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ARTICLE

Cross-incompatibility and self-incompatibility: unrelated phenomena in wild and cultivated potatoes?

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Abstract: Knowledge of internal hybridization barriers is relevant for germplasm conservation and utilization. The two pre-zygotic barriers are pollen–pistil self-incompatibility (SI) and cross-incompatibility (CI). To ascertain whether SI and CI were phenotypically related phenomena in potatoes, extensive intra- and interspecific, both intra- and interploidy breeding relationships were established, without previous assumptions on the compatibility behavior of the studied germplasm. Pollen–pistil relationships were analyzed at the individual genotype/accession/family level. In two seasons, 828 intra- and interspecific genotypic combinations were performed, using accessions of the wild potatoes *Solanum chacoense* Bitter ($2n = 2x = 24$), *S. gourlayi* Hawkes ($2n = 2x = 24$; $2n = 4x = 48$), and *S. spegazzinii* Bitter ($2n = 2x = 24$), full-sibling (hereinafter "full-sib") families ($2n = 2x = 24$) within/between the latter two diploids, and *S. tuberosum* L. ($2n = 4x = 48$) cultivars. Pollen–pistil incompatibility occurred in the upper first third of the style ($I_{1/3}$) in all selfed diploids. In both the intra- and interspecific combinations, the most frequent relationship was compatibility, followed by $I_{1/3}$, but incompatibility also occurred in the stigma and the style (middle third and bottom third). We observed segregation for these relationships in full-sib families, and unilateral and bilateral incompatibility in reciprocal crosses between functional SI genotypes. Cross-incompatibility in potatoes is, apparently, controlled by genes independent of the *S*-locus or its *S*-haplotype recognition region (although molecular evidence is needed to confirm it), with segregation even within accessions.

Key words: hybridization barriers, pollen-pistil relationships, cross-incompatibility, self-incompatibility, wild and cultivated potatoes.

Résumé : La connaissance des barrières internes à l'hybridation est importante pour la conservation et l'utilisation de germoplasmes. Les deux barrières pré-zygotiques sont l'auto-incompatibilité (AI) et l'incompatibilité croisée (IC) pollen–pistil. Afin de déterminer si l'AI et la CI constituent des phénomènes phénotypiquement reliés chez la pomme de terre, les relations de croisements intra- et interspécifiques, intra- et interploïdes ont été exhaustivement établies, sans présupposer de la compatibilité des germoplasmes étudiés. Les relations pollen–pistil ont été analysées au niveau individuel du génotype/accession/famille. Au cours de deux saisons, 828 combinaisons génotypiques intra- et interspécifiques ont été réalisées, en utilisant les accessions de pomme de terre sauvage *Solanum chacoense* Bitter ($2n = 2x = 24$), *S. gourlayi* Hawkes ($2n = 2x = 24$; $2n = 4x = 48$) et *S. spegazzinii* Bitter ($2n = 2x = 24$), les familles pleins germains ($2n = 2x = 24$) à l'intérieur ou entre les deux derniers diploïdes, et des cultivars de *S. tuberosum* L. ($2n = 4x = 48$). Une incompatibilité pollen–pistil survenait dans le premier tiers supérieur du style ($I_{1/3}$) chez tous les diploïdes S1. La relation la plus fréquente chez les combinaisons intra- et interspécifiques était la compatibilité, suivie par $I_{1/3}$, mais l'incompatibilité survenait aussi dans le stigmate et le style (tiers du milieu et dernier tiers). Les auteurs ont observé une ségrégation pour ces relations chez les familles pleins germains, et une incompatibilité unilatérale et bilatérale chez les croisements réciproques entre des génotypes fonctionnels AI. L'incompatibilité croisée chez la pomme de terre est apparemment contrôlée par les gènes de manière indépendante de la région de reconnaissance du locus *S* ou son haplotype *S* (même si des données

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moléculaires sont requises pour le confirmer), avec une ségrégation même à l'intérieur des accessions. [Traduit par la Rédaction]

Mots-clés : barrières d'hybridation, parentés pollen–pistil, incompatibilité croisée, auto-incompatibilité, pommes de terre sauvages et cultivées.

Introduction

The two species concepts most frequently used in plant and animal taxonomy are the Taxonomic Species Concept (TSC) and the Biological Species Concept (BSC), which are based, respectively, on morphological phenotypes and breeding relationships (Grant 1981). Higher plants are usually classified according to the TSC, by comparing the morphological phenotypes of individual plants with holotypes (physical samples or illustrations of complete, or part of, single specimens that were used when the species was described for the first time) or other types when holotypes are not available (McNeill et al. 2012). Molecular characterization is sometimes considered in addition to morphology (Soltis and Soltis 1995).

Under the TSC, taxonomists artificially establish limits between species based exclusively on phenotype, with an underlying assumption that "species" are at the end of their evolutionary process and, therefore, are static and invariable over time. According to the BSC, however, species are groups of actually or potentially interbreeding natural populations that are reproductively isolated from other such groups (Mayr 1963). The BSC provides a good approximation to the actual situation in nature, even though asexual reproduction is excluded and limits between species are not easy to establish. It is also of greater value than the TSC for devising breeding strategies aimed at increasing the genetic variability of crop species through the introgression of desirable traits from their wild relatives (crop wild relatives, CWR).

In higher plants, reproductive isolation is the consequence of the action of several breeding barriers. These can be either external (e.g., geographical, temporal, mechanical, among others) or internal. The latter, which are expressed in the plant tissues themselves, can be either pre-zygotic or post-zygotic, depending on whether they occur before or after fertilization (Hadley and Openshaw 1980; see Camadro et al. 2004 for examples in potatoes). The two pre-zygotic hybridization barriers that act at the pollen–pistil level, preventing pollen tubes from reaching the eggs, are self-incompatibility (SI) and cross-incompatibility (CI). The former promotes allogamy and the second establishes limits to gene flow, which could eventually lead to (or reinforce) speciation (de Nettancourt 2001).

Self-incompatibility

In plants with SI systems, inbreeding is restricted by self-discrimination between pollen and pistil, in a process genetically regulated by one or more highly polymorphic loci (de Nettancourt 2001). The *S*-locus is the most extensively studied because it is present in valuable species of the Rosaceae, Solanaceae, and Gramineae (see de Nettancourt 2001). The *S*-locus reactions are classified as either sporophytic (SSI) or gametophytic (GSI), depending on whether the *S*-haplotype expressed by a pollen grain is determined, respectively, by the genotype of the pollen parent or by its own genotype (de Nettancourt 2001). GSI occurs in potatoes, which belong to the Solanaceae, where the *S*-locus is complex and includes tightly-linked genes: one *S-RNAse* and multiple *SLF* (see Entani et al. 2014).The incompatibility reaction occurs when pistils and pollen in a given genotypic combination co-express the same *S*-haplotype.

In the GSI system, pollen germination occurs normally, but pollen tube growth is arrested mainly in the upper portion of the style (Lush and Clarke 1997; see de Nettancourt 2001; Hayes et al. 2005; Covey et al. 2010). The GSI reaction can be broken down by mutations in the SI genes themselves (see de Nettancourt 2001), the action of genes like *Sli* (*S*-locus *i*nhibitor) (Hosaka and Hanneman 1998), and (or) by the phenomenon of "competitive interaction" in diploid (or higher ploidy) pollen grains carrying two (or more) different *S*-haplotypes, which explains the ability of polyploids with GSI to produce seed after selfing (de Nettancourt 2001).

Cross-incompatibility

CI has been defined as any relationship (or its absence) between pollen and pistil that prevents hybrid zygote formation in crosses between two fertile species (de Nettancourt 2001). This phenomenon has been proposed to occur from a lack of genetic information in one of the partners of a given genotypic combination about either the structure or the physiology of the other partner (Hogenboom 1973), or for pistil recognition of pollen with a foreign origin (Kermicle and Evans 2010). CI can be either unilateral or bilateral, depending on whether a given genotypic combination is, respectively, incompatible in only one direction or in both directions of the cross. Arrest of pollen tube growth in CI can occur at various sites of the pistil (stigma; upper, middle or bottom part of the style) or in the ovary, as observed in various members of the Solanaceae family: wild potatoes (Camadro and Peloquin 1981; Hayes et al. 2005), wild tomatoes (Baek et al. 2015), and peppers (Onus and Pickersgill 2004).

The "SI × SC" rule

Most CI studies have been focused on the unilateral type and designed by taking into consideration Lewis and Crowe's (1958) SI × SC rule, under the assumption that the *S*-locus is involved in the CI reaction (see Liedl et al. 1996; Covey et al. 2010; Li and Chetelat 2010; Tovar-Méndez et al. 2014; Baek et al. 2015). According to this rule, pollen–pistil relationships between SI and SC individual plants/species/populations are dependent on the crossing direction, being incompatible in the SI × SC direction but compatible in the reciprocal cross. In tomatoes and tobacco (Solanaceae), evidence for the involvement of S-RNases in pollen rejection has been provided under this crossing scheme (Murfett et al. 1996; McClure et al. 2000; Cruz-Garcia et al. 2003; Covey et al. 2010; Tovar-Méndez et al. 2014; Baek et al. 2015). The success of interspecific crosses in various plant groups can be explained by the SI × SC rule. However, there are many exceptions, such as the finding of compatibility in SI × SC crosses (see Eijlander et al. 2000), and incompatibility in other SC–SI combinations (Hermsen and Ramanna 1976; Camadro and Peloquin 1981; de Nettancourt 2001; Hayes et al. 2005; Baek et al. 2015). The SI × SC rule fails to explain bilateral CI; moreover, CI has been reported in allogamous species without a GSI *S*-locus system, such as maize (Kermicle and Evans 2005, 2010) and carrot (Ibañez and Camadro 2015), and in a dioecious species, garden asparagus (Marcellán and Camadro 1996).

Potatoes

The common potato, *Solanum tuberosum* subsp. *tuberosum* L. (tbr[1], $2n = 4x = 48$), has a narrow genetic base but approximately 100 (Spooner et al. 2014) to 200 (Hawkes 1990) CWR, with a wide geographical and ecological distribution in the American continent. These species form a ploidy series with $2n = 2x$, $3x$, $4x$, $5x$, and $6x$ ($x = 12$) (Hawkes 1990). Owing to their adaptation to a range of habitats, wild potatoes are an important source of genetic diversity for cultivar breeding by conventional crossing methods (Jansky et al. 2013), because genome differentiation in the group is scarce (Matsubayashi 1991).

Samples of cultivated potatoes and their CWR are available at germplasm banks, where they are conserved and multiplied as accessions, usually under taxonomic species status (Jansky et al. 2013). Ex-situ multiplication by sexual reproduction is conducted under the assumption that plants within an accession can be freely intercrossed. However, SI and CI can either hinder or prevent hybridization, not only between accessions but also within accessions (see Camadro et al. 2004).

Wild and cultivated potatoes have a multialellic *S*-locus GSI system. Diploid cytotypes are obligate outcrossers (unless they carry rare self-compatibility alleles), polysomic polyploids are outcrossers that can self-pollinate (due to competitive interaction), and disomic polyploids are autogamous, apparently because competitive interaction occurs between *S*-haplotypes of the different genomes (Camadro et al. 1992), in a manner resembling Mac Key's (1970) fixed heterozygosity concept in polyploid wheats.

Knowledge of internal hybridization barriers is relevant for devising germplasm conservation and utilization strategies in crops. Whereas SI has been studied extensively, much remains to be known about CI. Thus, the objectives of this work were to (*i*) detect the presence of pre-zygotic breeding barriers in wild and cultivated potatoes in an extensive network of breeding relationships, without previous assumptions on the SI or SC behavior of the plant materials; (*ii*) discern whether the incompatible reactions at the phenotypic level could be attributed to the action of the *S*-locus or other (as yet unknown) locus (loci); (*iii*) select genotypic combinations for conducting future studies on the molecular basis of the CI phenomena in this plant group. Herein we report the results of controlled crosses, both intra- and interspecific (according to the current taxonomic status of the plant materials), and intra- and interploidy, which were analyzed at the level of individual genotypes/accessions/families, to understand the relationship between the two pre-zygotic reproductive barriers at the phenotypic level.

Materials and methods

Plant materials

Crossing was conducted in two seasons. In the first season (season 1: summer, December 2006 to March 2007), plants were obtained from both botanical seeds (one plant = one genotype) of the taxonomic species *S. gourlayi* Hawkes (grl[1]; $2n = 2x = 24$ and $2n = 4x = 48$) and *S. spegazzinii* Bitter (spg[1]; $2n = 2x = 24$), provided by the Potato Germplasm Bank of E.E.A. Balcarce, INTA, Argentina, and tubers of five commercial cultivars (one cultivar = one cloned genotype) of *S. tuberosum* subsp. *tuberosum* L. (tbr[1]; $2n = 4x = 48$). In the second season (season 2: summer 2009/2010) plants were obtained from botanical seeds of (*a*) two accessions of the taxonomic species *S. chacoense* Bitter (chc[1]; $2n = 2x = 24$) and (*b*) full-sib families obtained in season 1 from compatible intra- and interspecific crosses between individual plants of grl and spg accessions, and from tubers of three commercial tbr cultivars (Table 1). Ten or more plants were used per accession, when possible, in each season. Individual genotypes were identified according to the denomination of the accession or family to which they belong, followed by a dot and a number, e.g., "OL4911.19" corresponds to genotype 19 of accession OL4911, and "(OL4911.19 × Oka5649.16).4" corresponds to genotype 4 of the intraspecific (OL4911.19 × Oka5649.16) family.

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[1] Abbreviations according to Simmonds (1963).

Table 1 (A and B). Accessions, full-sibling families, and commercial cultivars used in crosses in season 1 (summer 2007/2008) and season 2 (summer 2009/2010), with taxonomic status, ploidy, and geographic origin of accessions.

Genetic Material							
Species	Ploidy[a]	Denomination	Plants (n)	Location in Argentina	Geographic coordinates		Elevation (m a.s.l.)
A. Season 1							
Accession							
grl	2x	ORHL4841	17	La Poma, Salta	24°33′S	66°12′W	3900
		Oka7518	3	San Carlos, Salta	25°20′S	65°53′W	3500
		OL4858	20	Rosario de Lerma, Salta	24°20′S	66°05′W	3500
	4x	Oka7547	20	Tambalaya, Jujuy	23°36′S	65°35′W	3300
		Oka7558	12	San Salvador de Jujuy, Jujuy	23°58′S	65°38′W	3200
		Oka7565	19	San Salvador de Jujuy, Jujuy	23°58′S	65°38′W	3500
		Oka7588	15	Tilcara, Jujuy	23°30′S	65°27′W	2700
		Oka7594	17	Humahuaca, Jujuy	23°10′S	65°11′W	3500
spg	2x	OL4911	27	Chicoana, Salta	25°10′S	65°52′W	3340
		OL4916	11	Chicoana, Salta	25°10′S	65°52′W	3300
		Oka5649	17	Chicoana, Salta	25°10′S	65°52′W	3000
		Oka5662	27	La Poma, Salta	24°49′S	66°10′W	2620
		Oka6147	27	Belén, Catamarca	27°29′S	67°05′W	1920
Cultivar							
tbr	4x	Calén INTA	—	—	—	—	—
		Kennebec	—	—	—	—	—
		Pampeana INTA	—	—	—	—	—
		Serrana INTA	—	—	—	—	—
		Shepody	—	—	—	—	—
B. Season 2							
Accession							
chc	2x	APEC1	13	Balcarce, Buenos Aires	37°45′S	58°17′W	128
		HHR4039	10	Castelar, Buenos Aires	34°40′S	58°40′W	20
Full-sibling family							
grl	2x	ORHL4841.9×Oka7518.5	11	—	—	—	—
		OL4858.13×ORHL4841.16	10	—	—	—	—
		ORHL4841.14×OL4858.17	6	—	—	—	—
		OL4858.13×Oka7518.3	23	—	—	—	—
spg	2x	OL4911.19×Oka5649.16	15	—	—	—	—
		OL4911.2×OL4911.5	12	—	—	—	—
		OL4911.19×OL4916.7	16	—	—	—	—
		OL4911.18×Oka5649.12	10	—	—	—	—
spg×grl	2x	OL4916.13×ORHL4841.2	6	—	—	—	—
grl×spg	2x	ORHL4841.3×OL4916.12	5	—	—	—	—
		ORHL4841.2×OL4916.13	7	—	—	—	—
Cultivar							
tbr	4x	Calén INTA	—	—	—	—	—
		Pampeana INTA	—	—	—	—	—
		Huinkul MAG	—	—	—	—	—

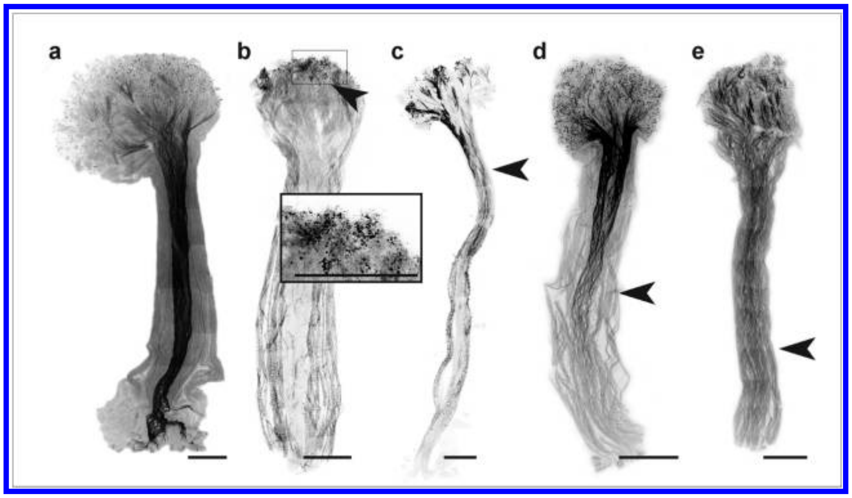
Note: —, not applicable; grl, *Solanum gourlayi*; spg, *S. spegazzinii*; chc, *S. chacoense*; tbr, *S. tuberosum* subsp. *tuberosum*.

[a] x = 12.

Crossing procedures

Seeds were placed in a gibberellic acid (GA_3) solution (1500 ppm) overnight and then sown in Petri dishes for germination. At the 2–3 leaf stage, the plantlets were transplanted into pots in a screenhouse, where they were grown under similar management practices for performing the crossing work. At flowering, and depending on the number of available flower buds, each plant to be used as a pistillate parent was crossed to one or more plants (pollen parent) to generate various genotypic combinations. Only buds emasculated one day before anthesis were pollinated early in the morning, either with fresh pollen (if available) or with pollen stored at 4 °C (viability ≥ 60%), following an incomplete diallel mating design, because the number of flower buds per genotype was limited and there were differences in flowering time among the genotypes. The genotypic combinations were intraspecific and interspecific, classified according to the current status of the progenitors for ex-situ conservation in the germplasm bank. They were performed

Fig. 1. Examples of pollen–pistil relationships in wild and cultivated potatoes: compatibility (*a*) and incompatibility at stigma (*b*), top (*c*), middle (*d*) and bottom third (*e*) of the style. Pollen tubes in black; black arrows indicate the site of arrest of pollen tube growth. Scale bars = 0.1 cm.



under similar environmental conditions, both within and between families or accessions; whenever possible, reciprocal crosses were also generated. To study pollen–pistil relationships, at least three pistils were pollinated for each genotypic combination on the same day, when possible, then fixed in FAA (1:1:8 *v*/*v*/*v*, formalin – glacial acetic acid – 96% ethanol) 48 h after pollination.

Pollen–pistil relationships

Fixed pistils were processed and stained with a solution of 0.1% aniline blue in 0.1 N K_3PO_4, following the methods of Martin (1959), mounted with a drop of glycerol on a glass slide, gently squashed with a cover slip, and observed under an optical microscope with UV light. Pollen–pistil relationships were classified as either compatible or incompatible depending, respectively, on whether pollen tubes reached the ovary or not. The compatible class included both fully compatible and partially compatible combinations to avoid erroneous interpretations, because abundant pollen was placed on the stigmas and not all pollen tubes reach the ovary even in fully compatible combinations. Incompatible relationships were classified according to the site of pollen tube arrest (Camadro and Peloquin 1981): in the stigma (I_S), and from stigma to ovary; in the upper third of the style ($I_{1/3}$); second third of the style ($I_{2/3}$); and the bottom third of the style ($I_{3/3}$). Pistils with ungerminated pollen were not considered in the analyses; those in which more than one site of pollen tube arrest was observed were classified according to the site at which the majority of the pollen tubes was arrested.

Results

Pollen–pistil relationships

A total of 828 (673 in season 1 and 155 in season 2) genotypic combinations were analyzed over two seasons, and included the microscopic observation of 2681 pollinated pistils. Compatible and incompatible pollen–pistil relationships were observed in both seasons (Fig. 1). Compatibility (in 615 combinations) and $I_{1/3}$ (in 136 combinations) were the most frequent pollen–pistil relationships observed in both intraspecific (including self-pollinations) and the interspecific crosses. In all of the self-pollinated pistils of the diploid genotypes, pollen tube growth was arrested in the upper third of the style. In both intraspecific (both within and between accession/family) and interspecific genotypic combinations, I_S, $I_{2/3}$, and $I_{3/3}$ were observed (Tables 2; Supplementary data, Tables S1 and S2)[2].

When pollen–pistil relationships were analyzed by considering the ploidy level of the progenitors (Supplementary data, Tables S1 and S2)[2], compatibility was the most frequent relationship observed, regardless of whether the genotypic combinations were intra- or interploidy, and intra- or interspecific (Table 3). The results of the intraspecific and interspecific intraploidy $2x \times 2x$ combinations were similar (compatibility and incompatibility at the four sites, although the percentage of combinations that fell into each incompatibility category varied). In the intraploid $4x \times 4x$ genotypic combinations, the incompatibility sites were $I_{1/3}$ and $I_{2/3}$ in the intraspecific, and $I_{1/3}$, $I_{2/3}$, plus I_S in the interspecific crosses. For the interploid $2x \times 4x$ combinations, the only compatibility was observed in the intraspecific crosses, whereas compatibility followed by $I_{1/3}$ were the most frequent

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[2] Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjb-2017-0070.

Table 2. Number of genotypic combinations according to type of cross and pollen–pistil relationships conducted with wild potato accessions, full-sibling families, and commercial cultivars, over two seasons (pooled data).

		Pollen–pistil relationship (%)				
			Incompatible			
				Style		
Type of cross[a]	Genotypic combinations (*n*)	Compatible	Stigma	1/3	2/3	3/3
Intraspecific						
Within accession/family[b]	66	53.0	7.6	36.4	1.5	1.5
Between accession/family	219	85.8	3.7	6.8	3.2	0.5
Interspecific[c]	543	72.2	5.2	17.8	4.4	0.4
Total	828	74.3	4.9	16.4	3.9	0.5

Note: Season 1 (summer 2006/2007) included accessions of 2*x* and 4*x* *Solanum gourlayi* (grl), 2*x* *S. spegazzinii* (spg), and 4*x* *S. tuberosum* (tbr) cultivars; season 2 (summer 2009/2010) included accessions of 2*x* *S. chacoense*, 2*x* intra- (grl × grl, spg × spg) and interspecific (spg × grl and grl × spg) full-sib families, and 4*x* tbr cultivars.

[a] According to current taxonomy, based on morphological phenotypes.

[b] Family includes full-sibs obtained from crosses between accessions classified as either the same or different taxonomic species.

[c] Genotypic combinations between interspecific hybrid families and intraspecific hybrid families [e.g., (spg × grl) × (grl × grl)] are also included.

Table 3. Number of genotypic combinations according to pollen–pistil relationships in intra- and interspecific crosses, within and between ploidy levels, among wild potato accessions, full-sibling families, and commercial cultivars, over two seasons (pooled data).

		Pollen–pistil relationship (%)				
			Incompatible			
				Style		
Type of cross[a]	Genotypic combinations (*n*)	Compatible	Stigma	1/3	2/3	3/3
Intraspecific						
2*x*×2*x*	241	78.0	14.2	14.9	2.5	0.4
2*x*×4*x*	43	100.0	0.0	0.0	0.0	0.0
4*x*×2*x*	41	80.5	7.3	7.3	4.9	0.0
4*x*×4*x*	39	92.3	0.0	5.1	2.6	0.0
Interspecific[b]						
2*x*×2*x*	169	79.3	4.7	9.5	5.3	1.2
2*x*×4*x*	89	71.9	7.9	18.0	2.2	0.0
4*x*×2*x*	70	52.9	7.1	35.7	2.9	1.4
4*x*×4*x*	136	58.8	5.9	27.9	7.4	0.0

Note: Season 1 (summer 2006/2007) included accessions of 2*x* and 4*x* *Solanum gourlayi* (grl), 2*x* *S. spegazzinii* (spg), and 4*x* *S. tuberosum* (tbr) cultivars; season 2 (summer 2009/2010) included accessions of 2*x* *S. chacoense*, 2*x* full-sib families (grl × grl, spg × spg, spg × grl, and grl × spg), and 4*x* tbr cultivars.

[a] According to current taxonomy, based on morphological phenotypes.

[b] Genotypic combinations between interspecific hybrid families and intraspecific hybrid families [e.g., (spg × grl) × (grl × grl)] are also included.

relationships in the interspecific combinations, followed by I_S and $I_{2/3}$. In the interploid intraspecific 4*x* × 2*x* genotypic combinations, incompatibility was observed in all sites except $I_{3/3}$, whereas incompatibility was observed at each site, with $I_{1/3}$ being the most frequent, in the interspecific crosses.

IC gene segregation

In general, more than one pollen–pistil relationship was observed when the genotypic combinations were analyzed at the level of (*a*) individual genotypes (regardless of the accession and family to which they belong) (Table 4), (*b*) accession, and (*c*) family, when used as either the female or male parent (Supplementary data, Tables S1 and S2)[2]. Similar behavior was observed when analyzing individual genotypic combinations at the level of inter-accession/family/cultivar crosses (Table 5). Pollen–pistil relationships observed when individual genotypes participated in more than two genotypic combi-

Table 4. Number of observed pollen–pistil relationships, discriminated by site of pollen tube arrest, exhibited by single genotypes used as either the female or male parent in crosses with two or more genotypes over two seasons (pooled data).

	Pollen–pistil relationship					Single genotypes (n) crossed as	
Observed pollen–pistil relationships (n)	C	I_S	$I_{1/3}$	$I_{2/3}$	$I_{3/3}$	Female	Male
1	x					84	59
		x				1	—
			x			2	2
				x		1	—
2	x	x				7	2
	x		x			34	31
	x			x		7	14
	x				x	1	—
		x	x			2	2
		x		x		1	—
			x	x		1	—
			x		x	—	1
3	x	x	x			9	13
	x		x	x		4	6
	x		x		x	1	—
	x	x		x		3	1
4	x	x	x	x		3	5
	x		x	x	x	—	1
5	x	x	x	x	x	1	—

Note: Season 1 (summer 2006/2007) included accessions of 2*x* and 4*x* *Solanum gourlayi* (grl), 2*x* *S. spegazzinii* (spg), and 4*x* *S. tuberosum* (tbr) cultivars; season 2 (summer 2009/2010) included accessions of 2*x* *S. chacoense*, 2*x* full-sib families (grl × grl, spg × spg, spg × grl, and grl × spg), and 4*x* tbr cultivars. —, relationship not observed; C, compatible; I_s, incompatible in stigma; $I_{1/3}$, incompatible in upper third of style; $I_{2/3}$, incompatible in middle third of style; $I_{3/3}$, incompatible in bottom third of style.

nations are presented in Table 4. If these genotypes exhibited (*a*) only one relationship, it was invariably compatibility; (*b*) two relationships, the most frequent were compatibility and $I_{1/3}$, although I_S, $I_{2/3}$, or $I_{3/3}$ were also observed; (*c*) more than two relationships, I_S, $I_{2/3}$, and (or) $I_{3/3}$ were observed in addition to compatibility and (or) $I_{1/3}$. The genotypes that exhibited four types of pollen–pistil relationships participated in 5 to 32 genotypic combinations as either the male or female parent. The five types of relationships were observed in one genotype that had participated as the female parent in 41 genotypic combinations (data not shown). Various individual plants obtained in season 2 from fully compatible genotypic combinations (carried out in season 1) between progenitors that were also compatible in other intra- and interspecific genotypic combinations, exhibited I_S, $I_{2/3}$, and $I_{3/3}$ when used as either the female and (or) male parent in intra- and interaccession/family crosses (Table 6; Supplementary data, Fig. S1[2]). In addition, two full-sib genotypes derived from an interspecific spg–2*x* grl cross, (OL4916.13 × ORHL4841.2).4 and (OL4916.13 × ORHL4841.2).3, exhibited various pollen–pistil relationships when crossed with APEC1.5 (chc); however, upon self-pollination, these three diploid genotypes were $I_{1/3}$ (Fig. 2).

Reciprocal genotypic combinations

The number of reciprocal genotypic combinations that could be conducted was limited by the number of flowers and the flowering time of each genotype. In season 1, 107 reciprocal combinations were completed: 88 of these were compatible in both directions whereas 15 were compatible in one direction, one exhibited $I_{1/3}$ in both directions, and three exhibited incompatibility but at different sites in each direction (i.e., $I_{1/3}$ in one direction, and stigma in the reciprocal) (Table 7). In the 24 reciprocal genotypic combinations conducted in season 2, 11 were compatible in both directions, three exhibited $I_{1/3}$ in both directions, two exhibited $I_{2/3}$ in both directions, four were compatible in one direction, and two exhibited incompatibility at different sites (Table 7).

Discussion

Wild potatoes are genetically very complex. Their breeding system provides for overlapping generations, and hybridization is a frequent phenomenon within and between ploidy levels and populations (Hawkes and Hjerting 1969; Masuelli et al. 2009) because breeding barriers, if present, can either be incomplete or circumvented (see Camadro et al. 2012). Moreover, their natural populations can be composed of plants with more than one ploidy level. Populations with diploid and tetraploid

Table 5 (A and B). Number of genotypic combinations, discriminated by type of pollen–pistil relationships, observed in a sample of intra- and interspecific crosses among potato accessions, families, and cultivars.

Cross				Pollen–pistil relationship				
Species	Accession/family/cultivar ♀	Accession/family/cultivar ♂	Species	C	I_S	$I_{1/3}$	$I_{2/3}$	$I_{3/3}$
A. Season 1								
2*x* grl	Oka7518	OL4858	2*x* grl	4	—	—	—	—
	OL4858*	Oka7518		4	—	—	1	—
	Oka7518	ORHL4841		4	—	—	—	—
	ORHL4841*	Oka7518		7	—	—	—	—
	OL4858	ORHL4841		11	—	—	1	—
	ORHL4841*	OL4858		11	—	—	—	—
	OL4858	Oka7588	4*x* grl	5	—	—	—	—
	OL4858	OL4911	2*x* spg	6	2	—	1	—
2*x* spg	OL4911*	OL4858	2*x* grl	5	—	—	—	—
2*x* grl	OL4858	Kennebec	4*x* tbr	6	1	2	3	—
4*x* tbr	Kennebec*	OL4858	2*x* grl	1	—	6	2	—
2*x* grl	OLHR4841	OL4911	2*x* spg	8	—	2	2	—
	OLHR4841	Oka5662		4	1	2	1	—
2*x* spg	Oka5662*	OLHR4841	2*x* grl	4	—	—	1	—
4*x* grl	Oka7547	Oka7558	4*x* grl	1	1	—	—	—
	Oka7565	Oka4911	2*x* spg	7	—	1	—	—
	Oka7595	ORHL4841	2*x* grl	5	—	—	1	—
		Oka5649	2*x* spg	3	1	—	2	—
		Oka5662		3	1	—	2	—
		Pampeana	4*x* tbr	1	—	1	1	—
		OL4911	2*x* spg	14	—	—	2	—
2*x* spg	Oka5649	Oka4911		13	2	3	—	—
		Calén	4*x* tbr	4	1	1	—	—
	Oka5662	Oka4911	2*x* spg	21	1	2	—	—
		Oka5649		5	1	1	—	—
		OL4858	2*x* grl	6	2	—	—	—
	Oka6147	Oka4911	2*x* spg	8	—	1	1	—
	Oka4911	Oka5662		10	—	—	—	1
		Pampeana	4*x* tbr	5	—	2	—	—
	OL4916	OL4911	2*x* spg	9	—	—	—	—
		Oka5649		13	—	—	—	—
4*x* tbr	Calén	Oka7547	4*x* grl	4	—	1	—	—
B. Season 2								
2*x* chc	APEC1	APEC1	2*x* chc	10	5	10	—	—
		(OL4916.13×ORHL4841.2)	spg×grl	1	1	1	1	—
spg×grl	(OL4916.13×ORHL4841.2)	APEC1	2*x* chc	5	—	1	1	—
2*x* chc	APEC1	Huinkul	4*x* tbr	—	2	1	1	—
4*x* tbr	Huinkul*	APEC1	2*x* chc	1	1	6	—	—
spg×spg	(OL 4911.2×OL4911.5)			3	—	—	—	1
	(OL4911.19×OL4916.7)	(OL4916.13×ORHL4841.2)	spg×grl	5	—	—	—	—
		(OL4911.19×OL4916.7)	spg×spg	5	—	4	1	—
spg×grl	(OL4916.13×ORHL4841.2)	(ORHL4841.14×OL4858.17)	grl×grl	6	—	1	—	—

Note: Pooled data. chc, *Solanum chacoense*; grl, *S. gourlayi*; spg, *S. spegazzinii*; tbr, *S. tuberosum* subsp. *tuberosum*; —, relationship not observed; C, compatible; I_s, incompatible in stigma; $I_{1/3}$, incompatible in upper third of style; $I_{2/3}$, incompatible in middle third of style; $I_{3/3}$, incompatible in bottom third of style; *, reciprocal crosses.

plants, for example, may produce numerically unreduced (2*n*) pollen in addition to normal (*n*) pollen (respectively, $n = x$ and $2n = 2x$ pollen in the diploids and $n = 2x$ and $2n = 4x$ pollen in the tetraploids). In 2*x* and higher ploidy pollen grains, competitive interaction can lead to SC relationships among plants with a GSI system. Despite these complexities, potato accessions are ex-situ conserved and multiplied as Taxonomic Species (TS) (Camadro et al. 2012), and genetic experiments are designed under the assumption that potato TS and Biological Species (BS) are equivalent.

Pollen–pistil relationships and the species concept in potatoes

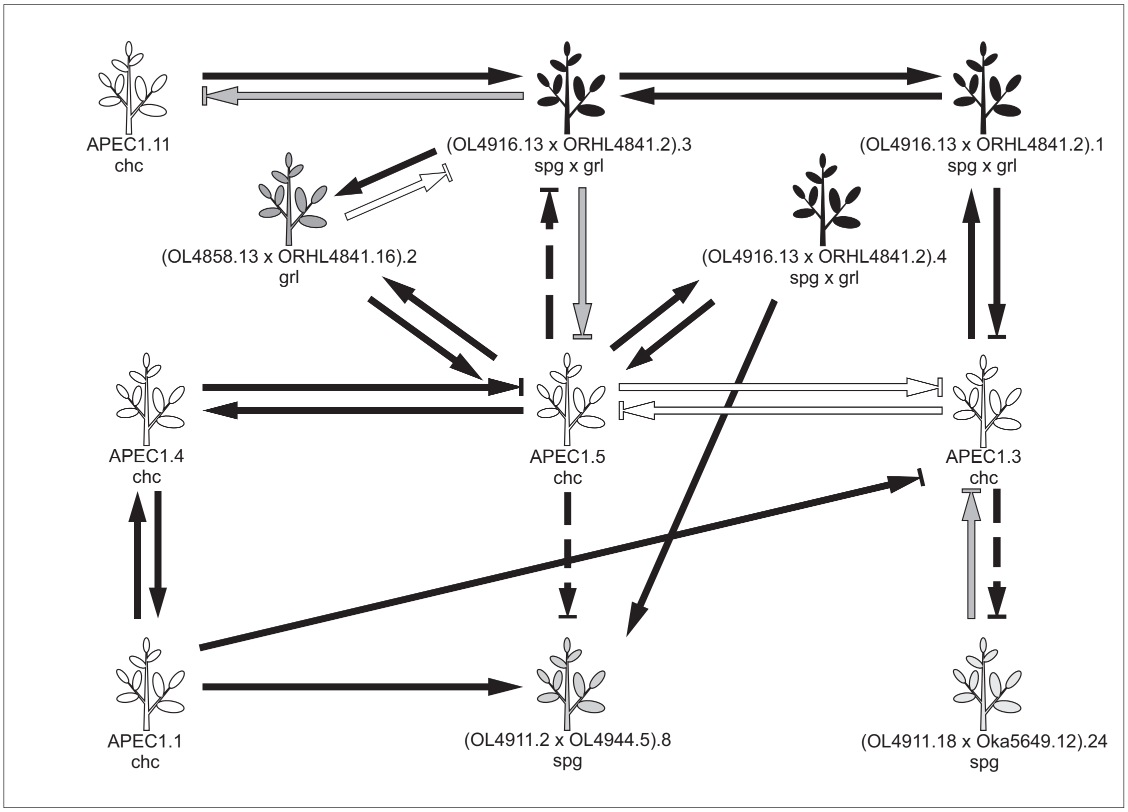
To pursue the objectives of this study, we created a network of breeding relationships with a wide genetic base, over two seasons. Compatibility was the most frequent pollen–pistil relationship observed, regardless of the origin and taxonomic status of the progenitors. In

Table 6. Pollen–pistil relationships exhibited by individual genotypes of one interspecific wild potato family (spg OL4916.13×grl ORHL4841.2), whose progenitors had exhibited only compatible relationships in, respectively, 15 and 20 intra- and interspecific genotypic combinations, upon crossing within the family and with genotypes from intraspecific *Solanum gourlayi* (grl) and *S. spegazzinii* (spg) families, and one *S. chacoense* (chc) accession.

Cross				Pollen–pistil relationship					
Species	Accession/family ♀	Accession/family ♂	Species	Genotypic combinations (*n*)	C	I_S	$I_{1/3}$	$I_{2/3}$	$I_{3/3}$
spg×2*x* grl	(OL4916.13×ORHL4841.2)	(OL4916.13×ORHL4841.2)	spg×2*x* grl	11	8	—	3	—	—
spg×2*x* grl	(OL4916.13×ORHL4841.2)	Various	spg or 2*x* grl	12	10	—	2	—	—
spg or 2*x* grl	Various	(OL4916.13×ORHL4841.2)	spg×2*x* grl	7	6	—	1	—	—
		APEC1	2*x* chc	9	8	—	—	1	—
2*x* chc	APEC1	(OL4916.13×ORHL4841.2)	spg×2*x* grl	5	1	2	1	1	—

Note: Pooled data. chc, *Solanum chacoense*; grl, *S. gourlayi*; spg, *S. spegazzinii*; —, relationship not observed; C, compatible; I_s, incompatible in stigma; $I_{1/3}$, incompatible in upper third of style; $I_{2/3}$, incompatible in middle third of style; $I_{3/3}$, incompatible in bottom third of style.

Fig. 2. Example of compatibility relationships in wild potatoes observed in season 2. Each plant represents one genotype; each shade represents one accession/family. Arrow direction = direction of pollination. Pollen–pistil relationships: compatibility (normal arrow); incompatibility (truncated arrow head); in black, stigma; white, 1/3 of style; grey, 2/3 of style; broken black arrow, 3/3 of style); chc, *Solanum chacoense*; grl, *S. gourlayi*; spg, *S. spegazzinii*.



season 1, viable seeds were obtained from all pollen–pistil compatible genotypic combinations, including those between grl and spg, which gave rise to the full-sib interspecific families used in season 2. These full-sib families, in turn, exhibited compatible pollen–pistil relationships in most genotypic combinations in which they were crossed, again regardless of the origin and taxonomic classification of the other progenitor. These results contrast with what was expected if TS and BS were equivalent in potatoes, and point out to the feasibility of gene flow among sympatric natural populations of different TS. In fact, various authors have reported the occurrence of natural interspecific wild potato hybrids (see Hawkes and Hjerting 1969; Camadro et al. 2004, 2012; Masuelli et al. 2009; Spooner et al. 2014), and morphological, genetic, and molecular data obtained by Bedonni and Camadro (2009) have revealed the occurrence of gene flow among sympatric populations of four TS.

In our study, most genotypic combinations were compatible; notwithstanding, incompatible reactions were observed upon selfing and intercrossing. Upon selfing of the diploid genotypes, pollen tube growth arrest invari-

Table 7. Pollen–pistil relationships observed in a sample of genotypic combinations among wild potatoes and cultivars and their reciprocals, excluding combinations that were compatible in both directions, carried out in two seasons.

Genotypic combination		Pollen–pistil relationship	
Female (F)	Male (M)	F×M	M×F
Season 1			
2*x* grl×4*x* tbr			
OL4858.3	Kennebec	C	$I_{2/3}$
ORHL4841.8		C	$I_{2/3}$
ORHL4841.11		C	$I_{1/3}$
ORHL4841.15		C	$I_{1/3}$
ORHL4841.17		C	$I_{1/3}$
ORHL4841.18		C	$I_{2/3}$
OL4858.8		I_S	$I_{1/3}$
OL4858.10		$I_{1/3}$	$I_{2/3}$
OL4858.16		$I_{2/3}$	$I_{1/3}$
2*x* grl×2*x* grl			
OL4858.16	ORHL4841.9	$I_{2/3}$	C
2*x* grl×2*x* spg			
ORHL4841.2	Oka5662.14	$I_{1/3}$	C
2*x* spg×2*x* spg			
OL4911.6	OL4911.9	I_S	C
Oka5649.6	Oka5662.25	C	I_S
4*x* grl×4*x* tbr			
Oka7547.10	Calén	C	$I_{1/3}$
Oka7547.13		C	$I_{1/3}$
Oka7565.20		$I_{1/3}$	C
Oka7565.11	Kennebec	C	$I_{1/3}$
2*x* spg×4*x* tbr			
OL4911.5	Kennebec	C	$I_{1/3}$
Oka5649.2	Calén	$I_{1/3}$	$I_{1/3}$
Season 2			
2*x* chc×2*x*(spg×grl)			
APEC1.5	(OL4916.13×ORHL4841.2).3	$I_{2/3}$	$I_{2/3}$
APEC1.11		$I_{1/3}$	C
APEC1.3	(OL4916.13×ORHL4841.2).1	I_S	C
2*x* chc×2*x* (spg×spg)			
APEC1.2	(OL 4911.02×OL4911.5).3	$I_{1/3}$	C
2*x* chc×2*x* chc			
APEC1.5	APEC1.4	I_S	C
APEC1.5	APEC1.3	$I_{1/3}$	$I_{1/3}$
2*x* chc×2*x* (grl×grl)			
APEC1.3	(OL4858.13×Oka7518.03).24	$I_{2/3}$	$I_{2/3}$
2*x* chc×4*x* tbr			
APEC1.1	Huinkul	$I_{1/3}$	$I_{1/3}$
APEC1.2		$I_{2/3}$	$I_{1/3}$
APEC1.5		$I_{1/3}$	$I_{1/3}$
2*x* spg×2*x* spg			
(OL4911.19×OL4916.07).10	(OL4911.19×OL4916.07).16	I_S	$I_{1/3}$

Note: chc, *Solanum chacoense*; grl, *S. gourlayi*; spg, *S. spegazzinii*; tbr, *S. tuberosum* subsp. *tuberosum*; C, compatible; I_s, incompatible in stigma; $I_{1/3}$ incompatible in upper third of style; $I_{2/3}$, incompatible in middle third of style; $I_{3/3}$, incompatible in bottom third of style.

ably occurred in $I_{1/3}$, a reaction reported as characteristic of the GSI system in the Solanaceae family (Lush and Clarke 1997; de Nettancourt 2001; Hayes et al. 2005; Covey et al. 2010). Upon intercrossing, the incompatible reaction sites in intra- (intra- and interaccession) and interspecific genotypic combinations were similar: $I_{1/3}$, reported as characteristic of both GSI and CI (Ascher and Peloquin 1968; Camadro and Peloquin 1981; Lush and Clarke 1997; Camadro et al. 2004; Hayes et al. 2005; Covey et al. 2010); and I_S, $I_{2/3}$, and $I_{3/3}$, reported as characteristic of CI (Ascher and Peloquin 1968; Camadro and Peloquin 1981; Camadro et al. 2004; Hayes et al. 2005; Baek et al. 2015).

Pollen–pistil hybridization barriers were incomplete in our plant material, although they are ex-situ conserved under TS status. Population systems between early and late stages of speciation, at various stages of divergence and reproductive isolation, and interconnected by a reduced amount of interbreeding and gene flow are referred to as semispecies (Grant 1981). If this were the case in wild potatoes, segregation for genes controlling hybridization barriers would be present within accessions, as we observed in our materials, highlighting the previously discussed lack of agreement between the TS and the BS in this plant group. Concomitantly, segregation for morphological traits is expected to occur. The appearance of morphologically "off type" plants during the ex-situ multiplication process at germplasm banks (see Camadro 2012) gives additional support this assertion.

GSI vs. CI at the phenotypic level

A first clue to phenotypically differentiate GSI from CI would be given by the results of reciprocal crosses at the individual genotypic combination level. In this regard, the number of reciprocal crosses that could be carried out in our study was limited by the number of flowers available for pollination. Even though cloning in potatoes could be an alternative to circumvent this limitation, the number of plants needed to test all of the possible genotypic combinations could become unmanageable. Thus, we used an incomplete diallel crossing design with reciprocal crosses when possible. Based on the action of the GSI *S*-locus, reciprocal crosses are expected to be either fully or partially compatible (both recorded as compatible in our study), or incompatible ($I_{1/3}$) in both directions. But, in addition to the expected reactions, we observed either unilateral incompatibility, or bilateral incompatibility with different reaction sites in each crossing direction, both in intraspecific intraploid (SI × SI) and interspecific intraploid (SI × SI and SC × SC) crosses, as well as in interploid (SI × SC) ones. These results cannot be explained by the action of the *S*-locus alone or, at least, of its specificity. The expression of $I_{1/3}$ in the interspecific combinations is another reaction that cannot be explained by the action of the *S*-locus alone. It is highly unlikely that genetic materials of such a wide geographic provenience, as the ones we used, would have shared the *S*-haplotypes. Moreover, the offspring from compatible genotypic combinations exhibited CI (in addition to compatibility and (or) $I_{1/3}$) upon crossing with other genotypes. These results can be explained by assuming segregation for the gene(s) controlling pollen–pistil barriers, as it was previously hypothesized by Camadro and Peloquin (1981), Eijlander et al. (2000), and Hayes et al. (2005) for other wild and cultivated potato materials.

The SI × SC rule

In potatoes, Hermsen et al. (1974, 1977) approached the study of interspecific crosses between *S. tuberosum* haploids (2*x*, SI) and the wild potato *S. verrucosum* (2*x*, SC) from the SI × SC rule. They observed unilateral incompatibility as predicted but, exceptionally, some of the SI genotypes acted as "acceptors" of the SC pollen. In a line of reasoning similar to that of Camadro and Peloquin (1981) and Hayes et al. (2005), these authors and Eijlander et al. (1997) proposed the action of a dominant allele not belonging to the *S*-locus (and expressed in the absence of an inhibitor gene) to explain this behavior. In a detailed analysis of male and female fertile backcross progenies from these materials, Eijlander et al. (2000) observed SC in F_1 populations, with segregation into SC and SI genotypes in later generations although in skewed ratios. Thus, they proposed a complex model involving at least four different loci to explain the appearance and disappearance of SC in the offspring generations. In this regard, our study included crosses in all 2*x*–4*x* intra- and interspecific combinations, and variable and unpredictable pollen–pistil relationships were observed. Under the assumption that each 2*x* is SI and that each 4*x* could be SC due to the competitive interaction, it can be concluded that the SI × SC rule does not work in our materials. Also, the observation of variable, although similar, pollen–pistil relationships in all 2*x*–4*x* interspecific combinations could not be explained by the specificity of the GSI *S*-locus. For example, if the results of 4*x* × 2*x* (SC × SI) and 2*x* × 4*x* (SI × SC) combinations in our materials would have depended on the action of the GSI S-locus, more compatible pollen–pistil relationships would have been expected in the second type of combination, owing to competitive interaction in the 2*x* *S*-heterozygous pollen from the 4*x* parent. However, we observed the same variability for pollen–pistil relationships in both crossing directions.

In tomatoes, which also belong to the Solanaceae, Liedl et al. (1996), Covey et al. (2010), Li and Chetelat (2010), and Baek et al. (2015) studied CI at the level of accessions of the same or different TS. Pollen–pistil relationships in intra- and interspecific crosses were studied under the SI × SC rule. They used species/accessions as the experimental units, and therefore only one type of relationship was reported for each combination. This rule presupposes that all genotypes belonging to the same accession would exhibit the same reaction upon crossing with another accession/species (that is, the character is fixed) and that this reaction can be predicted if the SI or SC of the materials is known.

Level of analysis

Because of their obligate allogamy, SI plants in general are expected to be heterozygous and their populations heterogeneous, whereas SC plants are expected to be homozygous and their populations more or less homogeneous, depending on whether they have a percentage of allogamy (Camadro 2012). Thus, genetic experiments carried out with a few genotypes per accession and (or) few accessions per TS species entail the idea that accessions/populations are genetically homogeneous, and that strong breeding barriers have developed between sympatric populations, preserving the genetic identity of the species.

Potatoes are genetically very complex and, as previously stated, competitive interaction in diploid and higher ploidy pollen grains provides for SC in plants with a GSI system. Therefore, to avoid underestimation of population variability for genes controlling pre-zygotic barriers, we analyzed pollen–pistil relationships at the level of individual genotype × genotype combinations. This approach revealed that a single genotype could exhibit various pollen–pistil relationships, depending on the genotype of the other parent and whether it was used as either the male or the female parent. Similar behavior was observed when the analysis was conducted with individual genotypes at the level of accession/family/cultivar. In this regard, by increasing the number of genotypic combinations in which a given genotype was involved, the probability of detecting different types of pollen–pistil relationships was increased, independently of the accession/species to which it belonged. These results provide further evidence of the inconsistency of the taxonomical classification based on morphological phenotypes, because pollen behavior upon pollination depended on the interaction between the genotypes involved in a given cross rather than on the accession/species to which they belonged, as Camadro and Peloquin (1981) and Raimondi and Camadro (2003) had reported. They also indicate the need of working at the level of individual genotypic combinations.

Conclusions

The results of the extensive breeding network reported in this paper allow us to conclude that TS and BS are not equivalent in potatoes. They also allow us to hypothesize that SI and CI in this plant group are two different phenomena at the phenotypic level, and that the *S*-locus (or at least its specificity) is apparently not involved in the latter because its action cannot be explained by the observed segregation for sites of pollen tube arrest. However, to exclude the *S*-locus action from the CI phenomenon in potatoes, it is necessary to conduct molecular studies at the level of gene expression in compatible and incompatible genotypic combinations. To that end, it is first necessary to perform a phenotypic study at the individual plant level to select the appropriate genotypes for the crossing work.

For applied purposes, the discrepancies between TS and BS in this plant group, the observed variability for pollen–pistil relationships within accessions, and the fact that genotypes within a given accession can exhibit CI upon intercrossing should be considered for both selection of parents and strategies to facilitate the breeding process, reducing operational costs, and devising ex-situ conservation and multiplication strategies to avoid or minimize the loss of genetic diversity "captured" from the natural populations in the accessions, as a main objective of active germplasm banks (see Camadro 2012).

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