; of 13,020 transcripts, 4,051 (31.1%) were higher in T, while 2,957 (22.7%) were higher in D (Fig. 6A). Inversely, the highest similarity was obtained between TD and DT (Fig. 6B). 85.8% of transcripts did not show

Table 10 Summary of obtained data

Sample	T	D	TD	DT
No. of bases (kilo bases)	3,175,152	3,204,206	3,146,108	3,035,230
No. of mapped reads	21,355,449	23,318,726	21,475,383	17,246,376
No. of mapped transcripts	12,595	12,846	13,018	13,016
Total RPKM values	2,037,300.4	1,968,924.6	2,005,482.3	2,003,634.7
Mean RPKM value	156.5	151.2	154.0	153.9
(Standard deviation)	(592.99)	(523.65)	(564.65)	(568.49)
No. of transcripts with < 1,000 RPKM value	9,463	9,037	9,349	10,064

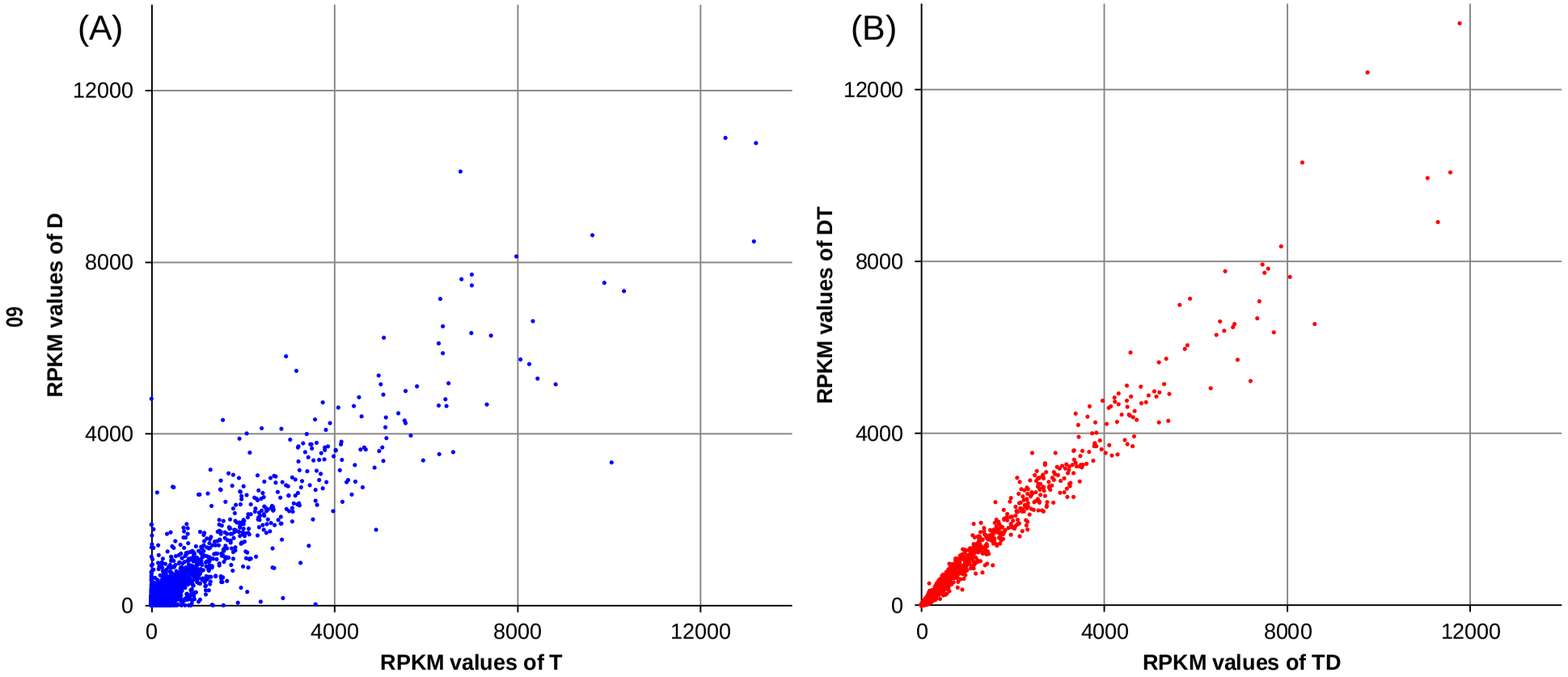

Fig. 6 Comparisons of RPKM values between T and D (A) and between TD and DT (B)

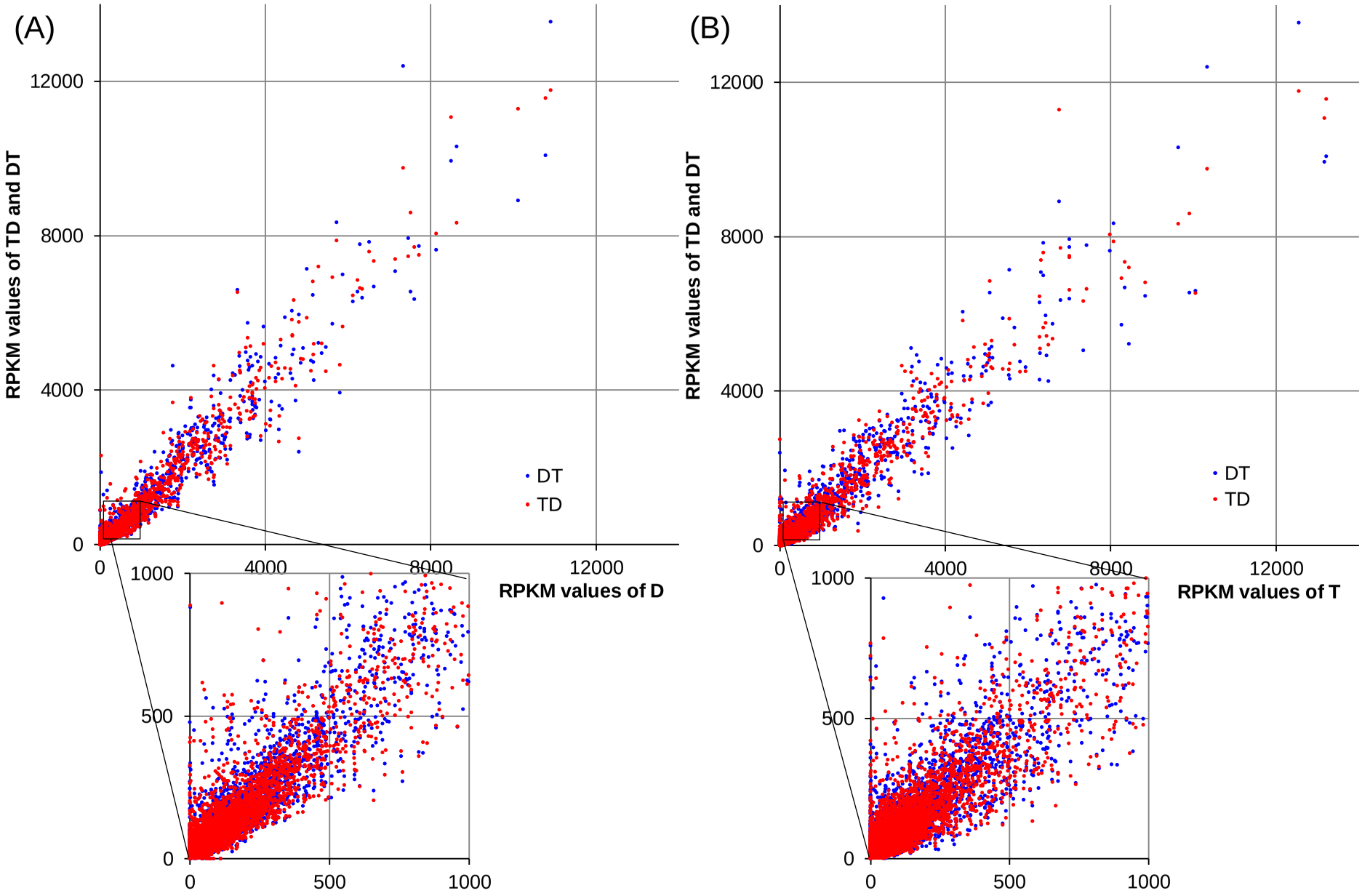

Fig. 7 Comparisons of RPKM values between D and the progeny TD and DT (A) and between T and the progeny TD and DT (B)

Table 11 Pairwise comparison among T, D, TD, and DT using x^2 test against 1 : 1 between RPKM values of each transcript ($P < 0.05$)

Difference	No. of transcripts
T vs. D	
T > D	4,051 (31.1%)
T = D	6,012 (46.2%)
T < D	2,957 (22.7%)
T vs. TD	
T > TD	2,202 (16.9%)
T = TD	7,538 (57.9%)
T < TD	3,280 (25.2%)
T vs. DT	
T > DT	2,983 (22.9%)
T = DT	6,542 (50.2%)
T < DT	3,498 (26.9%)
D vs. TD	
D > TD	2,289 (17.6%)
D = TD	8,490 (65.2%)
D < TD	2,241 (17.2%)
D vs. DT	
D > DT	2,349 (18.0%)
D = DT	8,857 (68.0%)
D < DT	1,814 (13.9%)
TD vs. DT	
TD > DT	1,128 (8.7%)
TD = DT	11,173 (85.8%)
TD < DT	719 (5.5%)

significant differences in RPKM values between TD and DT. In comparison between parents and the progeny (Fig. 7), both TD and DT showed larger numbers of the same level transcripts with D parent (65.2% and 68.0%, respectively) than with T parent (57.9% and 50.2%, respectively) (Table 11). In addition, D parent-progeny plots (Fig. 7A) were less scattered from a y = x line than T parent-progeny plots (Fig. 7B), indicating transcription levels of the progeny were more alike to D parent than to T parent.

Although TD and DT showed the highest similarity, there were 1,847 transcripts significantly different between TD and DT; 1,128 were higher in TD and 719 were higher in DT (Table 11). Among them 11 transcripts of 8 loci were mapped to chloroplast genome, while 3 transcripts of 3 loci were mapped to mitochondrial genome. The mean RPKM values of these 1,847 transcripts were 534.9 in TD and 538.8 in DT, which were much lower that those of all transcripts, indicating that most of transcripts with high transcription levels did not contribute to the significant difference between TD and DT.

Differential transcription patterns in 1,847 transcripts

For the 1,847 transcripts exhibiting significant differences in RPKM values between TD and DT, transcription patterns were investigated by x^2 test against 1:1 for all combinations among RPKM values of T, D, TD and DT (Table 12). Among 1,847 transcripts, 455 (24.6%) of TD and DT did not show significant differences from those of the parents. RPKM values in 514 (27.8%) transcripts of TD and DT were intermediate between those of T and D. 237 (12.8%) transcripts showed uniparental tendencies; TD and DT were at the same transcription levels with the respective maternal parents in 128 and with the respective paternal parents in 109 transcripts.

Table 12 Differential transcription patterns among RPKM values of T, D, TD and DT, for the transcripts exhibiting significant differences between TD and DT

Differential transcription pattern	No. of transcripts	Ct-like[a]	Mt-like[a]
1. No difference from parents[b]	455		
2. Intermediate between parents			
T > TD > DT > D	63		
T > TD > (DT, D)	34		
T > DT > TD > D	73	1 (1)	
T > DT > (TD, D)	34		
(T, TD) > DT > D	38		
(T, DT) > TD > D	31		
D > TD > DT > T	44		
D > DT > TD > T	75		
D > TD > (DT, T)	16		
D > DT > (TD, T)	22		
(D, TD) > DT > T	34		
(D, DT) > TD > T	50		
Subtotal	514		
3. Uniparental			
(T, TD) > (DT, D)	81		
(D, DT) > (TD, T)	47		
(T, DT) > (TD, D)	47		
(D, TD) > (DT, T)	62		
Subtotal	237		

4. Over-transcription of TD			
TD > (DT, T, D)	223		1 (1)
TD > DT > (T, D)	23	3 (3)	
TD > (DT, T) > D	40	1 (1)	
TD > (DT, D) > T	58	3 (1)	2 (2)
TD > T > DT > D	6	2 (1)	
TD > D > DT > T	4		
TD > T > (DT, D)	5		
TD > D > (DT, T)	9		
Subtotal	368		
5. Over-transcription of DT			
DT > (TD, T, D)	39		
DT > TD > (T, D)	29		
DT > (TD, T) > D	12		
DT > (TD, D) > T	20		
DT > T > TD > D	10		
DT > T > (TD, D)	16		
DT > D > (TD, T)	5		
Subtotal	131		
6. Over- and under-transcription			
TD > D > T > DT	1		
TD > T > D > DT	1		
DT > T > D > TD	3		
DT > D > T > TD	3		
Subtotal	8		

7. Under-transcription of DT		
(TD, T, D) > DT	26	
(T, D) > TD > DT	5	
(TD, D) > T > DT	2	
T > (TD, D) > DT	10	
D > (TD, T) > DT	8	
T > TD > D > DT	9	
D > TD > T > DT	1	
Subtotal	61	
8. Under-transcription of TD		
(DT, T, D) > TD	32	
(T, D) > DT > TD	4	
(DT, T) > D > TD	3	
T > (DT, D) > TD	14	1 (1)
D > (DT, T)> TD	14	
T > DT > D > TD	3	
D > DT > T > TD	3	
Subtotal	73	
Total	1,847	

[a] No. of transcripts mapped to chloroplast or mitochondrial genome. No. of loci in parenthesis

[b] TD and DT were significantly different from each other but not from both parents

However, none of transcripts mapped to chloroplast or mitochondrial genome showed uniparental transcription patterns, indicating that these transcripts are orthologues residing in the nuclear genome.

Interestingly, the rest of transcripts (34.7%) exhibited either over- or under-transcription in TD or DT than the both parental transcription (Table 12). Especially, over-transcription of TD [TD > (DT, T, D)] was prominent and found in 368 (19.9%) transcripts. Over-transcription of DT [DT > (TD, T, D)] was found in 131 (7.1%) transcripts, which was approximately one third of those found in TD but much larger number than those showing under-transcription in TD (4.0%) and DT (3.3%).

The extent of difference between TD and DT in 1,847 transcripts was measured by an RPKM ratio (=RPKM value of TD / RPKM value of DT), which ranged from 0.211 to 11.93. As shown in Table 13, a larger number of transcripts was found in ratios of > 1, indicating TD had more number of transcripts with larger RPKM values than DT. More than four times higher RPKM values of TD than those of DT were found in 62 transcripts, while more than four times higher RPKM values of DT than those of TD were found in two transcripts. These 64 transcripts were mostly TD over-transcription types (85.9%) and their RPKM values were all below the mean RPKM values of differential 1,874 transcripts. In the following analysis, only these 64 transcripts were used for comparison.

SNP-genotyping of transcripts

Each transcript might be composed of multiple transcript variants with differences of a maximum of two nucleotides. The 64 transcripts with more than four times RPKM value differences between TD and DT were analyzed for SNPs to distinguish transcript variants. Eight transcripts had no SNPs, while 56 (containing two transcripts

Table 13 Number of transcripts exhibiting differential transcription between TD and DT

RPKM ratio[a]	No. of transcripts
< 1/6	0
1/6~ 1/4	2
1/4 ~ 1/2	25
1/2~ 1	692
1~ 2	670
2 ~ 4	396
4~ 6	40
6 ~ 8	11
8 ~10	6
> 10	5
Total	1,847

[a] RPKM ratio = RPKM value of TD / RPKM value of DT

derived from one locus) were distinguished into 169 transcript variants of 55 loci. By further comparisons with parental transcripts, it was determined that, out of 169 transcript variants, 51 of 37 loci were derived from T, and 75 of 51 loci from D (Table 14). The remaining 43 transcript variants of 39 loci were found in both parents. Thus, each locus was composed of, on average, 3.1 transcript variants with at least 0.93 (ranging from 0 to 4) T-derived and 1.36 (ranging from 0 to 5) D-derived transcript variants (parent-specified transcripts).

Differential transcription of parent-specified transcripts

Intra-locus transcription levels of transcript variants in each locus (member transcripts) were compared by a ratio of an observed number of reads for a member transcript / the mean read number of member transcripts in a locus. No T- or six D-derived transcripts in TD showed two times higher transcription than the mean transcription levels (ratio of > 2), while two T- and six D-derived transcripts did so in DT (Table 14). Among them, three D-derived transcripts were present in both TD and DT. Thus, 8.7% of parent-specified transcripts showed over-transcription compared with the other member transcripts of a locus. In addition, 50.0% of parent-specified transcripts showed ratios of lower than 0.5, indicating under-transcription compared with the other member transcripts of a locus. Therefore, 58.7% of parent-specified transcripts showed differential transcription within each locus.

To investigate whether these parent-specified transcripts showing differential transcription within a locus also contributed to the differential transcription between TD and DT, a ratio of an observed read number difference (TD – DT) of a transcript / a mean read number difference (TD – DT) of member transcripts in a locus was calculated (Table 15). Of 32 T-derived transcripts showing intra-locus

Table 14 Number of parent-specified transcripts and those exhibiting intra-locus over- or under-transcription measured by a ratio of an observed read number of a member transcript / the mean read number of member transcripts in each locus[a]

Parental origin	No. of transcripts	Ratio in TD		Ratio in DT	
		> 2	< 0.5	> 2	< 0.5
T	51 (37)	0 (0)	25 (23)	2 (2)	22 (20)
D	75 (51)	6 (6)	21 (21)	6 (6)	24 (23)
Both	43 (39)	11 (11)	7 (7)	8 (8)	5 (5)
Total	169 (55)	17 (17)	53 (40)	16 (16)	51 (37)

[a] Number of loci in parenthesis

Table 15 Number of parent-specified transcripts exhibiting differential transcription within a locus and between TD and DT measured by a ratio of an observed read number difference (TD – DT) of a transcript / the mean read number difference (TD – DT) of member transcripts in a locus

Transcription pattern within a locus	No. of transcripts	Ratio	
		> 2	< 0.5
T-derived transcript			
Over-transcription in both TD and DT	0	-	-
Over-transcription only in TD	0	-	-
Over-transcription only in DT	2	0	0
Under-transcription in both TD and DT	15	0	15
Under-transcription only in TD	10	0	10
Under-transcription only in DT	7	0	1
D-derived transcript			
Over-transcription in both TD and DT	3	2	0
Over-transcription only in TD	3	3	0
Over-transcription only in DT	3	0	0
Under-transcription in both TD and DT	14	0	12
Under-transcription only in TD	7	0	7
Under-transcription only in DT	10	0	0

Table 16 Number of reads for the parent-specified transcripts showing more than two times over-transcription (asterisked) compared with the other member transcripts and between TD and DT

Locus	Rank	RPKM values				Parent-specified transcript[a]	Number of reads[b]			
		T	TD	DT	D		T	TD	DT	D
7361	12	24.6	131.6	17.5	14.5	T-derived	54	81	10	-
						T-derived	12 (u)	5 (u)	1 (u)	-
						D-derived	-	64	11	6
						D-derived	-	17 (u)	2 (u)	2 (u)
						D-derived*	-	157 (o)	5	26 (o)
7307	17	1.8	45.8	6.9	9.5	T-derived	4	13 (u)	1 (u)	-
						D-derived*	-	73 (o)	10 (o)	21
						T and D-derived	5	15 (u)	3	3 (u)
7792	22	1.8	45.9	7.5	11.5	T-derived	4	3 (u)	1 (u)	-
						D-derived*	-	92 (o)	12 (o)	29
						D-derived	-	13 (u)	1 (u)	2 (u)

5912	37	8.0	22.8	4.7	5.8	T-derived	23	3 (u)	1	-
						D-derived*	-	22 (o)	3	15
						T and D-derived	1 (u)	6	1	7
6582	49	9.6	33.3	7.4	7.7	T-derived	4	5 (u)	4	-
						T-derived	8	5 (u)	1 (u)	-
						D-derived*	-	27 (o)	3	17
						D-derived	-	12	3	1 (u)

[a] Asterisked parent-specified transcripts showed ratios [= an observed read number difference (TD – DT) of a transcript / the mean read number difference (TD – DT) of member transcripts in a locus] of > 2

[b] Read numbers shown with (o) or (u) indicate intra-locus over- (> 2) or under-transcription (< 0.5), respectively

under-transcription, 26 showed ratios lower than 0.5 and no transcript showed a ratio higher than 2, indicating no meaningful contribution to the difference (TD – DT). Likewise, 31 D-derived transcripts showing intra-locus under-transcription did not contribute to differential transcription between TD and DT. However, 5 of 9 D-derived transcripts showing intra-locus over-transcription did show ratios higher than 2, indicating apparent contribution to differential transcription between TD and DT.

For these five transcripts, their locus data were shown in Table 16. These five loci were those showing high RPKM value differences between TD and DT (ranked 12 - 49). In all the loci, one of D-derived transcripts overcome the other member transcripts and greatly increased RPKM values of TD. Interestingly, however, these D-derived transcripts also exhibited relatively high read numbers in D parent (Table 16).

Homology search

Homology search using BLASTX were performed for 64 transcripts with more than four times RPKM value difference between TD and DT. 59 of them were revealed as known sequences and their functional roles were estimated (Table 17). Six transcripts matched with late embryogenesis abundant protein (LEA), and five matched with putative methyltransferase PMT27. At least nine transcripts had functional roles related to stress response and ten related to transmembrane transport. In addition, SSK1 (SLF-interacting Skp1-like 1) protein (transcript number of 8185) was found.

Table 17 Homology search results for transcripts exhibiting more than four times larger RPKM value differences between TD and DT. Only transcripts that matched genes or proteins by BLASTX searching are listed

Rank	Trans-cript[a]	RPKM values		Matched gene or protein [species] accession number	e-value[b]	Function		Transcription pattern
		TD	DT			Biological process	Molecular function	
1	6697	42.5	3.6	Cold-regulated 413-plasma membrane 2 [*Arabidopsis thaliana*] NP_190652	6e-41	Response to stress	Binding, elemental activity	TD > (T, D, DT)
2	6950	27.4	2.4	Putative vicilin [*Solanum demissum*] AAT40548	3e-14	-	Nutrient reservoir activity	TD > T > (D, DT)
3	4987	62.1	5.7	Putative methyltransferase PMT27 [*Arabidopsis thaliana*] NP_190676.1	7e-43	Methylation	Methyltransferase activity	TD > T > (D, DT)
4	8185	52.9	5.1	SSK1 (SLF-interacting Skp1-like 1) [*Petunia* × *hybrida*] ACT35733.1	8e-40	Ubiqutin-dependent protein catabolic process, protein polymerization	Protein complex	TD > (T, D, DT)
5	6805	23.4	2.2	Beta-tubulin [*Glycine max*] AAA20243.1	2e-44	-	Chitin binding	TD > (T, D, DT)
6	7355	47.2	4.9	Putative methyltransferase PMT27 [*Arabidopsis thaliana*] NP_190676.1	2e-70	Methylation	Methyltransferase activity	TD > (T, D, DT)
7	7322	30.6	3.2	Putative vicilin [*Solanum demissum*] AAT40548.1	2e-26	-	Nutrient reservoir activity	TD > (T, D, DT)
8	6629	33.9	3.6	Putative methyltransferase PMT27 [*Arabidopsis thaliana*] NP_190676.1	3e-51	Methylation	Methyltransferase activity	TD > (T, D, DT)
9	7657	27.6	3.0	60S ribosomal protein L27-3 [*Arabidopsis thaliana*] NP_001078392	8e-45	Translation	Structural constituent of ribosome	TD > (T, D, DT)
10	3772	50.1	5.5	Putative methyltransferase PMT27 [*Arabidopsis thaliana*] NP_190676.1	1e-45	Methylation	Methyltransferase activity	TD > (T, D, DT)
11	9224	39.9	4.7	Late embryogenesis abundant domain-containing protein [*Arabidopsis thaliana*]	0.01	Embryo development ending in seed dormancy	-	TD > (DT, D) > T

				NP_177355.1				
12	7361*	131.6	17.5	Late embryogenesis abundant (LEA) protein [*Arabidopsis thaliana*] NP_195378.5	8e-20	Embryo development ending in seed dormancy	-	TD > (T, D, DT)
13	3565	118.7	15.8	Late embryogenesis abundant protein (LEA) family protein [*Arabidopsis thaliana*] NP_198692.1	0.55	-	-	TD > (DT, T) > D
14	9231	45.2	6.4	Wound-induced protein [*Solanum lycopersicum*] AAB49688.1	7e-24	Defense response to bacterium, defense response to fungus jasmonic acid, ethylene mediated signaling pathway	Chitin binding, chitinase activity	TD > (DT, D) > T
15	9357	53.8	7.8	Putative pathogen-induced protein - pi1 [*Solanum lycopersicum*] CAC81819	4e-67	Defense response to bacterium	Chitin binding	TD > (DT, D) > T
16	1320	31.7	4.7	RNA binding protein-like protein [*Solanum tuberosum*] ABA40453.1	1e-62	Response to osmotic stress and salt stress	ATP, RNA and copper binding	TD > (T, D, DT)
17	7307*	45.8	6.9	60S ribosomal protein L27 [*Solanum tuberosum*] CAB57298	7e-52	GTP, UTP, CTP biosynthesis	Nucleoside diphosphate kinase activity, ATP binding	TD > (T, D, DT)
18	7425	65.1	10.0	Mitochondrial import inner membrane translocase pom14 [*Solanum tuberosum*] CAA63967.1	9e-16	Transmembrane transport	Protein transporter activity	TD > (T, D, DT)
19	5584	35.4	5.5	Putative methyltransferase PMT27 [*Arabidopsis thaliana*] NP_190676.1	4e-29	Methylation	Methyltransferase activity	TD > (T, D, DT)
21	4806	85.1	13.7	Glyceraldehyde 3-phosphate dehydrogenase-like protein [*Solanum tuberosum*] ABB72804.1	2e-69	Glycolysis, response to oxidative stress	Glyceraldehyde-3-phosphate dehydrogenase activity, NADP activity	TD > (T, D, DT)
22	7792*	45.9	7.5	Nucleoside diphosphate kinase 1 [*Solanum*	2e-57	CTP, GTP, UTP biosynthetic	ATP and zinc ion binding	TD > (T, D, DT)

				tuberosum] ADF45668.1		process, nucleoside diphosphate phosphorylation		
23	5533	52.6	8.8	Inorganic phosphate transporter 1 [*Solanum tuberosum*] CAA67395.1	3e-43	Transmembrane transport	Transmembrane transporter activity	TD > (T, D, DT)
24	6417	69.0	11.6	Inorganic phosphate transporter 1 [*Solanum tuberosum*] CAA67395.1	2e-124	Transmembrane transport	Transmembrane transporter activity	TD > (T, D, DT)
25	9040	69.3	12.0	S-adenosyl-L-homocysteine hydrolase-like [*Solanum tuberosum*] ABB55380.1	1e-33	Embryo development ending in seed dormancy, methylation-dependent chromatin silencing, posttranscriptional gene silencing	Adenosylhomocysteinase activity, copper ion binding	TD > (DT, D) > T
26	7647	31.7	5.5	Cyclophilin ROC7-like [*Solanum tuberosum*] ABB86261.1	4e-46	Protein folding, protein peptidyl-prolyl isomerization	Peptide binding	TD > (T, D, DT)
27	2339	123.4	21.4	Late embryogenesis abundant (LEA) protein [*Arabidopsis thaliana*] NP_195378.5	4e-11	Embryo development ending in seed dormancy	-	TD > (T, D, DT)
28	6539	31.6	5.8	Xylose isomerase [*Arabidopsis thaliana*] NP_568861.3	2e-32	Carbohydrate metabolic process	Xylose isomerase activity	TD > (T, D, DT)
29	9348	121.0	23.5	Late embryogenesis abundant protein (LEA) family protein [*Arabidopsis thaliana*] NP_198692.1	0.12	-	-	TD > DT > (T, D)
30	4505	57.6	11.2	Pre-pro-cysteine proteinase [*Solanum lycopersicum*] CAA78403.1	3e-43	Response to osmotic stress and salt stress	Cysteine-type peptidase activity	(T,TD) > (DT, D)
31	1387	61.0	11.9	Phosphoglycerate kinase-like [*Solanum tuberosum*] ABB86242.1	2e-84	Glycolysis, phosphorylation	Phosphoglycerate kinase activity	TD > (T, D, DT)
33	7573	15.5	3.1	40S ribosomal protein S5-2 [*Arabidopsis*	2e-30	Translation	Structural constituent of	Not different

				thaliana] NP_850564.1			ribosome	
34	6852	18.8	3.8	Tonoplast intrinsic protein 1-1 [*Solanum nigrum*] ADW66151.1	8e-47	Response to salt stress, gibberellic acid mediated signaling pathway, transmembrane transport	Transmembrane transporter activity	TD > (T, D, DT)
35	1932	38.6	7.8	Cold-regulated 413-plasma membrane 2 [*Arabidopsis thaliana*] NP_190652.1	2e-28	Response to stress	-	TD > (T, D, DT)
36	7689	10.9	2.2	Glycoprotein-like protein [*Solanum tuberosum*] ABA40467.1	5e-29	Protein biosynthesis	Structural constituent of ribosome	Not different
37	5912*	22.8	4.7	Putative 60S ribosomal protein L34 [*Solanum demissum*] AAT39969.1	2e-46	Translation	Structural constituent of ribosome	TD > (T, D, DT)
38	1633a	135.3	28.8	Pre-pro-cysteine proteinase [*Solanum lycopersicum*] CAA78403.1	1e-136	Proteolysis	Cystein-type peptidase activity, hydrolase activity	TD > (T, D, DT)
40	4662	14.3	3.1	Hexose transporter protein [*Solanum lycopersicum*] CAA09419	1e-27	Transmembrane transport	Transmembrane transporter activity	(TD, T) > (D, DT)
41	4593	57.4	12.4	Adenosine kinase isoform 2T [*Nicotiana tabacum*] AAU14834.1	2e-50	Adenosine salvage, AMP biosynthtic process	Adenosine kinase activity	TD > (T, D, DT)
42	3398	41.0	8.8	Glyceraldehyde 3-phosphate dehydrogenase-like protein [*Solanum tuberosum*] ABB72804.1	2e-44	Defense response to bacterium, glycolysis, response to cadmium and oxidative stress	Copper ion binding, zinc ion binding	TD > (T, D, DT)
43	4366	77.8	16.9	Putative ATPase inhibitor [*Ricinus communis*] XP_002515764.1	0.17	Negative regulation of catalytic activity	ATPase inhibitor activity	TD > (DT, D) > T
44	1633b	119.7	26.1	Pre-pro-cysteine proteinase [*Solanum lycopersicum*] CAA78403.1	5e-132	Proteolysis	Cystein-type peptidase activity, hydrolase activity	TD > (T, D, DT)
45	7742	36.4	8.0	34 kDa outer mitochondrial membrane protein porin-like protein [*Solanum*	5e-60	Regulation of ion and anion transmembrane transport,	Voltage-gated anion channel activity, protein binding,	TD > (DT, D) > T

				tuberosum] ABC01904.1		response to bacterium		
46	4125	52.3	11.5	Phosphoglycerate kinase-like [*Solanum tuberosum*] ABB86242.1	1e-41	Glycolysis, phosphorylation	Phosphoglycerate kinase activity	TD > (T, D, DT)
47	5276	12.0	2.7	POM30-like protein [*Solanum tuberosum*] ABB16970	8e-99	Anion transport	Transmembrane transport, voltage-gated anion channel activity	(T, TD) > (DT, D)
48	7827	51.7	11.6	Cyclophilin ROC7-like [*Solanum tuberosum*] ABB86261.1	1e-49	Protein folding	Peptide binding	TD > (T, D, DT)
49	6582*	33.3	7.4	Inorganic phosphate transporter 1 [*Solanum tuberosum*] CAA67395.1	8e-55	Transmembrane transport	Phosphate transmembrane transporter activity	TD > (T, D, DT)
50	7795	72.5	16.5	S-adenosyl-L-homocysteine hydrolase-like [*Solanum tuberosum*] ABB55380.1	6e-16	Methylation-dependent chromatin silencing, embryo development ending in seed dormancy, posttranscriptional gene silencing	Adenosylhomocysteine hydrolase and deaminase activity, 5'-methyladenosine nucleosidase activity	TD > (T, D, DT)
51	4069	39.0	8.9	34 kDa outer mitochondrial membrane protein porin-like protein [*Solanum tuberosum*] ABC01904.1	3e-52	Regulation of ion and anion transmembrane transport, response to bacterium	Voltage-gated anion channel activity, protein binding,	TD > (DT, D) > T
52	3562	57.4	13.1	Adenosine kinase isoform 2T [*Nicotiana tabacum*] AAU14834.1	2e-88	Adenosine salvage, phoshorylation, AMP biosynthtic process, response to cadmium ion and trehalose	Adenosine kinase activity	TD > (T, D, DT)
54	1214	2.6	11.4	RNA-directed DNA polymerase (Reverse transcriptase) -Os08g0164800 [*Oryza sativa* Japonica Group] NP_001061072.1	2e-12	RNA-dependent DNA polymerase activity	RNA binding	D > (DT, T) > TD
55	5988	27.2	6.3	ABCG subfamily transporter [*Solanum tuberosum*] AEB65936.1	9e-25	Drug transmembrane transport	ATPase activity	TD > (T, D, DT)

56	3024	33.6	7.8	Ribosomal protein L27a-like protein [*Solanum tuberosum*] ABA40430.1	1e-63	Translation	Structural constituent of ribosome	TD > (T, D, DT)
57	4435	51.9	12.1	Tim17 domain-containing protein [*Arabidopsis thaliana*] NP_565968.1	5e-62	-	-	TD > (T, D, DT)
58	9093	74.0	17.3	Pre-pro-cysteine proteinase [*Solanum lycopersicum*] CAA78403.1	2e-45	Proteolysis	Cystein-type peptidase activity, hydrolase activity	TD > (DT, D) > T
59	7344	25.9	6.1	Succinate dehydrogenase (ubiquinone) flavoprotein subunit 1 [*Arabidopsis thaliana*] NP_201477.1	2e-32	Mitochondrial electron transport, succinate to ubiquinone	ATP binding, cobalt ion binding	TD > (T, D, DT)
60	7464	22.1	5.2	Sucrose-phosphate-synthase [*Solanum tuberosum*] ACD50895.1	5e-41	Biosynthetic process, metabolic process, sucrose metabolic process	Sucrose-phosphate synthase activity, transferase activity	TD > (T, D, DT)
61	1765	165.6	41.2	Late embryogenesis abundant protein (LEA) family protein [*Arabidopsis thaliana*] NP_198692.1	2e-19	-	-	TD > (DT, T) > D
62	5361	25.1	6.2	Ripening regulated protein DDTFR19 [*Solanum lycopersicum*] AAG49033	8e-31	Ribosome biogenesis, translation	Structural constituent of ribosome	TD > (T, D, DT)
63	6061	75.3	18.8	Postmeiotic segregation increased 2 (PMS2) (*Rattus norvegicus*) EDL89650.1	0.92	Mismatch repair, reciprocal meiotic recombination	ATP binding, mismatched DNA binding	TD > (DT, T) > D

[a] Asterisked transcripts contained D-derived transcripts which contributed to differential transcription between TD and DT. See Table 16

[b] Indicating the degree of homology

Discussion

In this study, a high-throughput sequencer generated 12.6 billion bases from pollen mRNA, which were aligned into 13,020 transcripts. Interspecific differences at a transcription level were found in 53.8% of transcripts between *S. tuberosum* and *S. demissum*. Reciprocal differences between TD and DT hybrids were also found in 1,847 (14.2%) transcripts. Recently, genome-wide transcription levels were investigated for seedlings of reciprocal inter-varietal hybrids between two homozygous lines C24 and Columbia of *Arabidopsis thaliana* (Andorf et al. 2010) and for seedlings of reciprocal inter-subspecies hybrids (He et al. 2010) between, or for developing endosperm and embryo (Luo et al. 2011) in reciprocal crosses of, homozygous lines Nipponbare (ssp. *japonica*) and 93-11 (ssp. *indica*) of *Oryza sativa*. All these disclosed significant amounts of transcripts exhibiting reciprocal differences though examined organs or tissues were different among authors. In the previous chapter (Chapter III), I found at least six distinct AFLP bands (< 0.1%) showing reciprocal differences in DNA or DNA methylation status. At a transcription level, more dynamic differences were disclosed in pollen.

Transcription levels in hybrids were often deviated from the mid-parent levels of their homozygous parents as disclosed by a genome-wide survey (Vuylsteke et al. 2005; Guo et al. 2006; Swanson-Wagner et al. 2006; Zhuang and Adams 2007; Guo et al 2008; Wei et al. 2009; Andorf et al. 2010; He et al. 2010; Riddle et al. 2010). In this study, among reciprocally different 1,847 transcripts, 52.5% showed intermediate between or near parental RPKM values, which were likely resulted by additive or dominance/recessive genetic effects. 34.7% of 1,847 transcripts exhibited over- or under-transcription in the hybrids compared with parental transcription levels (Table 12).

Particularly, TD over-transcription was prominent (19.9%). He et al. (2010) reported that, among 20,638 genes analyzed in reciprocal inter-subspecies rice hybrids, 3,261 in the Nipponbare × 93-11 hybrid and 3,229 in 93-11 × Nipponbare hybrids exhibited non-additive transcription patterns, in which 20.2-39.5% indicated over-transcription and 16.4-34.6% indicated under-transcription. Since reciprocal hybrids share the same nuclear genetic background, a cytoplasmic effect was suggested as a causal genetic effect for inconsistent over- or under-transcription patterns between reciprocal hybrids (He et al. 2010).

Alternative causes for differential transcription between TD and DT might be a direct maternal effect (non-genetic) and imprinting (differential expression of maternal or paternal alleles in either a preferentially or exclusively uniparental manner). A possibility of a direct maternal effect, however, had to be minimal and could be neglected because plants were grown in nearby rows in the same field and the pollen itself was a mass collection from the progeny population.

For the top 55 loci showing highly differential transcription profiles, the parental origins of transcript variants were determined (Table 14). Each locus was composed of 3.1 transcript variants with at least 0.93 T-derived and 1.36 D-derived transcript variants. Considering that transcription levels of the progeny were more alike to D parent than to T parent (Table 11, Fig. 7), a higher number of D-derived transcripts than T-derived transcripts is likely due to the genomic dosage in interspecific hybrids (three genomes derived from *S. demissum* and two from *S. tuberosum*). 58.7% of parent-specified transcripts showed intra-locus differential transcription (Table 14), implying these transcripts originated from imprinted genes. However, only five D-derived transcripts contributed to over-transcription between reciprocal hybrids (Table 15), and these also exhibited relatively high read numbers in D parent (Table 16). Thus, these five

D-derived transcripts were not transcribed in a parent-of-origin manner but in an allelic bias manner (intrinsic differential abilities of alleles within a locus, or described as allele-specific expression variation by Guo et al. 2004). Guo et al. (2004) found that among 15 genes analyzed, 11 showed allele-specific expression variation in maize hybrids. Since maternal or paternal transmission had little effect on the allele-specific transcript ratio, they suggested that parent-of-origin effect was minimal (Guo et al. 2004). Furthermore, Luo et al. (2011) reported that, although most imprinted genes were expressed in various tissues in the rice plant, their expression in a parent-of-origin manner was limited in endosperm and embryo. Therefore, it is suggested that imprinted genes were not involved in the interspecific reciprocal difference in pollen.

In this study, transcription levels of chloroplast and mitochondrial genes were unable to analyze, because mRNA from chloroplast and mitochondrial genes does not include poly(A) tails except in degradation (Forner et al. 2007; del Campo 2009). Sixty-eight transcripts showed sequence homology to either chloroplast and mitochondrial genes, but they were likely orthologues encoded in the nuclear genome because their transcription indicated biparental inheritance patterns. Thus, direct cytoplasmic effects on differential transcription between reciprocal hybrids remain unknown. However, as already shown in the crossing results of Chapter I, irrespective of being crossed as male or female, F_1 and BC_1 progenies with T cytoplasm always showed higher berry-setting rates (the average of 2.04 times) than those with the D cytoplasm. Therefore, it can be concluded that interactions between cytoplasmic genome and the nuclear chromosomal genes played an important role affecting transcription levels, particularly over- or under- transcription, which possibly resulted in phenotypic differences between reciprocal hybrids.

I found several interesting genes or proteins contributing to the reciprocal difference. SSK1 (transcript 8185, Table 17) is expressed specifically in pollen and acts as an adaptor in an SCF (Skp1-Cullin1-F-box)^{SLF} complex, which is required for cross-pollen compatibility in *S*-RNase-based self-incompatibility system (Zhao et al. 2010). Putative methyltransferase PMT27 is a member of the *TUMOROUS SHOOT DEVELOPMENT2* (*TSD2*) gene family in *Arabidopsis* with the essential role in cell adhesion and coordinated plant development (Krupková et al. 2007). Late embryogenesis abundant (LEA) proteins are responsive to water deficit; accumulated in dry seeds and disappeared during germination, relating to hydration and dehydration (Colmenero-Flores et al. 1997). In the pollen, these genes might function in adhesion to stigma and hydration for pollen germination. Therefore, further investigation of these transcripts may provide a clue to elucidate differential crossability.

Chapter IV

Band 1 is a useful indicator of the cytoplasm of *Solanum demissum*

Introduction

Unilateral incompatibility, or cytoplasmic male sterility is common in potatoes and their relatives. The common potato (*S. tuberosum*) shared at least seven different cytoplasmic sterility factors ([ASF^s], [Fm^s], [In^s], [SM^s], [Sp^s], [TA^s] and [VSA^s]) that cause sterilities in the presence of dominant chromosomal genes (*ASF*, *Fm*, *In*, *SM*, *Sp*, *TA* and *VSA*) (Grun et al. 1977). The cytoplasmic genome of potato is characterized by possessing T-type chloroplast DNA (Hosaka 1986) and β-type mitochondrial DNA (Lössl et al. 1999). Although the cytoplasmic sterility factors likely reside on mitochondrial DNA (Hosaka et al. 1988; Lössl et al. 2000), β-type mitochondrial DNA so far shows complete association with T-type chloroplast DNA (hereinafter, T/β cytoplasm) (Lössl et al. 2000). The T/β cytoplasm is predominant in the common potato (Hosaka and Hanneman 1988; Waugh et al. 1990; Powell et al. 1993; Bryan et al. 1999; Provan et al. 1999; Lössl et al. 2000). So, sterility problems are unavoidable as far as T/β cytoplasm is accompanied. Besides such intrinsic sterility, some specific male sterility associated with cytoplasmic genome has been known. Varieties carrying Ry_{sto} (an extreme resistance gene to *Potato virus Y*), released mainly in Germany (Ross 1986), show male sterility caused by association with the characteristic mitochondrial genome derived from *S. stoloniferum* (W/γ cytoplasm) (Lössl et al. 2000). In these cases with T/β and W/γ cytoplasms, sterilities are always characterized by visible abnormalities such as no pollen, no or poor pollen-shedding, or various deformities of

anthers (Grun 1979). In contrast, F_1 and back-crossed progenies carrying *S. demissum* cytoplasm (W/α) produce abundant and normal-looking pollen but this pollen is non-functional onto *S. tuberosum* (Dionne 1961). According to Lössl et al. (2000), the W/α cytoplasm occupied 40% of German varieties.

In Chapter I, the unilateral incompatibility between *S. tuberosum* and *S. demissum* was reconfirmed. In addition, I found that the reciprocal interspecific hybrids showed differential crossability. The further investigation of the pollen DNA indicated six distinct sequences different between the reciprocal hybrids (Chapter II). One of the six bands, named Band 1 was very interesting and worthy of further investigation. In this chapter, outside regions of Band 1 were sequenced to disclose its cellular origin whether it is nuclear, chloroplast or mitochondrial DNA. Further, the presence/absence of Band 1 was surveyed for three *S. demissum* accessions, 168 accessions of 38 cultivated and closely related wild species, and in addition 158 varieties and breeding lines.

Materials and Methods

Plant materials

Saikai 35 (a *S. tuberosum* breeding line) and 5H109-5 (*S. demissum* PI 186551) (referred to as T and D, respectively) were reciprocally crossed, deriving DT (D as female) and TD (T as female) F_1 populations (6H37 and 6H38 families, respectively). One TD plant (6H38-19) and one DT plant (6H37-6) were backcrossed with the pollen of Saikai 35, deriving (TD)T and (DT)T BC_1 populations (7H1 and 7H2 families), respectively. Since *S. demissum* is highly self-pollinated in nature and homozygous, we assumed all seedlings derived by selfing were genetically identical to each other and

to the parental clone 5H109-5, and used as D. Saikai 35 has S/ε cytoplasm, while 5H109-5 has W/α cytoplasm.

The presence/absence of Band 1 was examined for three *S. demissum* accessions and 168 accessions of 38 species (Table 18), which covered all 164 different cytoplasms previously distinguished among cultivated species and wild species closely related to cultivated ones except distantly related *S. pinnatisectum* Dun. and *S. stoloniferum* (Hosaka and Sanetomo 2010). A further survey was conducted for 158 varieties and breeding lines.

Extending Band 1 sequence

The sequenced region was extended from both ends of Band 1 using LA PCR™ *in vitro* Cloning Kit (Takara Bio Inc., Japan) by the manufacturer's protocol briefly described below. Total DNA of D was extracted from fresh leaves by the method of Hosaka and Hanneman (1998), digested separately with various restriction enzymes, and ligated for appropriate adapters to the end of restriction digests. Using a pair of primers (one assigned to internal sequence of Band 1 and the other to the adapter sequence), PCR was performed. If a single band was obtained, it was directly sequenced by Takara Bio Inc.

PCR detection of Band 1

The extended Band 1 sequence was divided to three overlapped regions, Region 1, 2 and 3, and the three primer sets were designed to amplify these regions (Table 19). PCR reaction was performed using a volume of 5 μl consisting of 1 μl of template DNA (approximately 5 ng/μl), 2.5 μl of Ampdirect® Plus (Shimadzu Co., Japan), 0.125 units *Taq* DNA polymerase (BIOTAQ™ HS DNA Polymerase, Bioline Ltd., UK) and 0.3 μM

Table 18 Wild and Andean cultivated potatoes surveyed by Band 1. Accessions having W/α cytoplasm are shown by asterisks

Taxonomic series and species	Accession
Series *Pinnatisecta* (Rydb.) Hawkes	
S. pinnatisectum Dun.	PI 184764, PI 275230
Series *Yungasensa* Corr.	
S. chacoense Bitt.	PI 537025, chc 525-3
S. tarijense Hawkes	PI 498399*
Series *Megistacroloba* Cárd. et Hawkes	
S. boliviense Dun.	PI 498215, PI 545964*
S. megistacrolobum Bitt.	PI 265874, PI 473356, PI 473361, PI 545999
S. raphaniforium Cárd. et Hawkes	PI 473371
S. sogarandinum Ochoa	PI 230510
Series *Conicibaccata* Bitt.	
S. chomatophilum Bitt.	PI 266387, PI 365327
S. irosinum Ochoa	PI 568985
Series *Piurana*	
S. acroglossum Juz.	PI 498204
S. blanco-galdosii Ochoa	PI 442701
Series *Tuberosa* (Rydb.) Hawkes (Wild species)	
S. acroscopicum Ochoa	PI 365314, PI 365315
S. brevicaule Bitt.	PI 498110*, PI 498111*, PI 498112*, PI 498113*, PI

	498114*, PI 498115*, PI 498218, PI 545967, PI 545968, PI 545970*
S. bukasovii Juz.	PI 210042, PI 210051, PI 265876, PI 275271, PI 283074, PI 310937, PI 365304, PI 365318, PI 365321, PI 365349, PI 365350, PI 365355, PI 414155, PI 442698, PI 458379, PI 473447, PI 473450, PI 473453, PI 473491, PI 473492, PI 498219, PI 498220, PI 568932, PI 568933, PI 568939, PI 568944, PI 568949, PI 568954
S. canasense Hawkes	PI 246533, PI 283080, PI 310938, PI 310956, PI 473346, PI 473347, PI 473348
S. candolleanum Berth.	PI 498227, PI 545972, PI 568969
S. coelestipetalum Vargas	PI 473354, PI 590904
S. dolichocremastrum Bitt.	PI 498234
S. immite Dun.	PI 365330, PI 498245
S. leptophyes Bitt.	PI 283090*, PI 320340*, PI 458378, PI 473342*, PI 473343*, PI 473344*, PI 473445, PI 473451, PI 473495*, PI 545895, PI 545896, PI 545985*, PI 545986*, PI 545987, PI 545988*, PI 545990, PI 545991*, PI 545992*, PI 545993*, PI 545995
S. marinasense Vargas	PI 210040, PI 310946
S. medians Bitt.	PI 210045, PI 442703, PI 473496
S. multidissectum Hawkes	PI 210043, PI 210044, PI 210052, PI 210055, PI 473349, PI 473353, PI 498304
S. multiinterruptum Bitt.	PI 275272, PI 498267*
S. oplocense Hawkes	PI 435079*, PI 442693*, PI 458390*, PI 498067, PI 545876, PI 545908*, PI 545910*
S. pampasense Hawkes	PI 275274, PI 442697
S. sparsipilum (Bitt.) Juz. et Buk.	PI 498136*, PI 498138*, PI 498139*, PI 498140*, PI

	498305*
S. × *sucrense* Hawkes	PI 473506
S. vernei Bitt. et Wittm.	PI 458373, PI 458374, PI 473306, PI 473311, PI 500067, PI 545884*, PI 558148, PI 558151
(Cultivated species)	
S. ajanhuiri Juz. et Buk.	CIP 702677
S. curtilobum Juz. et Buk.	CIP 700273, CIP 702455
S. juzepczukii Buk.	CIP 700895
S. phureja Juz. et Buk.	CIP 703275
S. stenotomum Juz. et Buk.	CIP 701165, CIP 701985, CIP 702583, CIP 703088, CIP 703710, CIP 703808, CIP 703933, CIP 707297
S. tuberosum L. ssp. *andigena* Hawkes	PI 243363, PI 246497, PI 255508, PI 265882*, PI 281080, PI 281105, PI 292089, PI 365345, PI 473285*, PI 473391*, PI 473393*, PI 498076, PI 498294, PI 498310, PI 546017*, PI 546023, CIP 700790, CIP 703268
S. tuberosum L. ssp. *tuberosum*	CIP 703252
Series *Acaulia* Juz.	
S. acaule Bitt.	PI 210030, CIP 761143
S. albicans (Ochoa) Ochoa	PI 266381, PI 365306
Series *Longipedicellata* Buk.	
S. stoloniferum Schlechtd. et Bché.	PI 186544*, PI 195167*
Series *Demissa* Buk.	
S. demissum Lindl.	PI 175411, PI 186551, PI 498012

Table 19 Primers used in this study

Target	Primer (5'-3' sequence)	Size (bp)
Band 1	Band 1-F (GCCTATGGCTCTCATCTTCAA)	163
	Band 1-R (GGACCAGATCCAGAAGGTAACG)	
Region 1	Band 1-F11 (CGGGAGGTGGTGTACTTTCT)	527
	Band 1-R6 (ACGGCTGACTGTGTGTTTGA)	
Region 2	Band 1-F8 (AACTTGGAAGCGAAAGCTCA)	434
	Band 1-R9 (ATTGCCGATGTCCAAGTAGG)	
Region 3	Band 1-F9 (CCCTTTGTTTGAGCCCTTGT)	446
	Band 1-R3 (GCTCCCGTTTCCCACTATTT)	
GBSS	GBSS-01 (ATGGCAAGCATCACAG)	981
	GBSS-02 (CAAAACTTTAGGTGCCTC)	
β-tubulin	Tubf (ATGGATCTAGAGCCTGGTACTATG)	525
	Tubr (CAAACAGCAAGTAACACCACTC)	

Primer sequences for amplification of GBSS and β-tubulin were obtained from Takeuchi et al. (2009) and Turra et al. (2009) respectively

primers (Table 19). Thermal cycling was performed using Veriti® 96-well thermal cycler (Applied Biosystems) (one cycle of 10 min at 95 °C, followed by 35 cycles of 30 sec at 94 °C, 30 sec at 60 °C and 1 min at 72 °C, and then, terminated with one cycle of 5 min at 72 °C). PCR products were separated by electrophoresis on a 1.4% agarose gel in 1× TAE buffer (40 mM Tris-acetate and 1 mM EDTA pH 8.0), stained in 2.5 µl of Midori Green DNA Stain (Nippon Genetics Europe GmbH, Germany) per 100 ml of 1× TAE buffer for 30 min with gentle shaking, followed by de-staining in used 1× TAE buffer for 30 min with gentle shaking. Photographic images were captured over UV lamp.

For a wide survey of wild and cultivated potatoes, Region 2 of extended Band 1 was amplified by the same protocol described above except that the granule-bound starch synthase I gene (GBSS) marker (Table 19) was included at 0.3 µM concentration in the reaction as a positive control to check whether the PCR was performed correctly or not.

Southern-hybridization

Approximately 15 µg of total DNA of T and D were digested with a restriction enzyme *Msp*I, *Hin*dIII or *Eco*RI, and Southern-hybridized with a PCR product of Band 1 (primers shown in Table 19) as probe DNA by the method described earlier (Hosaka and Hanneman 1998).

Transcription analysis

Fresh leaves were sampled from T, D, TD and DT plants, quick-frozen in liquid nitrogen and ground to powder with a mortar and a pestle. Total RNA was extracted using the RNeasy® Plant Mini Kit (QIAGEN, ME, USA). To eliminate possible

contaminated genomic DNA, all RNA samples were treated for 20 min at 37°C with DNase (TURBO DNA-*free*™, Ambion, TX, USA). RNA concentration was measured by a fluorometer (QuantiFluor™-P, Promega, WI, USA) using Quanti-iT™ RiboGreen® RNA Assay Kit (Invitrogen). The reverse transcription was performed from 1 μg of total RNA primed with an oligo $(dT)_{21}$ primer using SuperScript®III First-Strand Synthesis SuperMix (Invitrogen). The synthesized cDNA was diluted to 1/10 and subjected to PCR amplification for the three regions. PCR reactions were as described above. The following thermal profile was used: 10 min at 95°C, 30 cycles of 30 sec at 95°C, 30 sec at 60°C and 1 min at 72°C, followed by a final extension step of 5 min at 72°C. To confirm the absence of genomic DNA contaminations in cDNA samples, PCR was carried out under the same condition with primers Tubf and Tubr (Table 19), which amplified a 525 bp β-tubulin gene fragment from cDNA instead of an approximately 1,600 bp fragment from genomic DNA containing an intron in potato (Turra et al. 2009).

Determination of chloroplast and mitochondrial DNA types

Their chloroplast DNA types were determined by restriction fragment length polymorphism (RFLP) analysis of chloroplast DNA as described in Sukhotu et al. (2004). Mitochondrial DNA type was determined by PCR using primers ALM_4 and ALM_5, which amplified a 2.4 kb, 1.6 kb or no band from α-, β- or γ-type mitochondrial DNA, respectively (Lössl et al. 2000); however, if a 1.6 kb band was associated with S or A-type chloroplast DNA, the mitochondrial DNA type was deduced to be ε type, based on circumstantial information from Lössl et al. (1999).

Results

Extended Band 1 sequence

The 170 bp of previously sequenced region of Band 1 was extended to a total of 1,032 bp containing 170 bp Band 1 (Fig. 8A). Homology search was carried out for the extended Band 1 sequence using the BLASTN program. However, it did not show high homology to any known sequences, even to those of the latest potato genome database, including chloroplast and mitochondrial genome sequences (carried out by courtesy of Drs. S. Huang and Z. Zhang, Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences).

Inheritance and specificity of Band 1

Using three primer sets for amplification of Region 1 to 3 (Fig. 8B), or a primer set that specifically amplified Band 1 (Table 19), D, T, 5 DT, 5 TD, 90 (DT)T and 38 (TD)T plants were examined for the presence/absence of Band 1. As shown in Fig. 9, DNA fragments with expected sizes were present in D and all DT and (DT)T plants, but not in T and all TD and (TD)T plants. By Southern-hybridization analysis, hybridization signals were obtained as single bands from all three restriction digests of D, whereas no signal was obtained from T, indicating lack of the homologous sequence to Band 1 in T (Fig. 10).

Transcription of Band 1

Leaf mRNA was extracted and cDNA was amplified for the Band 1 region (Fig. 11). Compared with a band of β-tubulin amplified from genomic DNA, only a smaller band (525 bp) was amplified from cDNA, indicating that an intron was removed and no

(A)

```
*********1*********2*********3*********4*********5*********6*********7*********8*********9*********0
CGGGAGGTGGTGTACTTTCTTCCTAACAAAGCCATAAGGATAAGGCAAGGAAGTGAAGCTAAAGGCCCGCCATTTGAGTAGCCAACTTCCTAACGAAATC
ACAGTTTGATGCTAACTACTGCAGCCCCTGCTTCTTCGGGTGGAACTCCTTGCTTTCACCTCAATCCCCCTCGTCAGGAGCTTGACAGAAGGCTACATCA
AAGAGGTAGTTCTCCCTTAGGCTAAGAGAAAGGTACGAAATGAGTTGAACATAGTGAGACTAAGGAACTATGAAACTTCAATTGGAACTGCAACCGACGA
AAATGGCTCGCAAGGAGAAAACATTAGATTAGCAATGGCTGATAAAAGGGAACCCCTTTCACCTACCTATACCTAACTTGGAAGCGAAAGCTCACTTGCA
TAATTATAAATTTTAGTTATTATTATTATTATTGTATTACTTAGCATGGTATTTCGGCCTTGTCATCTTTTTAGGGAGCCTGTGAATTCAGCCTATGGCT
CTCATCTTCAAACACACAGTCAGCCGTGGCAGCCAGGCTGTGGAGTCGTGCTTTCCTTTCTGCTGTATATATGGAATAGGGCTCAGCCCTTTGTTTGAGC
CCTTGTAAGTGTAAGAAAGAGGCTTAGTCATCGTTACCTTCTGGATCTGGTCCGGGCCAGACAGTAAAATTACTTACGGCCTATACATCTCTAACACACA
CAGTTTTTAAGGTGGGTGTATCAACCTCATCGATGATTGGACTGGCTCTCGTCGGTCTCCCTTCTCCATCTGGAGGAGAATTAAAGGGCCTACTTGGACA
TCGGCAATCAGCCTGCCTCTTGCTGGCCTTCTCGAGTTATCATAAGCGGTGATGTACACGGTAGAAGGAGCCAGAGAAATGTCAAGGGCGCGCAGTGTAG
CGTGGATAGAGGAACGAAGTCGCTCGAAGAGGTACGAAGTAGCTTTGAAGAAAGGTACGAAGTGGCTAGAGATTCGTTTCACTATGTGAAACTCATTCGG
TTTCCTTACTTTAAATAGTGGGAAACGGGAGC
```

(B)

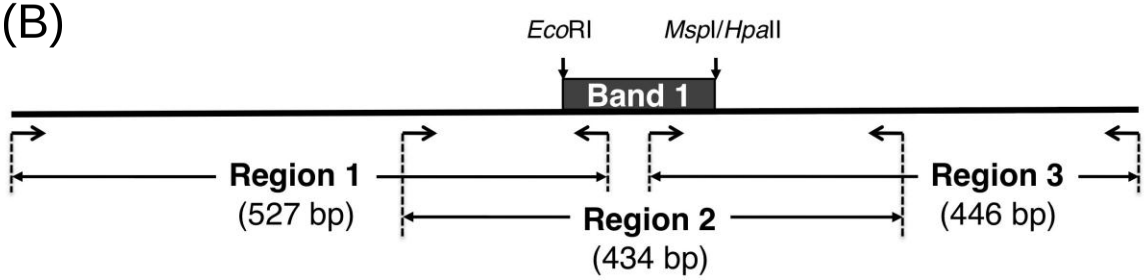

Fig. 8 A DNA fragment of 1,032 bp harboring Band 1 (underlined in A). Three pairs of primer sequences for amplification of Region 1, 2 and 3 are shown in (A) and the schematic representation in (B)

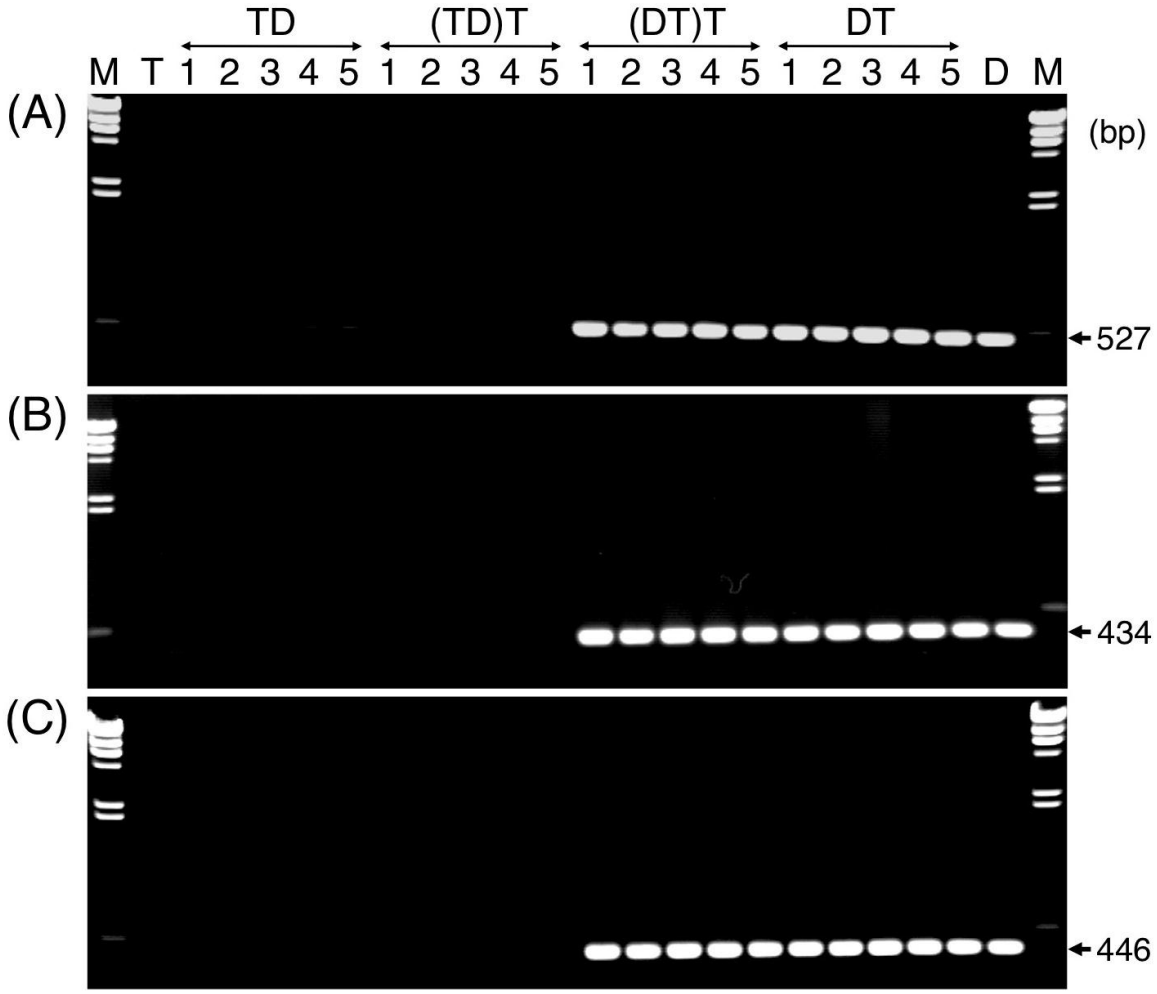

Fig. 9 Amplification of Region 1 (A), Region 2 (B) and Region 3 (C) from T, D, 5 reciprocal F_1 plants of TD and DT, and 5 BC_1 plants of (TD)T and (DT)T. M denotes λDNA *Hin*dIII digests

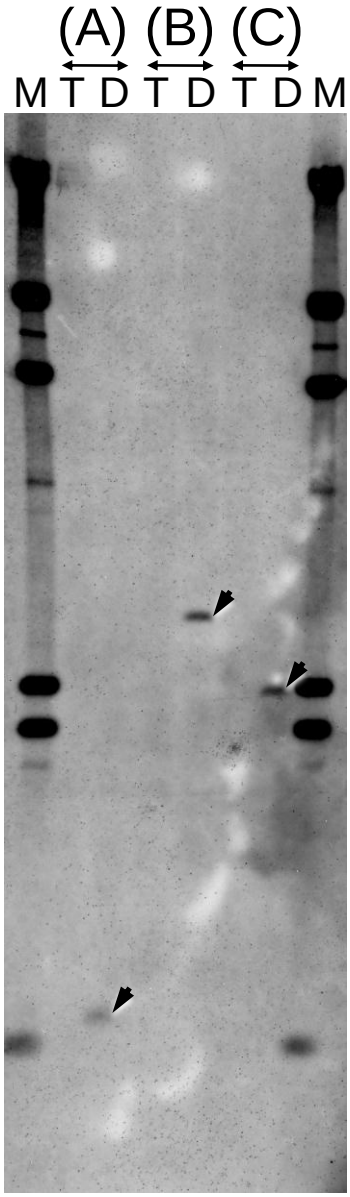

Fig. 10 Southern hybridization of *Msp*I (A), *Hin*dIII (B) and *Eco*RI (C) digests of T and D probed with Band 1. Arrows indicate hybridization signals. M denotes λDNA *Hin*dIII digests

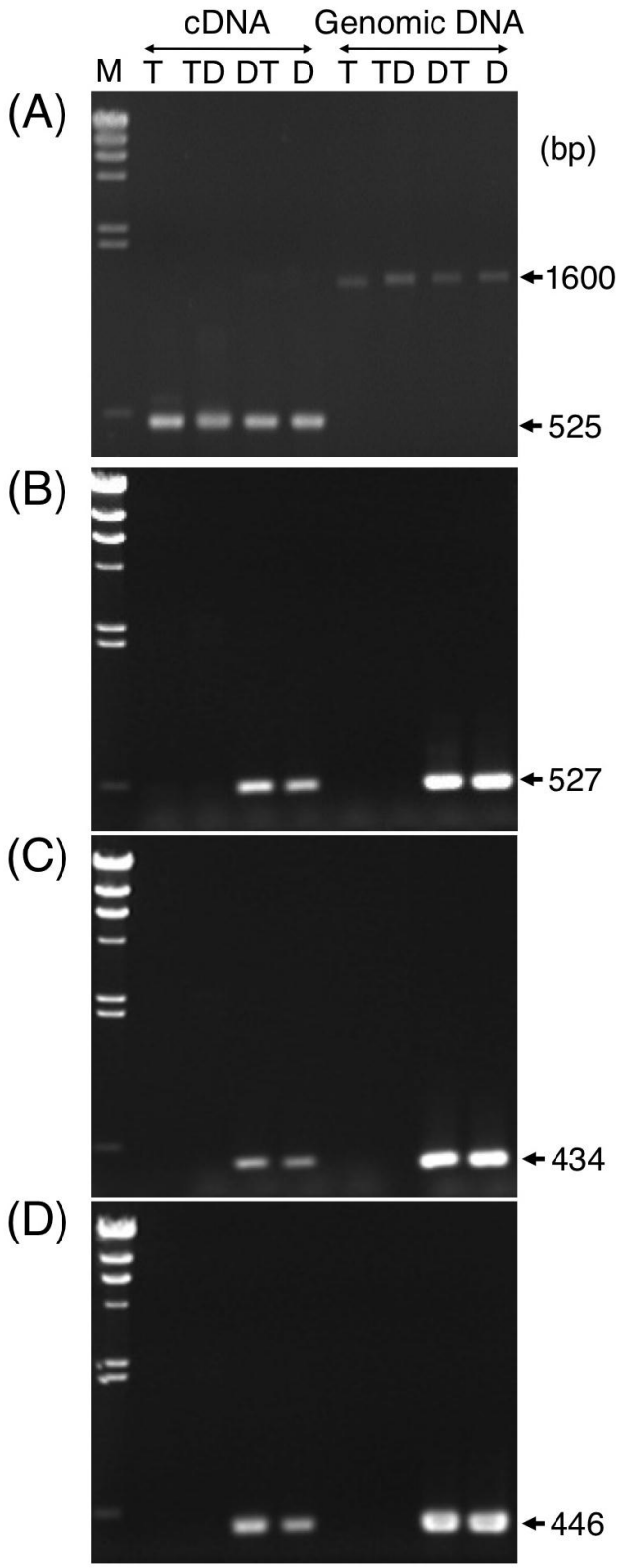

Fig. 11 Leaf cDNA and genomic DNA of T, TD, DT and D were amplified for Region 1 (B), Region 2 (C) and Region 3 (D). A β-tubulin gene amplified from leaf cDNA samples exhibited only 525 bp band (A), demonstrating no contamination with genomic DNA. M denotes λDNA *Hin*dIII digests.

genomic DNA was contaminated in cDNA samples (Fig. 11A). As shown in Fig. 11B-11D, three regions were all transcribed to mRNA in leaves of D and DT plants, but not in those of T and TD plants. The sizes of amplified bands from cDNA were similar to those from genomic DNA for all three regions. Furthermore, Region 1 PCR products from cDNA were sequenced, which showed similar sequences to that of genomic DNA. This indicates that the entire sequence of extended Band 1 is transcribed to mRNA and contains no intron.

Screening Band 1 against various cytoplasms

Band 1 was screened in three *S. demissum* accessions and 168 accessions of 38 species with various cytoplasms (Table 18). Although 43 accessions had W/α cytoplasm, none of them except three accessions of *S. demissum* (all had W/α cytoplasm) had Band 1.

Characterization of varieties and breeding lines

Cytoplasm types were determined for 158 varieties and breeding lines. One hundred and eight (68.4%) had T/β cytoplasm typical of *S. tuberosum* (Table 20). The W/α cytoplasm was found in 21 varieties and 12 breeding lines (20.9%) (Table 20). One of landraces, Nemuromurasaki, and two others had A/ε cytoplasm derived possibly from *S. tuberosum* L. ssp. *andigena* Hawkes. The S/ε cytoplasm, derived from *S. phureja*, and W/γ cytoplasm, derived from *S. stoloniferum*, were found in 12 and 2 varieties or breeding lines, respectively (Table 20). Among them, Band 1 was exclusively detected in varieties or breeding lines that had W/α cytoplasm (Fig. 12, Table 21). Band 1 was detected in Tunika and its three haploid clones (Table 21) and

Table 20 Varieties and breeding lines not possessing Band 1. Their cytoplasm types are given (^{A} = A/ε, ^{S} = S/ε, ^{W} = W/γ, no symbol = T/β)

Variety or breeding line
1. Japanese variety
Aino-aka, Aiyutaka, Beni-akari, Benimaru, Bifukabeni, Bihoro, Chitose, Dejima, Early Starch, Eniwa, Fugenmaru, Haru-akari, Haruka, Hatsufubuki, Hinomaru 1, Hokkai-aka, Hokkaikogane, Inka Gold^{S}, Inka-no-hitomi^{S}, Inka-no-mezame^{S}, Inka Purple, Inka Red, Kannanjiro, Kita-akari, Kitahime, Konafubuki, Konayuki, Myojo, Neodelicious, Nishiyutaka, Norin 1, Norin 2, North Chip, Ohotsuku-chip, Oojiro, Ranran-chip, Red Moon, Sakurafubuki, Saya-akane, Sayaka, Shimabara, Snow March, Star Ruby, Star Queen, Tachibana, Tarumae, Tokachikogane, Tokachi Pirika, Touya, Toyoshiro, Unzen, Waseshiro, White Flyer, Yellow Shark, Yukijiro, Yukirasha, Yukitsubura
2. Japanese land race
Ginzan-murasaki, Iya-imo (white), Kintoki-imo, Nagasaki-zairai B, Nemuromurasaki^{A}, Seinaiji-ki-imo
3. Foreign variety or breeding line
Agasize, Alowa, Alwara^{W}, Andover, Atlantic, Cherie, Chipeta, Cimbal's Pheonix, Corolle, Cynthia, Daisy, Desiree, Early Rose, Irish Cobbler, Jenny, Juliette, Kanona, Kennebec, Kexin No. 1, Matilda, May Queen, ND860-2^{S}, Norking Russet, Pike, Prevalent, Russet Burbank, Salem, Shepody, Snowden, Sylvia, V2^{A}, Vermont Gold Coin, Yankee Chipper
2. Breeding line
529-1, 10H15^{S}, 10H16^{S}, 10H17^{S}, Chokei 129^{S}, Chokei 131^{S}, Hokkai 50, Hokkai 56^{W}, Hokkai 82, Hokkai 87, Hoku-iku 11, Hoku-iku 14, Kitakei 31, Kon-iku 35, KW85091-21, KW85093-33, Saikai 30, Saikai 31, Saikai 34, Saikai 35^{S}, Saikai 37^{S}, Saikai 38, Shimakei 569, T05003-1^{A}, T03097-19, T09073-88, WB77025-3, WB88055-8, WB902209-1^{S}

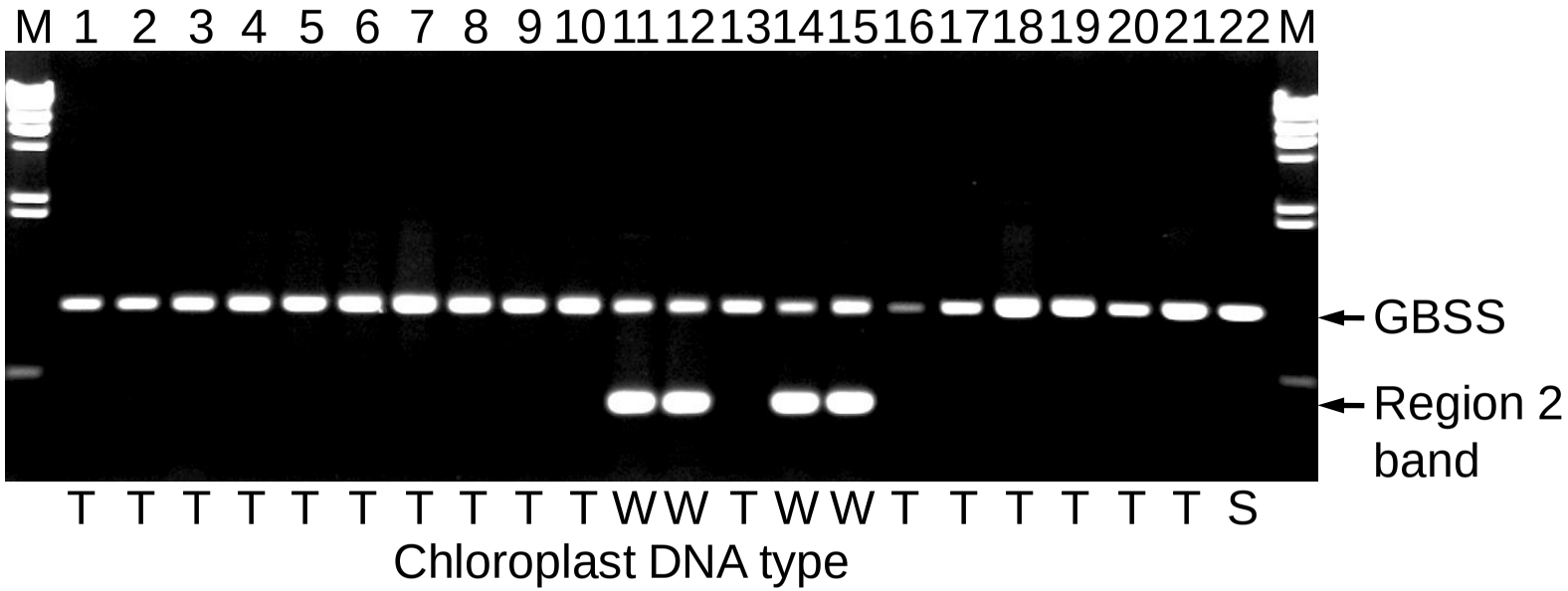

Fig. 12 Amplification of Region 2 together with GBSS as positive control from various varieties. Chloroplast DNA types are shown below. 1 Early Rose, 2 Irish Cobbler, 3 May Queen, 4 Desiree, 5 Russet Burbank, 6 Kennebec, 7 Atlantic, 8 Shepody, 9 Snowden, 10 Norking Russet, 11 Tunika, 12 Astarte, 13 Norin 1, 14 Rishiri, 15 Chijiwa, 16 Toyoshiro, 17 Nishiyutaka, 18 Hokkaikogane, 19 Konafubuki, 20 Kita-akari, 21 Touya, 22 Inca-no-mezame. M denotes λDNA *Hin*dIII digests

Table 21 Varieties and breeding lines possessing Band 1

Varieties and breeding lines	Year released	Pedigree (Female × male)	Cytoplasm	Cytoplasmic origin (No. of generations)
1. Japanese variety				
Yoraku	1958	41089-8 × Norin 1	W/α	*S. demissum* T-2 (3)
Rishiri	1960	41089-8 × Norin 1	W/α	*S. demissum* T-2 (3)
Chijiwa	1962	S54042-15 × Unzen	W/α	*S. demissum* T-2 (5)
Shiretoko	1967	Hokkai 24 × Shimakei 291	W/α	HLT-4 (2)
Setoyutaka	1977	Saikai 10 × Unzen	W/α	*S. demissum* T-2 (7)
Meihou	1986	Chijiwa × Chokei 80	W/α	*S. demissum* T-2 (6)
Toyo-akari	1986	Tunika × WB61037-4	W/α	W-race (4)
Ezo-akari	1987	Tunika × Priekulsky Ranny	W/α	W-race (4)
Musamaru	1992	Tunika × Kon-iku 20	W/α	W-race (4)
Hanashibetsu	1997	W553-4 × R392-50	W/α	W553-4 (1)
Benihisashi	2001	W862207-1 × WB72209-5	W/α	W217H.2 (2)
Natsufubuki	2003	Musamaru × Shimakei 544	W/α	W-race (5)
Kitamurasaki	2004	Shimakei 571 × Shimakei 561	W/α	Nemuromurasaki (3)
Koganemaru	2006	Musamaru × Tokachikogane	W/α	W-race (5)
Northern Ruby	2006	Open-polination from Kitamurasaki	W/α	Nemuromurasaki (4)
Shadow Queen	2006	Open-polination from Kitamurasaki	W/α	Nemuromurasaki (4)
3. Japanese breeding line				
Chokei 108	-	Musamaru × Touya	W/α	W-race (5)
Hokkai 97	-	Meihou × Tokachikogane	W/α	*S. demissum* T-2 (7)
Hoku-iku 6	-	Kon-iku 27 × Hokkaikogane	W/α	HLT-4 (6)
Kitakei 29	-	Hanashibetsu ×	W/α	W553-4 (2)

		Tokachikogane		
Saikai 39	-	Aikei 125 × Saikai 35	W/α	*S. demissum* T-2 (10)
Shimakei 284	-	Open-pollination from Nemuromurasaki	W/α	Nemuromurasaki (1)
Shimakei 561	-	Shimakei 530 × ND860-2	W/α	W-race (5)
Shimakei 571	-	Shimakei 284 × 83015-47	W/α	Nemuromurasaki (2)
W553-4	-	A possible interspecific hybrid of unknown origin	W/α	
W794215-H33	-	2x Tunika	W/α	
W794215-H34	-	2x Tunika	W/α	
W794215-H35	-	2x Tunika	W/α	
2. Foreign variety				
Tunika	1967	Lü.56.186/21 N × Lü.51.183/2	W/α	W-race (3)
Astarte	1993	SVP RR 62-5-43 × VTN 62-69-5	W/α	MPI 19268 (4)
Eva	1999	Steuben × bulk pollen hybrids	W/α	B4494-3 (3)
Monticello	2003	Steuben × Kanona	W/α	B4494-3 (3)
Sassy	2004	G82TT137.1 × Promesse	W/α	?

Band 1 was inherited maternally from *S. demissum* even after 10 times backcrossing (see Saikai 39 in Table 21).

Homology between the Band 1 sequences of different origins

PCR products amplified from Band 1 of Rishiri (the cytoplasm originally derived from *S. demissum* T-2), Hanashibetsu (from W553-4) and Kitamurasaki (from Nemuromurasaki), and those from Region 1 of Rishiri, Tunika (from W-race) and Astarte (from MPI 19268), were sequenced, which perfectly fit to the corresponding sequences of D (Fig. 8A).

Discussion

According to Lössl et al. (2000), W/α cytoplasm is specific to the *S. demissum*-derived varieties. This was true as indicated in Table 21, because the W/α cytoplasm found in varieties and breeding lines was likely all descended from *S. demissum* alone. However, W-type chloroplast DNA was defined by RFLP analysis of chloroplast DNA and found in many wild species (Hosaka 1986; Sukhotu et al. 2004; Hosaka and Sanetomo 2010). According to the raw data used in Hosaka and Sanetomo (2010), among 164 different cytoplasms distinguished in Andean cultivated potatoes and closely related species, 73 cytoplasms had W-type chloroplast DNA, while 49 cytoplasms had α-type mitochondrial DNA. Consequently, 40 cytoplasms were disclosed to be W/α type, which was found in many wild species including *S. demissum* (shown by asterisks in Table 18). Thus, the W/α cytoplasm is not specific to *S. demissum.* Alternatively, as discussed later, Band 1 was perfectly associated with the *S.*

demissum cytoplasm, so that it becomes a useful DNA marker identifying the *S. demissum* cytoplasm.

Varieties and breeding lines with W/α cytoplasm had exclusively Band 1. Their cytoplasms were descended from at least eight parents: *S. demissum* T-2, W-race and MPI 19268 (both are famous backcross progenies from *S. demissum*, Ross 1986), Nemuromurasaki, HLT-4 (from USDA), W553-4, W217H.2 and B4494-3 (Table 21). The cytoplasm of W-race was incorporated into Japanese varieties via Tunika (introduced from former East Germany in 1981), from which a potato cyst nematode resistance gene (*H1*) was introduced to several Japanese varieties (Mori et al. 2007). Shimakei 571, Kitamurasaki, Northern Ruby and Shadow Queen had Band 1. According to the pedigrees, they were maternally descended from Shimakei 284, which was developed from seedlings of an open-pollinated berry set on Nemuromurasaki (Table 21). However, the parent of Shimakei 284 was apparently misreported, because Nemuromurasaki is the oldest variety with A-type chloroplast DNA (Table 20) typical to *S. tuberosum* ssp. *andigena*, and was thought to be one of relic potatoes of the early European potato (Hosaka 1993). Although W553-4 was recorded as *S. tuberosum* ssp. *andigena* by Dr. Y. Irikura, who collected this clone in Colombia in 1977, now it has been recognized as an inter-specific hybrid of unknown origin because of extremely wide segregation observed in the progeny. As W553-4 shows a high level of late blight resistance, it might be a backcross progeny from *S. demissum*. The other maternal parents that conferred W/α cytoplasm were not able to trace their pedigrees furthermore. In conclusion, since W/α cytoplasm in varieties and breeding lines were perfectly associated with Band 1 and all were derived likely from *S. demissum*, Band 1 or an extended DNA fragment harboring Band 1 would become a precise indicator of the *S. demissum* cytoplasm.

I demonstrated that Band 1 was a *S. demissum*-specific DNA fragment and maternally inherited from *S. demissum* to *S. tuberosum* through backcrosses. However, Band 1 did not show high homology to any known sequences including complete sequences of potato chloroplast DNA (Chung et al. 2006) and mitochondrial DNA of the related genus *Nicotiana* (Sugiyama et al. 2005). It is generally known that plant chloroplast DNA evolves very slowly and is highly conservative in size, structure, gene content, and linear order of genes (Palmer et al. 1988; Downie and Palmer 1992). Band 1 or Region 1 sequences, maternally inherited through many generations from different source accessions of *S. demissum*, were all similar to those of *S. demissum* used in this study, demonstrating highly conservative nature of Band 1. However, *S. demissum*-specific insertion with a size of over 1 kbp was not detected in the chloroplast DNA (Hosaka 1986), so that it is less likely that Band 1 is a part of chloroplast DNA. By comparison among several completely sequenced angiosperm mitochondrial DNAs, it is known that, although identified genes and introns are rather well conserved, intergenic regions are highly variable in sequence, even between two close relatives (Handa 2003; Kubo and Mikami 2007; Alverson et al. 2010). Therefore, it is highly probable that Band 1 is a part of mitochondrial DNA of *S. demissum*. However, mRNAs with poly(A) tails from chloroplast and mitochondrial genes are generally found only in degradation and are thus expected only at a minor fraction of the steady state pool (Forner et al. 2007; del Campo 2009). Thus, alternative possibilities would be that Band 1 is 1) located on one of *S. demissum* chromosomes and exclusively transmitted maternally, or 2) something else such as plasmid-like DNA maternally inherited. Linear or circular plasmids have been frequently reported in mitochondria in higher plants (Turpen et al. 1987; Handa et al. 2002, Allen et al. 2007). Further investigation on the intra-cellular origin of Band 1 is necessary.

Conclusion

In this study, the UI between *S. demissum* and *S. tuberosum* was reconfirmed and disclosed as a quantitative trait, and the possible cause was concluded to be a post-zygotic failure of seed formation by the imbalance of EBN between the two species (*S. tuberosum* EBN > *S. demissum* EBN).

The most important observation in this study is that reciprocal interspecific F_1 hybrids of *S. tuberosum* × *S. demissum* showed differential crossability. Irrespective of being crossed as male or female, F_1 and BC_1 progenies with the *S. demissum* cytoplasm always showed lower berry-setting rates than those with the *S. tuberosum* cytoplasm (Table 4). Thus, the cytoplasmic contribution is unambiguous. Although the reciprocal F_1 hybrids share identical nuclear genetic backgrounds, at least six distinct DNA bands were different between the reciprocal hybrids (Table 9). As expected, one difference possibly occurred in chloroplast DNA and another one in mitochondrial DNA. Two others including Band 1 were also inherited maternally (Table 8). These maternally inherited DNA fragments or DNA methylation might contribute to the crossability.

It is easy to suspect organelle DNA contributing by some way to female germ line because maternal organelle DNA is included into the egg and central cells. However, as generally recognized, paternal organelle DNA is not delivered with sperm cells into egg cell nor central cell. Thus, it can be hardly understood why the cytoplasmic information from *S. demissum* delivered via pollen to central cell and resulted in lower berry-setting rates. Sperm nuclei in pollen are modified differentially by DNA methylation or histone modification from vegetative nucleus during pollen maturation

(Gutiérrez-Marcos et al. 2006; Okada et al. 2006; Singh et al. 2008; Ribeiro et al. 2009). Borges et al. (2008) carried out transcriptome analysis of *Arabidopsis* sperm cells at a genome-wide level and showed that sperm had a distinct and diverse transcriptional profile. Ning et al. (2006) analyzed gene expression profiles of tobacco eggs, zygotes and sperm cells, and found specific mRNAs to both sperm cells and zygotes, although it has not been confirmed that these mRNAs in the zygote are transcribed *de novo* or transmitted by fertilization with sperm cells. Recently, Slotkin et al. (2009) demonstrated that small RNA was accumulated in sperm cells likely generated from reactivated *Athila* transposable element transcripts from the vegetative nucleus in *Arabidopsis thaliana* pollen. Thus, these implied that some proteins or RNA other than DNA could be delivered into the central cell and function in the endosperm development. This implication is now enforced by the transcript analysis of pollen mRNA from reciprocal hybrids. Drastic differences between the reciprocal hybrids were found in 14.2% of pollen transcripts as revealed by a genome-wide survey using a high-throughput sequencer (Table 11). Therefore, pollen of reciprocal hybrids, though carrying the same nuclear genetic information, contains qualitatively and quantitatively different transcripts, some of which might be delivered to central cell and functioned differently in endosperm development.

In conclusion, reciprocal differences observed in interspecific F_1 hybrids would be attributed to the cytoplasmic effect. Differential expression of cytoplasmic genes, or more likely by interactions between cytoplasmic genes of *S. demissum* and the nuclear genes of the hybrids contributed to the crossability, resulting in reduced berry-setting rates in all crosses with *S. demissum* cytoplasm. Cytoplasmic male sterility, caused by interaction between nuclear and mitochondrial genes (Grun 1979; Chase 2006), could be a typical case for organelle DNA contributing to crossability.

The cytoplasmic contribution of *S. demissum* to crossability is supported by a general and empirical recognition that parental lines with the *S. demissum* cytoplasm produce abundant, but non- or less functional pollen. We often tend to use genotypes producing abundant pollen as pollen parents. However, if a desirable genotype produces abundant pollen and has *S. demissum*-derived cytoplasm, we should not use it as male to avoid a difficulty in obtaining hybrid seeds. Thus, it is very important to identify the *S. demissum*-derived cytoplasm in breeding programs. In the past, a set of PCR primers flanking a 241 bp deletion that defined T-type chloroplast DNA (Hosaka et al. 1988; Kawagoe and Kikuta 1991) was developed (Lössl et al. 2000; Hosaka 2002), which has been used frequently worldwide for various purposes (Gavrilenko et al. 2007; Spooner et al. 2007; Ames and Spooner 2008; Chimote et al. 2008). I found that Band 1 is a useful PCR marker for precise identification of the *S. demissum*-derived cytoplasm (Chapter IV). Therefore, the *S. tuberosum* cytoplasm and the *S. demissum*-derived cytoplasm, the two most prevalent cytoplasms in the common potato, can now be accurately identified, which promotes to design efficient mating combinations in breeding programs.

Summary

Interspecific crosses frequently fail, or lead to abnormalities in the growth of hybrid seeds. Recent studies at molecular levels in model plants indicated that imprinted genes and a balance between maternally contributed Polycomb repressive complex 2 proteins and paternally contributed AGAMOUS-LIKE Type-1 MADS domain transcription factors play important roles for seed development in interspecific or inter-ploidy crosses. While in potato, a conceptual explanation, known as the Endosperm Balance Number (EBN) hypothesis (Johnston et al. 1980) can foresee success or failure of a given interspecific cross, although the underlying molecular basis is poorly studied. To elucidate the seed formation mechanism in interspecific crosses of potato, I investigated the unilateral incompatibility (UI) between the common potato *Solanum tuberosum* L. (2*n*=4*x*=48) and a hexaploid Mexican wild species *S. demissum* Lindl. (2*n*=6*x*=72) in this study.

(1) Four hundred and eighty-eight pollinations on 110 plants of *S. demissum* (25 accessions) with pollen of *S. tuberosum* (a breeding line Saikai 35, referred to as T) produced 395 berries with the mean berry-setting rate of 81.2%, while the reciprocal crosses on 232 flowers of T with *S. demissum* pollen produced only 24 berries, resulting in the 18.7% berry-setting rate. This indicated that the UI was clearly confirmed between the two species. Furthermore, the *S. demissum* × T crosses produced the significantly lower number of seeds per berry (34.0) and the heavier mean seed weight (0.94 mg) than the reciprocal crosses (113.2 and 0.39 mg, respectively). Based on the EBN hypothesis, a slight excess of maternal dosage will produce small seeds, while a

slight excess of paternal dosage will produce large seeds. Early seed abortion would likely drop berries, lowering the berry-setting rate. Thus, it is concluded that the UI was caused as a post-zygotic barrier by the imbalance of EBN between the two species, *S. demissum* having a slightly lower EBN than *S. tuberosum*.

The interspecific F_1 hybrid DT from *S. demissum* (PI 186551 and the selfed progeny, collectively referred to as D) as female × T as male and the reciprocal TD hybrid (T female × D male), and their BC_1 plants from (TD) × T, (DT) × T, (TD) × D and (DT) × D were selfed, sib-crossed, and backcrossed with both T and D. Based on the observation of the berry-setting rates, seeds/berry, seed size and pollen tube growth, I found at least three factors involved in these crosses for normal seed formation: 1) a cytoplasmic factor, and nuclear genome-encoded factors functioned 2) in female gametophyte and 3) in pollen. Among these factors, the cytoplasmic factor, or a maternally inherited factor was prominent. Irrespective of being crossed as male or female, F_1 and BC_1 progenies with T cytoplasm always showed higher berry-setting rates (the average of 2.04 times) than those with D cytoplasm. Thus, D cytoplasm always suppressed the hybrid seed formation.

(2) Reciprocal differences in crossability were found between TD and DT. Particularly when TD and DT were crossed as pollen parents onto D, a significantly lower berry-setting rate was obtained in DT (24.2%) compared with TD (64.9%). To reveal this reciprocal differences, DNA sequences and methylation status of pollen DNA were compared between TD and DT using methylation-sensitive amplified polymorphism (MSAP) analysis. Using 126 primer combinations, 23,527 and 23,525 bands were amplified from bulked pollen DNA samples of TD and DT, respectively, and 23,530 and 23,533 bands from bulked leaf DNA samples, respectively. The

methylation rates of pollen and leaf DNA were almost similar to each other (8.85-8.88% and 8.83-8.87%, respectively), suggesting that the sporophyte and the male gametophyte DNA are methylated to the same degree at least in a genome-wide view.

TD and DT bulked pollen DNA samples showed almost the same MSAP banding patterns and the same methylation rates (8.88% in TD and 8.85% in DT, respectively). Yet, 57 bands from 43 primer pairs revealed differences between TD and DT bulked pollen DNA samples. Individual examination of 16 TD and 9 DT plants disclosed at least six distinct DNA bands uniformly different between TD and DT. As expected, one band shared high homology with chloroplast DNA, and another one with mitochondrial DNA. However, one band that was apparently different at DNA sequence level and maternally transmitted from D showed no homology with any known sequence (named Band 1). The remaining three bands were DNA methylation level differences with no or uncertain homology to known sequences. To our knowledge, this is the first report detecting reciprocal differences in DNA sequence or DNA methylation other than those in cytoplasmic DNA.

(3) A genome-wide transcript analysis was performed to the pollen mRNA of T, D, TD and DT using a high-throughput sequencer in order to disclose interspecific reciprocal differences at a transcription level. A total of 3,035,230 - 3,204,206 kilo base (72.5 - 80.3 %) sequences per sample were generated by a single run of 75 cycles using Illumina Genome Analyzer IIx, which resulted in a total of 13,020 transcripts of 9,366 loci. 7,008 (53.8%) showed significant transcription level differences between T and D, and 1,847 (14.2%) between TD and DT. 52.5% of 1,847 transcripts showed intermediate RPKM values (indicating normalized transcription levels) between or near parental ones, which were likely resulted by additive or dominance/recessive genetic

effects. 34.7% of 1,847 transcripts exhibited over- or under-transcription in the hybrids compared with parental transcription levels. Particularly, TD over-transcription was prominent (19.9%). For the top 55 loci showing highly differential transcription profiles, the parental origins of transcript variants were determined. Each locus was composed of 3.1 transcript variants with at least 0.93 T-derived and 1.36 D-derived transcript variants. 58.7% of parent-specified transcripts showed intra-locus differential transcription, among which only five D-derived transcripts contributed to over-transcription between reciprocal hybrids and these also exhibited relatively high read numbers in D parent. Thus, these five D-derived transcripts were not transcribed in a parent-of-origin manner but in an allelic bias manner.

(4) A novel DNA fragment was found and named Band 1, which was originally detected as a 170-bp DNA fragment in DT hybrids and the maternal parent D. The region harboring Band 1 was further sequenced up to 1,032 bp. Nevertheless, it did not show any homology to known sequences. The extended region harboring Band 1 was divided into three overlapping regions (Region 1-3), all of which were amplified from D, 5 DT and 90 (DT)T plants, but not from T, 5 TD and 38 (TD)T plants. This indicated that these regions were maternally inherited from D to F_1 and BC_1 populations. In addition, these regions were all transcribed to mRNA in leaves of D and DT plants, but not in those of T and TD plants. The sizes of amplified bands from cDNA were similar to those from genomic DNA, indicating that the extended Band 1 sequence is a part of an expressed gene and contains no intron at least within the sequenced region. Band 1 was screened in three *S. demissum* accessions and 168 accessions of 38 species with various cytoplasms. Although 43 accessions had W-type chloroplast DNA and

α-type mitochondrial DNA (W/α cytoplasm), none of them except three accessions of *S. demissum* (all had W/α cytoplasm) amplified Band 1. One hundred fifty-eight varieties and breeding lines were characterized for chloroplast and mitochondrial DNA types and for the presence of Band 1. The W/α cytoplasm was found in 21varieties and 12 breeding lines which exclusively amplified Band 1. According to the available pedigree information, these W/α cytoplasms found in varieties and breeding lines were all descended from *S. demissum*. Therefore, Band 1 was perfectly associated with the *S. demissum*-derived W/α cytoplasm.

The interspecific F_1 hybrids between *S. demissum* and *S. tuberosum* and their derived BC_1 progenies with *S. demissum* cytoplasm resulted in reduced berry-setting rates in all crosses, irrespective of being crossed as male or female. Although the interspecific F_1 hybrids possess identical nuclear genetic backgrounds, they exhibited reciprocal differences in at least six distinct DNA bands and in 14.2% of transcripts. Therefore, I suggest that the cytoplasmic contribution is unambiguous in interspecific hybrid seed formation, although differential gene expression of chloroplast or mitochondrial genes was not evident. Band 1 can serve as an indicator of the *S. demissum* cytoplasm, which would help breeders to design efficient mating combinations in breeding programs.

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