



REVIEW ARTICLE

Diploid inbred-based hybrids: fast-forward breeding approach in potatoes

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Abstract

Following the identification of the self-compatibility gene (*Sli*) in diploid potatoes two decades ago, the breeding of inbred based diploid hybrid potatoes made its way. Tetraploid potatoes have a long history of cultivation through domestication and selection. Tetrasomic inheritance, heterozygosity and clonal propagation complicate genetic studies, resulting in a low genetic gain in potato breeding. Diploid hybrid TPS potato breeding, similar to the developments in hybrid maize, was pursued as an alternative to the genetic improvement of potatoes. However, several challenges, like self-incompatibility and high inbreeding depression associated with diploid potatoes, must be overcome to develop inbred lines in potatoes. Moreover, the inbred lines must retain good fertility and vigour for hybrid breeding. Good progress has been made by creating di-haploids of popular varieties, mapping self-incompatibility inhibitor gene, understanding the genetic basis of inbreeding depression, and identifying genomic regions for deleterious alleles and fertility. Further, the genome sequencing of diploid inbred lines has revealed the genetics of key traits associated with potato breeding. This article discussed these insights and summarized the progress of diploid hybrid TPS potato breeding. Recent advances in genetic and genomic research and genome editing technology have shown promise for this technology's success and far-reaching implications.

Keywords Diploid potato · F1 hybrids · Homozygosity · Heterosis · Inbred lines · Inbreeding depression · Self-compatibility · TPS

Introduction

Many countries throughout the world rely heavily on potatoes as a staple diet. It has a long history of agriculture in its native Peruvian Andean region. Due to its high heterozygosity and polyploid nature, it is propagated by tubers to ensure cultivar integrity. Cultivated potatoes are self-compatible and auto-tetraploid. However, they exhibit substantial inbreeding depression because of high heterozygosity in parental lines and the expression of lethal genes during selfing (Zhang et al. 2021). Consequently, the development of homozygous lines in tetraploid potatoes was not feasible, and tubers are the only means by which varieties and

advanced breeding material could be maintained. Pests and diseases, especially viruses, accumulate due to clonal multiplication used to maintain varieties and other breeding lines in the form of tubers, which also multiply with each cycle of clonal propagation. This reduces the productivity and acceptability of varieties. True potato seeds, i.e., botanical seeds, have not been employed commercially (on a large scale) in the multiplication of cultivars that are otherwise simple to manage, transport, store, genetically manipulate, and devoid of disease and pest inoculums.

In potatoes, different ploidy groups exist, varying from diploid to hexaploids. Although tetraploids are mainly used in commercial cultivation, most species are diploid, and some diploids, like *Solanum phureja* are grown and consumed in Peru and Europe. Many essential genes from diploid wild species have been transferred to tetraploid cultivated potatoes through unreduced gametes in diploids. Several new alleles were added to the cultivated potatoes, but the favourable alleles could not be fixed due to deleterious counterparts in autotetraploid heterozygous potatoes

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(Vos et al. 2015). Due to complex polysomic inheritance, genome reshuffling cannot be accomplished in heterozygous tetraploid potatoes. This is the primary reason for slow genetic gains in potato breeding across the globe (Douches et al. 1996; Sood et al. 2022). Many previous efforts were made to incorporate a similar breeding scheme in potatoes as maize, but could not be successfully implemented for commercial potato production (Jansky and Spooner 2017). Thus the problems of asexual reproduction, heterozygosity, seed degeneration, varietal maintenance and transportation remained as such.

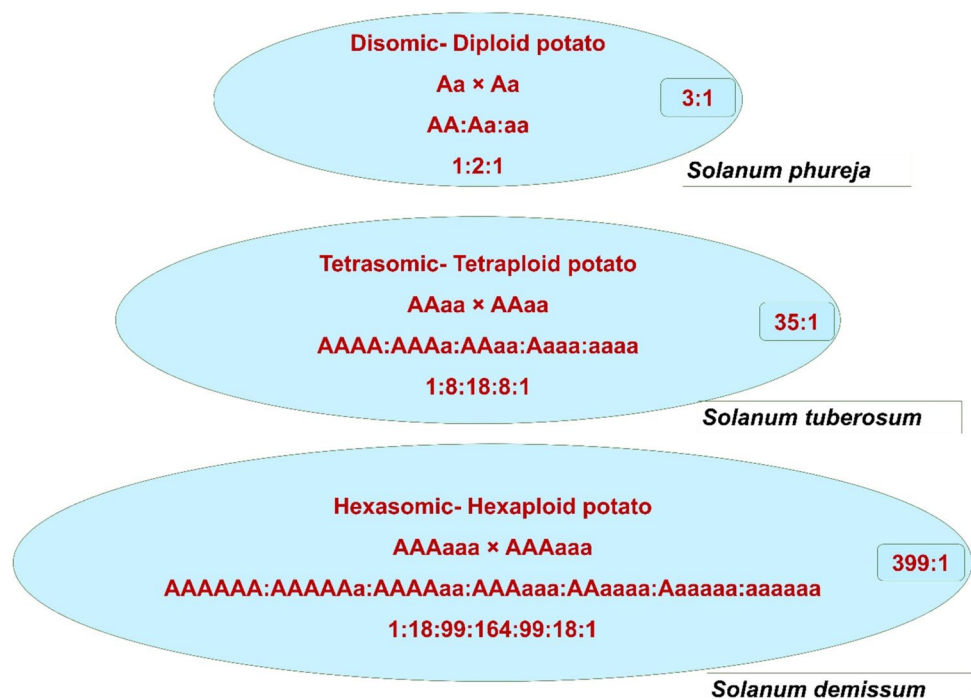
A revolutionary approach, “diploid hybrid TPS” (DHTPS), to counter these pitfalls of conventional potato breeding was proposed and practically demonstrated by Lindhout et al. (2011). The technology is similar to the breeding approach used in major cereal grains like rice and maize, where true seed *i.e.* botanical seed is used for propagation, Homozygous inbred lines are developed, and heterotic combinations are evaluated. However, the major hindrance in developing inbred or homozygous lines at the diploid level is gametophytic self-incompatibility in potatoes, which is widespread in diploid species and di-haploids of potatoes. The second major problem is high inbreeding depression and maintenance of fertility. The heterozygosity is carried forward for generations in potato breeding due to the selection of vigorous plants. This results in the accumulation of deleterious mutations as recessive alleles suppressed by dominant favourable alleles. The di-haploids of adapted tetraploid varieties also express deleterious mutations and require purging in each selfing generation to get

highly homozygous diploid lines. The review is focussed on research developments in transforming potato from tetraploid vegetatively propagated crop to diploid inbred based hybrid TPS crop.

Tetraploid empirical breeding versus diploid inbred hybrid TPS breeding

Potatoes have two main gene pools: Andigenum, which is adapted to short days in the uplands, and Chilotanum, which is adapted to long days in the lowlands (Spooner et al. 2007; Campos and Ortiz 2019). The tuberosum group originated from the Chilotanum group and comprises modern cultivars adapted to the Northern Hemisphere (Gavrilenko et al. 2013). In potatoes, phenotypic recurrent selection has been in practice for many years for developing improved varieties (Bradshaw 2009, 2017). Many improved varieties of potatoes have been released across the globe following clonal selection. Developing new cultivars takes about 13–14 years (Bradshaw 2009; Jansky and Spooner 2017), much longer than grain crops. The main reason for the long breeding cycle of potatoes is the low propagation coefficient (1:8), which delays the tuber multiplication for conducting field traits (Bonierbale et al. 2020). Another critical aspect of potato breeding is tetraploid genome. Chromosomal tetrasomic behaviour and complex genetic segregation hamper breeding research (Fig. 1). The complex inheritance, heterozygosity and vegetative propagation pose several hindrances in the crop's breeding advancement. Vegetative propagation in the form of tubers results in accumulation

Fig. 1 Complexity of zygotic outputs in diploid, tetraploid and hexaploids. Assuming a diallelic model with complete dominant A and recessive a at a locus, crossings from heterozygous parental genotypes show genotypic and phenotypic segregation. Ovals represent genotypic segregation, whereas squares represent phenotypic segregation. It clearly shows that an increase in ploidy requires a large progeny population for phenotypic selection



of pathogens and viruses, which results in seed degeneration over a period time (Thomas-Sharma et al. 2016). In potato breeding, epistasis and heterozygosity are major factors, although additive components contribute to quantitative features. There are four unique aspects of the tetrasomic inheritance of polyploids (Bradshaw 1994).

- One locus accommodates four alleles due to tetrasomic conditions. Thus, five genotype classes are feasible with a diallelic locus, A and a: AAAA, AAAa, AAaa, Aaaa, and aaaa. If an allele has an additive effect, one site may have significant quantitative segregation. (Fig. 1).
- Each allele, such as a1, a2, a3 and a4, may have a distinct function, resulting in a diversity of multiple alleles.
- These numerous alleles can combine to produce a variety of allelic interactions, including diallelic (a1a2a3a3), tri-allelic (a1a2a3a4) and tetrallelic (a1a2a3a4). Therefore, there are a variety of outcomes that might occur from an interaction between two alleles.
- Chromosome segregation may occur at a site distant from the centromere with a high likelihood of crossing over. Under the assumption that homologous chromosomes and chromatids are distributed randomly, a locus with genotype Aaaa and diallelic condition generates gametic output in a 1:1 ratio to Aa:aa through chromosomal segregation and 15AA: 12Aa: 1aa through chromatid segregation. A rare gametic genotype and zygotic progeny phenotype are therefore possible. A further instance is the AAaa genotype, which leads to a 3: 8: 3 AA: Aa: aa ratio with chromatid segregation and a 1:1 AA: aa ratio with chromosomal segregation. If AAaa genotypes are interbred, a phenotypic ratio of 208: 1 is possible.

All these examples show that tetrasomic tetraploid genetics is far more complex than diploid genetics, even with chromosomal segregation. Additionally, the high heterozygosity of tetraploid potato varieties and their outcrossing tendency make segregation more challenging. Potato breeding goals frequently include quantitative traits strongly impacted by the environment. A large true seed population is required in crosses to select the desired clones due to the vast range of phenotypic diversity at tetraploid level and environmental impacts.

To simplify genetics and potato breeding, efforts were made to develop homozygous lines at the tetraploid level in potatoes, but all efforts were unsuccessful (Jansky and Spooner 2017). It is difficult to express the deleterious alleles in the tetraploid background, i.e., a triplex, AAAa plant will not reveal the recessive phenotype; the only chance for the recessive phenotype to appear is the fertilisation of double reduction products in both the male and female gametes. The heterozygous plants were carried forward in the next generation due to their vigour

and fertility, making the selection biased. Tuber yield and vigour in S₆ generation plants was around 19% of the initial starting hybrids (Jansky and Spooner 2017). Seeing the bottlenecks of inbreeding in potatoes, the work in this direction almost stopped everywhere.

Many potato wild species carry important genes for resistance to pest diseases and abiotic stress tolerance (Sood et al. 2017). Till now, only a fraction of these valuable genes from wild species have been introgressed in cultivated potatoes through unreduced gametes (2n) in diploid species (Hardigan et al. 2017). Gene introgression is also accompanied by the undesirable genes or linkage drag. Several new alleles were added to the cultivated potato till the mid-twentieth century (Vos et al. 2015). The beneficial alleles from wild species could not be fixed because their deleterious counterparts could be removed through selection in highly heterozygous and autotetraploid potatoes (Vos et al. 2015). Due to complex polysomic inheritance, intragenomic and intergenomic repatterning cannot be accomplished in tetraploid potatoes. Therefore, it is difficult to advance breeding efforts through asexual reproduction in autotetraploid potatoes (Jansky and Spooner 2017). To incorporate a similar breeding scheme as followed in maize, a diploid analytical breeding scheme was proposed in potato, whereby ploidy will be reduced to half in tetraploid potato to introgress important genes from diploid species and afterwards, selected plants will be brought back to tetraploid level (Chase 1963). However, sexual polyploidization has been commonly used to transfer desirable alleles from diploid species via 2n gametes (Jansky and Spooner 2017). All these methods led to the improvement of simply inherited traits in potatoes, while the genetic gain for complex traits attained slow progress compared to major crops. For example, a slow genetic yield gain in tetraploid potatoes is attributed to high heterozygosity of the potato genome (AAAA, 2n = 4x = 48), sterility barriers, and inbreeding depression. The unfavourable alleles, which remain masked in the tetraploid genome in heterozygous condition, manifest themselves and delay genetic gain at each breeding cycle (Hojsgaard et al. 2024).

A groundbreaking answer to many pitfalls of conventional tetraploid potato breeding is a diploid hybrid true potato seed-based strategy (Lindhout et al. 2011; Jansky et al. 2016). Since the demonstration of proof of concept of DHTPS, efforts have been made globally to reinvent potato at the diploid level by ploidy reduction of tetraploid adapted varieties to diploid level, self-compatibility assessment and introgression, purging deleterious mutations during selfing, genomics assisted selection for stacking beneficial alleles and homozygosity assessment, and exploit heterosis using inbred lines (Table 1; Fig. 2).

Table 1 Major scientific accomplishments in diploid hybrid TPS potato breeding till date

Significant Milestone	References
Identification of self-compatible diploid potato clone in a wild species, <i>Solanum chacoense</i> , which could be continuously selfed for seven generations	Hanneman (1989)
The self-incompatible × Self-compatible cross resulted in segregating progeny for self-compatibility. This confirmed that a single dominant gene (<i>Sli</i>) is involved in dysfunction of the gametophytic self-incompatibility system	Hosaka and Hanneman (1998a)
Linkage analysis using DNA markers (RAPD and RFLP) mapped the <i>Sli</i> gene to the end of linkage group 12. This made it evident that <i>Sli</i> and the S locus are independent of each other and located on separate chromosomes	Hosaka and Hanneman (1998b)
Use of the <i>Sli</i> gene demonstrated that highly homozygous and seed-propagated diploid potatoes could be obtained by repeated selfing	Phumichai et al. (2005)
A diploid inbred line, M6 registered as new plant germplasm for use in breeding and genetics research	Jansky et al. (2014)
First genetic mapping study using diploid inbred line derived F2 population (DM 1–3 × M6). Identified genes for tuber pigmentation (PGSC0003DMG400013965), pigment colour (PGSC0003DMG400000425, PGSC0003DMG400003605), pigmented flesh intensity (PGSC0003DMG400012891), yellow flesh (PGSC0003DMG400010169),	Endelman and Jansky (2016)
Genetic basis of inbreeding depression deciphered in potato	Zhang et al. (2019)
Genome editing approach used to overcome self-incompatibility in diploid potato using CRISPR-Cas9 by knocking out the <i>SRNase</i> gene	Enciso-Rodriguez et al. (2019)
First example of marker-assisted introgression of four different <i>Phytophthora infestans</i> resistance genes (<i>Rpi-avl1</i> , <i>Rpi-tar1</i> , <i>Rpi-cha1</i> , <i>Rpi-vnt1</i>) in selected highly homozygous, diploid potato lines	Su et al. (2020)
Generated a highly homozygous diploid potato (19H2) retaining male and female fertility and tuberization ability under long days after 10 generations of selfing using the S locus inhibitor (<i>Sli</i>) gene	Hosaka and Sanetomo (2020)
The study demonstrated that <i>Sli</i> is widespread and many clones can be used to breed self-compatible diploid potato. Haplotype-specific KASP markers of the <i>Sli</i> gene were designed and validated on a panel of diploid clones. Male fertility was presumed as the major limitation in diploid inbred based hybrid potato breeding	Clot et al. (2020)
A de-novo reference genome of highly homozygous, diploid, vigorous and self-compatible <i>Solanum tuberosum</i> cultivar ‘Solyntus’ was made available to potato researchers	van Lieshout et al. (2020)
First haplotype resolved genome assembly of heterozygous diploid potato clone (RH89-039–16) was decoded using a combination of multiple sequencing strategies. The two haplotypes showed ~2.1% intragenomic diversity, with 22,134 predicted deleterious mutations in 10,642 annotated genes	Zhou et al. (2020)
QTL mapping using linkage analysis showed that <i>PGSC0003DMG400016861</i> is the <i>Sli</i> gene, which encodes an F-box protein specifically expressed in pollen from self-compatible plants. A 533 bp insertion in the promoter of the gene leads to a gain of function mutation, which overcomes self-pollen rejection	Eggers et al. (2021)
A separate study identified non-S-locus F-box (<i>NSF</i>) gene in RH (RH89-039–16- a self-compatible tuberosum background diploid). <i>NSF</i> is identical to <i>Sli</i> gene and was found to interact with multiple allelic variants of the pistil-specific S-ribonucleases (<i>S-RNases</i>) to introduce self-compatibility to other self-incompatible lines	Ma et al. (2021)
Recurrent selection approach improved the self-compatibility, male fertility and tuber traits after six cycles of recurrent selection	Alsahlany et al. (2021)
Genome design for hybrid potatoes was proposed by breaking tight linkage of deleterious alleles and purging of deleterious mutations using genomics tools	Zhang et al. (2021)
Generated insights into the genome-wide incidence and properties of deleterious mutations and inferred that diploid lines with relatively high homozygous deleterious burden can be better starting material for inbred-line development	Wu et al. (2023)
Developed an optimal phenotyping method for self-compatibility associated with the <i>Sli</i> allele. The study further revealed that <i>Sli</i> is inherited gametophytically	Eggers et al. (2024)

Progress towards the development of diploid inbred lines

Dihaploids as an elite germplasm for diploid hybrid breeding

Diploid germplasm is the first and foremost requirement of breeding at the diploid level. It is well known that most

potato wild species are diploids and a reservoir of valuable genomic resources (Spooner et al. 2007). But they also carry a load of undesirable genes. Therefore, concerted efforts must be made to create a diploid germplasm pool of cultivated potato species, *S. tuberosum*, and must be prioritized for use. Di-haploids (haploids) of adapted tetraploid potato varieties make the best starting material for this approach. Maternal di-haploids in potatoes have been easily generated

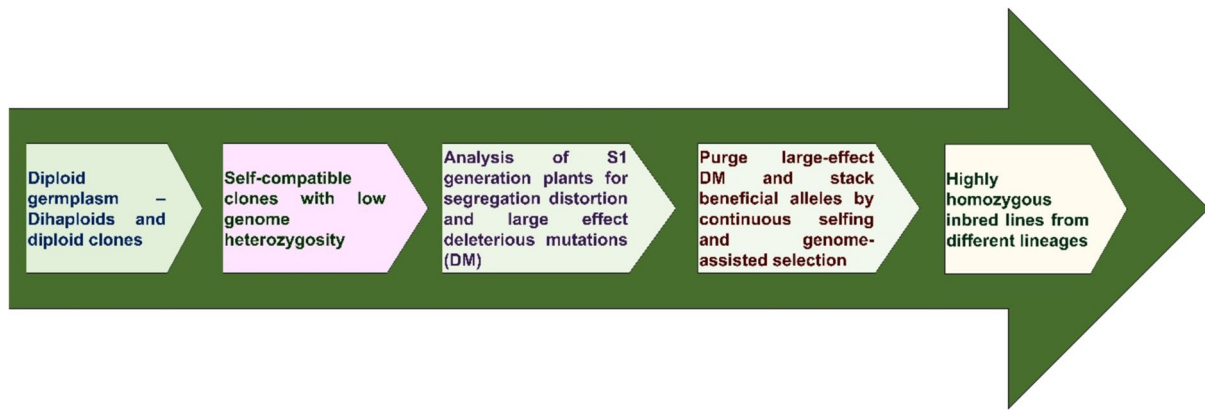


Fig. 2 Diploid Inbred Lines development in potato. The flow chart demonstrates the major steps and selection of desired clones in early selfing generations through genetic analysis to develop elite inbred lines

by interspecific hybridization of tetraploid cultivars with pollen of *S. phureja* via parthenogenesis (Peloquin et al. 1996). However, the haploid induction efficiency was low and varied from genotype to genotype. Earlier three *S. phureja* clones, viz., IVP35, IVP48 and IVP101, were widely used as pollinators, with the latter being the best (Hutten 1994). Recently, a new pollinator PL-4 (CIP596131.4) produced from a cross between IVP35 and IVP101 at the International Potato Centre (CIP), Peru, has shown better haploid induction efficiency of 11% compared to 6.8% in IVP101 (Ordoñez et al. 2021). PL-4 displays profuse flowering, good pollen shedding and pollen viability compared to its parents. Data from tetraploid breeding lines crossed with PL-4 and its parents (IVP101 and IVP 35) revealed that PL-4 generated twice as many seeds without embryo spot as IVP 101. Further, the confirmed haploids through flow cytometry and chloroplast counting in stomatal guard cells were also significantly higher in PL4 crosses compared to IVP101 and IVP35 crosses (Ortiz and Mihovilovich 2020).

Anther culture has also been practised in potatoes to obtain paternal haploids (Rokka et al. 1996). But, genes for androgenic competence are not present in all potato cultivars, hence the response to anther culture is poor. Moreover, the ploidy of androgenic plants varies broadly and regenerated plants can be monoploids, mixoploids or polyploids (Veilleux 2005). As a result, maternal haploids offer more advantages than paternal haploids in diploid breeding. Nonetheless, the majority of diploid species, dihaploid species, and their hybrids exhibit gametophytic self-incompatibility, and dihaploids are typically male sterile. This prevents selfing and the production of inbred lines for diploid inbred-based hybrid TPS breeding. In a nutshell, the diploid clones/lines are available for diploid hybrid TPS breeding. However, selecting the best germplasm will be necessary for high yield potential, resistance to biotic and abiotic stresses, and improving quality traits (Bradshaw 2022).

Overcoming self-incompatibility in diploid potato

Self-incompatibility (SI) in diploids and di-haploids is caused by a gametophytic SI system that is controlled by the interaction of a pistil S gene and a pollen S gene (s). Closely linked genes, *S-RNase* and *S-Locus F-box (SLF/SFB)*, which regulate female and male specificity, are present in the S locus and regulate gametophytic SI. Selfing, therefore, was impossible until the fertile hybrids were obtained between *S. phureja* and di-haploid of tetraploid andigena clone (Cipar et al. 1964). However, the first self-fertile plant was found in the *Solanum chacoense*, wild diploid species (Hanneman 1989). Several studies were conducted by Hosaka and his group on this self-compatible clone through selfing and crossing to study the inheritance, gene control and map location in the genome (Hosaka and Hanneman 1998a, b; Birhman and Hosaka 2000). Later, Hosaka and Hanneman (1998a) discovered a single dominant gene in *S. chacoense*, the S locus inhibitor gene (*Sli*), which causes self-incompatible plants to become self-compatible. The *Sli* gene is expressed sporophytically to block the action of the S allele in pollen.

In diploid potatoes, the self-incompatibility locus, or S locus, has been found on chromosome 1 (Gebhardt et al. 1991), while the *Sli* gene was mapped using DNA sequencing and linkage analysis through DNA markers at the distal end of chromosome 12 (Hosaka and Hanneman 1998b; Peterson et al. 2016; Clot et al. 2020). The existence of the S locus and *Sli* gene on different chromosomes shows their independence from each other. However, the *Sli* gene interacts with the gametophytic self-incompatibility system in pollen and results in self-compatible (SC) plants (Hosaka and Hanneman 1998b). Through bulked segregant K-mer mapping approach and allele mining of already available DNA sequence data of the M6 clone, the candidate region of the *Sli* gene was

narrowed down to 333 Kb from the 1.5 Mb region on chromosome 12 in the potato genome (Clot et al. 2020). Using an F₁ population recombinant screening approach, Eggers et al. (2021) also mapped the *Sli* locus on chromosome 12 to the same region and reduced the interval to 12.6 Kb region, which contained two genes. To further narrow down to the candidate gene, functional characterization through expression analysis and CRISPR knock-out revealed that the PGSC0003DMG400016861 is *Sli* gene (Eggers et al. 2021). However, expression analysis of the *Sli* gene showed that a self-compatible allele is explicitly expressed in pollen, which may be attributed to the presence of MITE in the *Sli* promotor region, which needs further confirmation (Eggers et al. 2021). Although (Hosaka and Hanneman 1998a) reported a close association of *Sli* with lethality, *Sli* gene homozygous lines M6 and Solyntus could be produced later, stating that lethality is not associated with *Sli* homozygosity or the lethal allele have been removed through recombination breeding in the development of *Sli* gene homozygous lines (Eggers et al. 2021). Another identical gene to *Sli*, a non-S-locus F-box (*NSF*), was identified in long-day adapted highly fertile and vigorous diploid line, RH89 (Ma et al. 2021). This *NSF* gene also functions like a general S-locus inhibitor to introduce self-distortion in the F₂ population; however, it suggests the association of the lethal allele with the *Sli* self-incompatibility allele. It is also unclear whether the *Sli* allele inhibits all S alleles' function for self-incompatibility needs further research to resolve this question. Recently, Eggers et al. (2024) devised the most accurate protocol to phenotype the self-compatibility associated with the *Sli* allele.

Further, using *S. chacoense* as a *Sli* gene donor has several disadvantages owing to undesirable traits in the species. The major undesirable traits in *S. chacoense* are very long stolons and toxic steroidal glycoalkaloids. Therefore, Ye et al. (2018) employed an alternative method, CRISPR-Cas9, to create self-compatible diploids devoid of wild DNA fragments. CRISPR–Cas9 system was successfully used to develop self-compatible diploid lines through loss-of-function mutation in the *S-RNase* gene of self-incompatible *S. tuberosum* Phureja (clones S15-65, S15-47 and S15-76) and *S. tuberosum* Stenotomum (clones S15-107 and S15-48). Likewise, Enciso-Rodriguez et al. (2019) also used the CRISPR–Cas9 technique to create targeted *S-RNase* gene knockout lines of hybrid clones viz., DRH-195 and DRH-310 of *S. tuberosum* Group Phureja, DM 1–3 516 R44 and *S. tuberosum* Group Tuberosum, RH 89–039-16. They found nine *S-RNase* knockout lines in the T₀ generation, transmitting self-compatibility in their progenies in the T₁ generation. The discovery of self-incompatibility and its inhibitor genes paved the way and opened new doors to explore diploid inbred lines

development in a diploid hybrid TPS potato breeding program (Fig. 2).

Marker assisted selection for self-compatibility

Diploid inbred-based hybrid TPS potato breeding requires the introgression of consistently transmitted self-compatibility to self-incompatible elite diploid material, primarily tuberosum background. Clot et al. (2020) developed 18 Kompetitive Allele Specific PCR (KASP)TM markers in Dutch germplasm encompassing a 224 kb region associated with *Sli*. Using six markers, Kaiser et al. (2021) established that M6 is homozygous for *Sli*. In addition, they discovered self-compatible *Sli* alleles in dihaploids of three lines viz., Atlantic, Superior and NY148. The results revealed that *Sli* genotyping has the potential to identify self-compatibility (SC) in *S. tuberosum* dihaploids. They also examined how *Sli* was transmitted in a diploid recurrent selection and back-cross breeding populations, designed to introgress SC while improving agronomic characteristics. During four cycles of recurrent selection, the homozygous self-compatible *Sli* genotype frequency increased as determined by six *Sli* markers. Out of six markers, five marker loci *Sli*_090, *Sli*_304, *Sli*_561, *Sli*_626 and *Sli*_898 on chromosome 12 (within an 80.8 kb region) perfectly predicted the SC phenotype (Kaiser et al. 2021). Similarly, 10 KASP markers of the *Sli* gene showed perfect association with the phenotype for self-compatibility (Sood et al. 2024). The results inferred that these KASP markers can be deployed for high throughput rapid screening of self-compatibility in diploid populations. Although KASP markers have shown their potential in identifying self-compatible diploid potato lines (Kardile et al. 2022), the gene-specific primers will be more accurate and advantageous. Therefore, efforts should be made to develop the *Sli* candidate gene (PGSC0003DMG400016861) markers for self-compatibility screening.

Homozygosity and inbreeding depression

The expression of the *Sli* gene was investigated in several genetic background in order to increase the gene's global applicability in potato breeding and genetics (Phumichai et al. 2006). The study revealed that the self-compatibility of the hybrid is significantly influenced by the cytoplasm type of the female parent and the genotype of the *Sli* gene in the pollen parent. Non-suppression of self-incompatibility in T and W type cytoplasm was attributed to inherent male sterility linked to cytoplasm type rather than failure of *Sli* gene function. Two *Sli* gene donor recommendations were 97H32-6 and 97H32-14 because of more cultivated genetic background and abundant pollen production (Phumichai et al. 2006). These diploids were selfed for three generations, and evaluation of inbreeding progress for

homozygosity using RFLP markers revealed higher than expected heterozygosity in the S_3 generation, suggesting an association between heterozygosity and fertility. Later, two inbred series diploid lines were produced from self-compatible *Sli* gene homozygous line 97H32-6, one of which could attain 90% homozygosity in the S_5 generation for the first time (Phumichai et al. 2006). The mean heterozygosity was reduced to 8.6% in S_5 and 10.7% in S_4 generations in two different inbred series lines from 100% in the S_0 generation. The heterozygosity reduction rate was around 38%, which is slower than the expected 50% reduction in each generation of selfing. The degree of heterozygosity, measured as the Inbreeding coefficient, reduces at 50% in each self-fertilization, assuming no mutation and selection (Allard 1999). The expected homozygosity through selfing was not practically achieved in each generation as the selection was skewed towards vigorous, fertile plants, favouring heterozygosity (Phumichai et al. 2006; Phumichai and Hosaka 2006). During these inbreeding studies, selection for tuber yield was not carried out, although individuals with good tuber yield were observed in the S_4 generation. The interest in developing homozygous diploid lines further increased after Lindhout et al. (2011) demonstrated the feasibility of the F_1 hybrid potato breeding method at the diploid level in the Netherlands. They transferred the *Sli* gene (from the *Sli* gene donor line provided by Hosaka, as stated above) in elite diploid lines and assessed the F_3 (S_3) generation plants for genome homozygosity using SNP markers. The homozygosity in F_3 families varied from 84 to 94%, while the range was 71 to 100% for individual plants across the populations. Among the tested plants, six plants showed 100% homozygosity based on 24 SNP markers. Likewise, another study reported 73.7 to 87.8% homozygosity in S_3 generation plants, assessed for 99 loci across the genome covering all 12 chromosomes (Sood et al. 2024). However, the tuber yield in S_3/F_3 generation plants varied from very low to over a kg per plant (Lindhout et al. 2011). A similar study in the US identified and developed the highly homozygous diploid inbred line, M6, after seven selfing generations of *Sli*-containing *S. chacoense* clone (Jansky et al. 2014). Self-compatibility is also found in *S. tuberosum* germplasm. Some examples are US-W4 and G254, dihaploids extracted from Minn. 20–20-34 and Gineke, respectively, and the diploid clone RH89-039–16 (Clot et al. 2020). At CIP, 20 *Sli*-bearing lines have been developed in the *S. tuberosum* groups Stenotomum and Phureja to create inbred lines (Ortiz and Mihovilovich 2020).

If we assume that at the beginning, every locus is heterozygous in diploid potato clones and the number of heterozygous tracts is 12, i.e., the haploid number of chromosomes. Then, after 16 generations, the proportion of lines homozygous at all loci will be 0.9949 (Bradshaw 2022). Similar results were practically shown in 14 S_{11} plants with

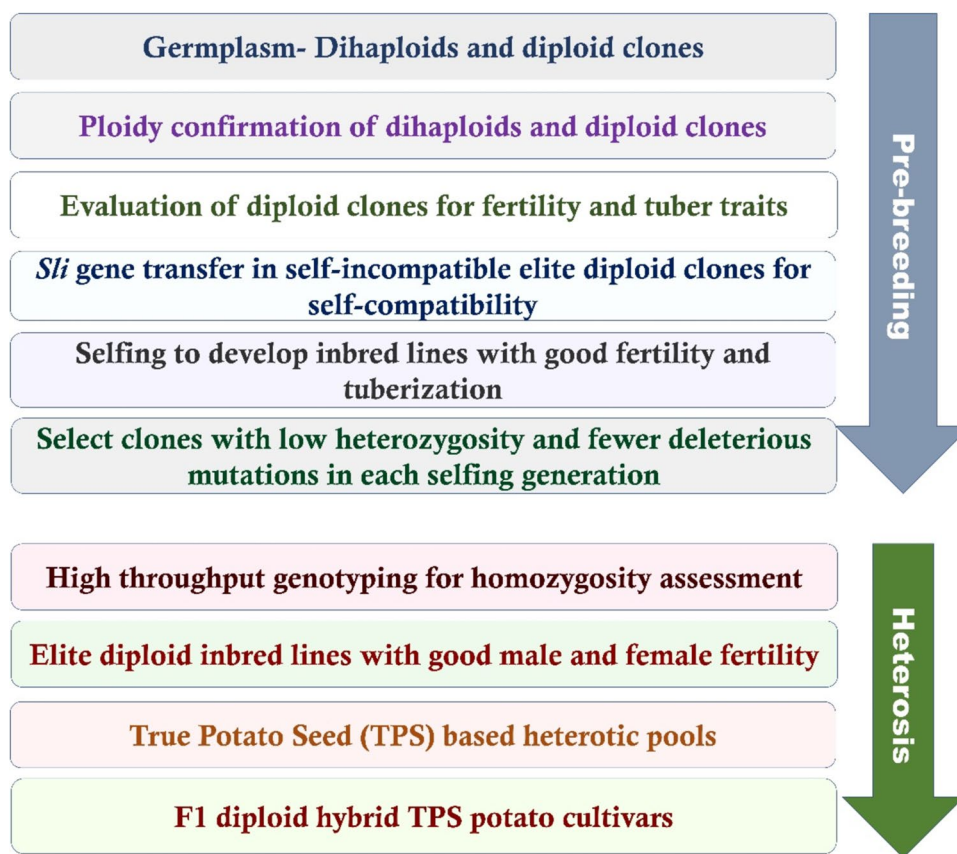
100% homozygosity, verified by the lack of genetic segregation at 19,366 SNPs (Hosaka and Sanetomo 2020). However, around 20% heterozygosity was observed in Solyntus, a F_9 inbred, through SNP rate of 200 SNPs per 30 Kbp as a threshold for heterozygosity evaluation (van Lieshout et al. 2020).

It is generally believed that inbreeding depression will be more severe in clonally produced plants than in seed plants, complicating the development of inbred lines. Many harmful or lethal alleles are expected to reside as heterozygotes in the potato genome, resulting in significant inbreeding depression and limiting continual selfing. These studies showed that inbreeding depression is expressed as reduced pollen viability and tuber yield, limiting further selfing. Inbreeding often reduces vigour and fecundity, continuous selfing eliminates the undesirable recessive alleles as the lines attain homozygosity, which also recovers fertility in advanced selfing generations. However, in diploid potatoes, fertility is not recovered even after 4–5 generations of selfing and most plants do not flower or produce pollen after 2–3 generations of selfing. The results suggested growing large population in each selfing generation to select desirable gene combinations and eliminate undesirable plants. This also prompted an alternative strategy of delaying the inbreeding through sib-mating in the middle of inbreeding generations for more effective selection before fixation of alleles on homozygosity. This has been observed in selecting self-compatible clone 97H32-6, where sib-mating worked rather than selfing in the S_2 generation (Phumichai et al. 2006). However, the clone 97H32-6 was further selfed for 11 generations, and after two decades of selection of 97H32-6, a completely homozygous diploid potato was created. Although it took a long time, continuous selfing removed all deleterious alleles and selected homozygous diploid male and female fertile potato plants with tuberization ability under long days (Hosaka and Sanetomo 2020). All the homozygous lines developed in the above studies showed poor agronomic performance due to severe inbreeding depression, which could be attributed to high heterozygosity, accumulation and maintenance of deleterious alleles in recessive conditions through clonal propagation. Although homozygous lines did not show good tuber traits, these studies set the foundation for genetic studies of inbred line development in diploid potatoes (Fig. 3).

Genetic basis of inbreeding depression in potato

A self-compatible diploid inbred line, namely 'Solyntus,' which is an F_9 inbred line, was identified to possess heterozygosity above 20% (van Lieshout et al. 2020). When there is more than 20% genome heterozygosity, too many traits will segregate, which will restrict the homogeneity and commercial viability of any ensuing F_1 hybrids (Stokstad

Fig. 3 Schematic representation of reinventing vegetatively propagated tetraploid potato into a diploid inbred based TPS hybrid crop



2019). The data suggests that the conventional method of selfing and selection is not sufficient to achieve the intended results in breeding. The haplotype-resolved genome map of heterozygous diploid clone RH89-039-16 (RH) was recently published by Zhou et al. (2020). The map demonstrated that deleterious alleles were distributed across both haplotypes, which introduced complexity to eliminate deleterious alleles or stack beneficial alleles via meiotic recombination. Even after numerous generations of selfing, phenotypic selection would make it impossible to break the linkage of two strongly coupled detrimental mutations in the repulsion phase, resulting in the high heterozygosity of inbred lines. To address these challenges in producing superior inbred lines, a thorough understanding of potato genomes is necessary.

Inbreeding depression reduces fitness in progenies of genetic relations. Potato suffers from severe inbreeding depression as a clonally produced crop, yet the genetic cause of inbreeding depression in potatoes is mostly unclear. The origin and consequences of several particular loci generating inbreeding depression were carefully explored in 151 diploid potatoes to acquire insight into inbreeding depression, and 344,831 projected harmful substitutions were obtained. Line-specific harmful mutations in potatoes are abundant in the pericentromeric areas. Zhang et al. (2019)

used three F2 populations to identify 15 genomic areas with substantial segregation abnormalities caused by selection during the gametic and zygotic phases. Most harmful recessive genes impacting survival and growth vigour were found in areas with high recombination rates. One of these detrimental alleles of *ar1* mutation in potatoes is a rare mutation that affects a gene needed for embryo development (*StSIEL*-SHORT-ROOT INTERACTING EMBRYONIC LEATHAL). It explains why past attempts to inbreed potatoes failed (Bradshaw 2009); nevertheless, the second line of evidence demonstrates that eliminating around half a dozen detrimental mutations per parent in one generation is possible (Zhang et al. 2019). It's still unclear how to resolve this seeming contradiction. This research lays the groundwork for the genomic design of potato inbred lines (Bachem et al. 2019; Zhang et al. 2019).

Another deleterious mutation affecting fertility, designated as floral bud abortion 1 (*fbal*), was located on chromosome 2 and is orthologous to the Arabidopsis *DYT1* gene. Similarly, white flesh allele (*y*) was also associated with a large effect deleterious mutation. Other deleterious mutant alleles were *yl1* (yellow leaf 1 associated with virescent phenotype) and *led1* (large-effect deleterious mutation 1) mapped on chromosome 12 (Zhang et al. 2021). Another study based on diverse diploid potato panel inferred 367,499

deleterious variants, 50% at non-coding and 15% at synonymous sites (Wu et al. 2023). These studies inferred that diploid lines with relatively high homozygous deleterious burden could be better founder material for inbred-line development.

Genome sequence of diploid inbred lines

The cultivated potato is a highly heterozygous and auto-tetraploid, making genome analysis and breeding difficult. Wild potato species are an important source of key genes which can be introduced into cultivated potatoes. The first genome to be made public was that of the wild cousin of the cultivated potato, *S. phureja* DM1-3 516 R44, a doubled monoploid often known as DM (Potato Genome Sequencing Consortium, 2011). This eventually resulted in a reference containing firmly orientated contigs along multiple unanchored super-scaffolds (Sharma et al. 2013). This initial reference genome was extremely useful for genetic research. However, the high frequency of repeated sequences and short read lengths may have led to problems in sequencing and assembly. Furthermore, substantial genetic divergence between DM and more economically advantageous potato genotypes has impeded thorough genome research (Uitendilligen et al. 2013).

In comparison to DM, the diploid inbred clone M6 of *Solanum chacoense* has remarkable qualities in terms of self-compatibility, tuber market quality and resilience to diseases. The sequencing and annotation of M6 clone resulted in 508 Mb of the assembled genome into 12 chromosomes with 49,124 high-confidence gene models representing 37,740 genes. Comparison of M6 genome to the genomes of six more Solanaceae species resulted in a core group of 158,367 *Solanaceae* genes and 1897 genes exclusive to three potato species were identified. The analysis of SNP across the M6 genome found that chromosomes 4, 8, and 9 have higher residual heterozygosity than the other chromosomes. Thus, the availability of the M6 genome facilitates the identification of pivotal genes associated with essential agronomic characteristics and will further aid in the development of diploid inbred lines ultimately accelerating the breeding process for potatoes (Leisner et al. 2018).

Another “pure potato” reference line ‘Solyntus’ was developed to facilitate genetic studies at the diploid level (van Lieshout et al. 2020). To provide a high-quality de novo assembled reference genome, the Solyntus genome was sequenced utilising an improved sequencing technique for high coverage and long reads in conjunction with ultra-deep short-read sequencing. Solyntus is a true *Solanum tuberosum* line that is highly homozygous, diploid, robust, and self-compatible. Each pseudochromosome was formed by assembling 116 contigs into scaffolds, with three

to seventeen contigs per chromosome (van Lieshout et al. 2020). The availability of very accurate genome sequence allows faster and more focused breeding, as it is easier to find at an early stage whether the inbred diploid potato lines have the desired traits, such as resistance to specific diseases.

To study the genome evolution and diversity, the genome sequencing of 9 non-inbred adaptable diploid potato clones was generated at Agriculture and Agri-Food Canada (Achakagari et al. 2022). De-novo genome assembly revealed the genome size ranged from 712 to 948 Mbp. The k-mer-based sequence-read analysis revealed a genome heterozygosity range of 1 to 9.04% amongst clones and the harmful alleles were spread across the genome, including areas with overlapping segregation abnormalities. The study also discovered allelic variations of the *StCDF1* gene that induce early tuberization among these clones. Similarly, Tang et al. (2022) assembled 44 high-quality diploid potato genomes from 24 wild and 20 cultivated accessions, identified 561,433 high-confidence structural variants and constructed a map of large inversions, which provided insights for improving inbred lines. The information generated through genome sequences of different diploid clones will help us understand the genetics of key traits associated with potato breeding at the diploid level.

Genetic improvement of diploid inbred lines

Although tetraploid potato breeding has been successful, it has required a substantial investment of resources because large populations must be raised over an extended period. The genetic studies using homozygous diploid lines are much more manageable and straightforward. Endelman and Jansky (2016) created the first diploid inbred line-derived F_2 population and showed its applicability for genetic mapping. The population was created by crossing the doubled monoploid potato DM1-3 as a female to M6 as a male parent, then self-pollinating a single F_1 plant. M6 is a S7 inbred line obtained from wild relative *S. chacoense*. To strengthen the physical anchoring of super scaffolds in the potato reference genome based on DM1-3, a genetic linkage map with 2264 single nucleotide polymorphisms (SNP) was created. Most tuber traits, such as flesh colour, skin colour, pigments, and tuber shape, showed segregation. They identified ten genes using the R/qtl software; seven were already mapped, and three were newly discovered. Likewise, F_2 population progenies from diploid inbred populations resulting from a hybrid between *S. tuberosum* (D2) and *S. chacoense* (DS) were utilised in another study to map numerous quality features, including tuber form, flesh and skin colour (Meijer et al. 2018). They identified QTLs for all the traits investigated, coinciding with some previously mapped and a few newly identified loci. To validate the QTLs and track the

overall increase in homozygosity, nine F_3 populations were employed.

Another study used GWAS, linkage mapping, and multi-allelic QTL detection using identity-by-descent (IBD) probabilities to identify QTLs for tuber fresh weight using multiple diploid potato F_3 families derived from a cross between an inbred *S. chacoense* and an outbred diploid *S. tuberosum*. The study discovered that IBD information may be interpreted more directly and precisely by tying QTL alleles to the founders (Korontzis et al. 2020).

The most homozygous inbred lines should be employed for genetic research on important potato qualities such as earliness, plant vigour, and tuber quality. Furthermore, resistance genes to *Phytophthora infestans* or other biotic stressors could be introgressed by marker-assisted backcrossing to improve the elite inbred lines. In 2–3 years, this process can enrich an existing diploid hybrid with resistance genes. Similarly, a mutant population could be developed from inbred lines to target and identify desirable mutations, which was lacking earlier in potatoes. The inbred lines can also be employed in research on plant development, biotic and abiotic stress tolerance, and estimate breeding value to enhance the genetic gain.

Introgression of genes in elite diploid inbred lines

The breeding program's success depends upon the speed of trait introgression. The trait introgression can be done through phenotypic and/or molecular marker-assisted selection. SNP markers make trait introgression and monitoring straight-forward and cost-effective. Very few markers are available for trait introgression in cultivated tetraploid potatoes. However, trait mapping and genomics-assisted breeding, especially marker-assisted backcrossing, can be applied in diploid inbred lines, similar to major crops like rice, maize, etc. The initial inbred diploid potato lines were not very good, but with persistent breeding, they were improved to the point where the first hybrid cultivars were acceptable (De Vries et al. 2016; Lindhout et al. 2018). The diploid inbred lines need to have key agronomic, stress tolerance and quality traits for the success of diploid inbred-based TPS hybrids (Fig. 3). The first instance of marker-assisted introgression of four distinct late blight resistance genes in selected highly homozygous, diploid potato lines was demonstrated by Su et al. (2020). With the help of two backcrosses and one selfing, the original line was able to regain its value by becoming resistant to *Phytophthora infestans*. After crossing two diploid lines with differing resistance genes, hybrids were produced and evaluated for late blight resistance in field experiments. The results revealed that hybrids with two resistance genes outperformed compared

to hybrids with only one of the two resistance genes (Su et al. 2020).

Hybrid breeding and heterosis using diploid inbred lines

Two complimentary breeding strategies can be applied when a pool of self-compatible germplasm is available: (1) recurrent selection, which focuses largely on tuber yield and attempts to preserve beneficial alleles, and (2) line development to sustain fertility. Alsahany et al. (2021) established the viability of recurrent selection within a diploid pool of the initial generation. On the contrary, inbreeding depression poses a significant obstacle in the development of diploid potatoes, necessitating the implementation of pedigree-based selection to prioritise fertility and vigour above other traits (Zhang et al. 2019). Thus, pedigree selection can generate fertile and healthy lines to reduce inbreeding depression. Then, for complex traits, recurrent selection may be used to recombine advantageous alleles (de Vries et al. 2023). The best-performing lines are thought to have the highest breeding value and can be crossed to produce experimental hybrids. If we look at the performance of diploid hybrid potatoes today, it is nascent, as maize was in the 1930s. An increase of 500% in Maize yields was observed from 1930 to 2000, mainly due to the introduction of single cross hybrids (Beumer and Edelenbosch 2019). Therefore, researchers are optimistic about the diploid hybrid TPS system in potato (Fig. 3).

Initial trials of diploid hybrids conducted by Solynta in Europe and Africa showed encouraging results compared to tetraploid controls. In China, two F_1 hybrids of inbred homozygous lines (A6-26 and E4-63) showed strong morphological heterosis over the parents (Li et al. 2024). A multi-omics method was used to obtain insight into the genetic and molecular basis of heterosis in potatoes, employing transcriptome, metabolome, and DNA methylation data at three developmental phases (seedlings, blooming, and tuber stage) (Li et al. 2022, 2024). The findings demonstrated that positive heterotic effects of yield-related QTLs and negative heterotic effects of metabolite QTLs (mQTLs) contribute to yield heterosis. In hybrids, gene-dominant complementation was evident in flower colour, male fertility, starch and sucrose metabolism. Additionally, they discovered 2,700 allele-specific expression genes at each stage. These genes are probably involved in potato heterosis and could be impacted by the methylation levels of the CHH allele (Li et al. 2022). Another study showed a higher magnitude of the general combining ability for yield phenotypes than the specific combining ability in diploid hybrid potato, which revealed the importance of additive effects for all traits and suggested that the mid-parent value

alone is helpful for hybrid potato evaluation (Adams et al. 2022). Li et al. (2024) identified a PME gene with a dominance heterotic effect that plays an important role in male fertility heterosis. More studies are required to assess the performance of different inbred lines and understand the genetics and molecular mechanism of F_1 hybrid heterosis in diploid potatoes. Concerted research efforts and the availability of cutting-edge new techniques, especially gene editing and genomic selection, can add value to a hybrid breeding programme.

Advantages and disadvantages of TPS

Following the founding of the ICAR-Central Potato Research Institute in 1949, TPS-based variety development work was prioritised because it looked to be the most appealing prospect in the lowland tropics and subtropics. However, non-uniformity of tubers, longer duration and difficulty in the maintenance of parental lines, as well as the inability to produce sufficient TPS, were major drawbacks of non-continuation of work on TPS in India. The work on TPS breeding at the International Potato Centre (CIP) also suffered similar glitches and could not get the thrust. Despite this, TPS potatoes have demonstrated remarkable efficacy in bolstering food security, specifically for small-scale farmers, in China, Bangladesh, India, Egypt, Nicaragua, Indonesia, Peru, southern Italy, the Philippines, and Vietnam (Ortiz et al. 1997; Chujoy and Cabello 2007). The results of on-farm research on TPS potatoes in India, Indonesia, Peru and Egypt were interesting (Chilver et al. 1994). Seedling tubers observed a high tuber yield of ~20t/ha, comparable to healthy seeds of competing clones. In the northeastern region of India, where TPS is still used as seed material, transplanted seedlings produced relatively good yields of 15–20 t/ha, although the production from clonally propagated seed tubers was 25–30 t/ha. The studies concluded that it is unlikely that large-scale adoption of TPS will occur in the immediate future, but sustained effort on TPS potatoes could be justified in India and China.

Nonetheless, the recent intervention of inbred-based diploid hybrids opened new avenues of research in this direction (Lindhout et al. 2011). The major benefit of TPS-based breeding is reduced seed costs due to much smaller amounts of planting material requirement, easy storage and transport, tuber uniformity, and disease-free planting material, except for some viruses transmitted sexually through TPS. Besides, sexual breeding provides the opportunity for more recombination events, development of mapping populations for genetic studies, trait introgression and improvement of existing varieties. There are several technical glitches of diploid inbred-based hybrid TPS like the mechanism of hybrid seed production on a large scale, agronomic management,

crop duration, direct TPS seeding or seedling transplanting or seedling tubers will be used as the planting material. It will take time to tackle these hurdles scientifically to make the new TPS technology as vibrant as the clonal tuber-based propagation methodology. Scientifically, the benefits of inbred-based diploid hybrid TPS technology outweigh the conventional clonal method, but farmers and seed stakeholders in countries like India will decide the fate of the TPS.

Future research directions

Diploid hybrid TPS potato breeding is a promising approach to potato breeding, but there is still much to learn and develop to realize its full potential. Here are some future research directions that could help to advance the field:

- *Genetic analysis and genome editing* Advances in genetic analysis and sequencing technologies can help identify and characterize the genetic makeup of diploid inbred lines, which can aid in selecting plants with desired traits. Moreover, diploid potato lines are useful for genome editing because they are simpler to work with than polyploid lines and agrobacterium mediated transformation can be used in diploid inbred lines to generate DNA free genome editing products (SDN1 and SDN2) by segregating out any insertions of foreign DNA through selection during selfing.
- *Genetic mapping and marker-assisted selection* Developing molecular markers linked to important traits could improve the efficiency and accuracy of diploid hybrid TPS potato breeding. Genetic mapping could also help to identify regions of the genome associated with important traits and could lead to the discovery of novel genes and genetic pathways.
- *Climate adaptation* As global climate changes, developing potato cultivars adapted to new and changing environmental conditions will be increasingly important. Diploid hybrid TPS breeding could play a key role in developing new cultivars with enhanced tolerance to abiotic stressors like drought and heat.
- *Disease resistance* Developing cultivars with enhanced resistance to pests and diseases is an ongoing challenge in potato breeding. Diploid hybrid TPS breeding could provide new opportunities for developing cultivars with novel disease-resistance genes and help identify new sources of resistance in wild and exotic germplasm.
- *Multi-trait selection* Combining multiple desirable traits in a single cultivar is a complex challenge that requires careful selection and breeding. Diploid hybrid TPS breeding could help overcome this challenge by enabling the selection of desirable traits at the diploid level,

reducing the number of generations required to produce a stable cultivar with multiple desirable characteristics.

- *Improving efficiency* Diploid hybrid TPS breeding is still a relatively new approach to potato breeding, and there is a need to develop more efficient and scalable methods for TPS extraction, hybridization, and selection. Developing new techniques and protocols for large-scale TPS production and selection could help to accelerate the development of new cultivars.

Overall, these future research directions highlight the potential for Diploid hybrid TPS breeding to contribute to developing new and improved potato cultivars adapted to changing environmental conditions and can help meet the increasing demand for food worldwide.

Glossary

TPS	True potato seed
<i>Sli</i>	Self incompatibility locus inhibitor
DHTPS	Diploid hybrid true potato seed
SC	Self-compatibility
MITE	Miniature inverted-repeat transposable elements
CHH	Cytosine methylation in the context of CHH, where H can be C, T, or A.
PME	A pectin methylesterase gene that affects pectin homeostasis in cell walls and regulates plant growth
Low propagation coefficient	Number of propagules multiplied from one propagule i.e. tuber in one generation.
Double reduction	Double reduction is a condition that occurs when sister alleles move to the same gamete during meiosis.
Solynta	Solynta is a Dutch biotechnology company that specializes in hybrid potato breeding.

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Declarations

Conflicts of interest The authors claim to have no conflicts of interest.

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