Research article

Development of near homozygous lines for diploid hybrid TPS breeding in potatoes

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ABSTRACT

Diploid inbred-based F_1 hybrid True Potato Seed (DHTPS) breeding is a novel technique to transform potato breeding and cultivation across the globe. Significant efforts are being made to identify elite diploids, dihaploids and develop diploid inbred lines for heterosis exploitation in potatoes. Self-incompatibility is the first obstacle for developing inbred lines in diploid potatoes, which necessitates the introgression of a dominant S locus inhibitor gene (*Sli*) for switching self-incompatibility to self-compatibility. We evaluated a set of 357 diploid clones in different selfing generations for self-compatibility and degree of homozygosity using Kompetitive Allele Specific PCR (KASP) Single Nucleotide Polymorphism (SNP) markers. A subset of 10 KASP markers of the *Sli* candidate region on chromosome 12 showed an association with the phenotype for self-compatibility. The results revealed that the selected 10 KASP markers for the *Sli* gene genotype could be deployed for high throughput rapid screening of self-compatibility in diploid populations and to identify new sources of self-compatibility. The homozygosity assessed through 99 KASP markers distributed across all the chromosomes of the potato genome was 20–78 % in founder diploid clones, while different selfing generations, i.e., S_0, S_1, S_2 and S_3 observed 36.1–80.4, 56.9–82.8, 59.5–85.4 and 73.7–87.8 % average homozygosity, respectively. The diploid plants with ~80 % homozygosity were also observed in the first selfing generation, which inferred that homozygosity assessment in the early generations itself could identify the best plants with high homozygosity to speed up the generation of diploid inbred lines.

1. Introduction

Potato is a major staple food all around the globe and is consumed in both fresh and processed forms. Due to its high heterozygosity and polyploid genome, it is reproduced clonally through tubers to preserve the purity of cultivars. The cultivated potatoes are self-compatible auto-tetraploids. However, selfing in heterozygous tetraploid potato clones results in significant inbreeding depression [1]. As a result, the homozygous lines could not be developed in tetraploid potatoes, and the varieties and advanced breeding material are only maintained as tubers. The clonal propagation through tubers leads to the accumulation of pests and viruses, which keeps multiplying during each cycle of clonal propagation. As a result, the production and acceptance of varieties decreases. True potato

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seeds or botanical seeds have never been employed commercially as a method of propagation of potato varieties that is otherwise easy to maintain, transport, store, accessible to genetic alteration, and devoid of disease or pest inoculums.

Most tuber-bearing potato species are diploids and are valuable sources of resistance genes and allelic diversity to improve quantitative traits. Different methods have been used to transfer important genes from diploids to tetraploids [2]. The process is cumbersome, non-targeted and does not allow the recreation of the same genotypes through sexual reproduction and/or the transfer of a few genes in the same background. This shifted the focus on identifying elite diploid clones, inbred development and heterosis exploitation in potatoes.

Lindhout et al. [3] devised and empirically tested a novel strategy called "Diploid Inbred Hybrid TPS" to avoid these shortcomings of standard potato breeding. Propagation of potatoes through diploid hybrid true potato seed (DHTPS) is a new avenue in potato breeding, which could lead to a "green revolution" in potato cultivation across the globe [4]. The method is comparable to the breeding approach utilized in important cereal crops like rice and maize, where homozygous inbred lines are developed and heterotic combinations are assessed. Several groups have been working on transforming potato cultivation from tetraploid tuber form to DHTPS for targeted trait gene discovery and introgression in a fast and iterative process [3–7]. However, gametophytic self-incompatibility, common in diploid species and dihaploids of potatoes, is the main obstacle to developing inbred lines at the diploid level. A pioneering work on the identification of natural self-compatible diploid clone (chc 525-3) for the first time in the species *Solanum chacoense* carrying S-locus inhibitor gene (*Sli*), a dominant locus to induce selfing in hitherto self-incompatible diploid lines laid the foundation of inbreeding in diploid potatoes [8]. Later, the same self-compatible clone chc 525-3, was selfed for seven generations to develop near homozygous line, M6 by Ref. [9].

Similarly, the self-compatibility was also observed in *S. tuberosum* clones, i.e., US-W4 and G254, the dihaploids extracted from Minn. 20-20-34 and Gineke, respectively [10,11]. The *Sli* gene was mapped on the distal end of chromosome 12 in many independent studies [8,12–14], which opened the avenues for marker-assisted selection for self-compatibility in potatoes [15]. In addition to *S. chacoense*, some plants in other diploid germplasm lines were also found to set spontaneous berries, which broadened the genetic base of transferring self-compatibility in developing inbred lines for DHTPS breeding [15]. Nevertheless, besides self-compatibility, homozygosity assessment is also of practical importance in identifying highly homozygous diploid inbred lines in early selfing generations [4].

The evaluation of self-compatibility requires growing all plants till flowering and assessing each plant for selfing followed by berry set. The development and availability of *Sli*-specific KASP markers from the candidate *Sli* region on chromosome 12 have simplified the selection of self-compatible plants for inbreeding in the DHTPS approach [15,16]. Moreover, markers distributed across the genome could be used to identify highly homozygous lines in the early selfing generations [4]. Thus, the present study aimed to validate 10 *Sli* KASP markers for self-compatibility in diploid germplasm collection, design and validate 100 new KASP markers for homozygosity/heterozygosity in different inbreeding generation progenies, and investigate the KASP markers efficacy for marker-assisted selection in speeding up the inbred development in diploid potatoes.

2. Materials and methods

2.1. Plant materials

The materials included founder diploid accessions and diploid populations in different inbreeding generations. This included 132 founder diploid clones of different genetic backgrounds, 18 S_0, 62 S_1, 95 S_2 and 50 S_3 generation diploid lines. The 132 diploid clones included diploid breeding clones (derived from diploid TPS) and dihaploids (derived from tetraploid potato varieties) (Table S1). The S_0 generation had 15 F_1 crosses of elite diploid lines with *Sli* donor lines, three diploid populations viz., DM × M18, DM × M6, USW4 × M6. The S_1 and S_2 generations had selfed plants of three diploid populations, i.e., DMxM18, DM × M6, USW4 × M6, while the S_3 generation had selfed plants of only two populations (DM × M18, DM × M6). In each generation, the diploid TPS was grown in trays and 25–30 days old seedlings were transplanted in pots for clone formation and selfing. The diploid accession PI 654351 (*S. chacoense* clone 97H32-6) was used as source of the *Sli* gene for self-compatibility.

DM is a double monoploid used for genome sequencing [17], while M6 is a highly fertile vigorous inbred of S. chacoense developed through seven generations of selfing [9]. Likewise, M18 is a USW4 inbred line and USW4 is a self-compatible dihaploid of Minn 20-20-34 [18]. The clones M6 and M18 are homozygous while USW4 is heterozygous for *Sli* gene. Three F_1 populations (DM × M18, DM × M6, USW4 × M6) were received as 50 TPS each from the US Potato genebank. The F_1 seed of these three populations were grown and individual plants were selfed to generate segregating progenies in selfing generations.

All the plants in an F_1/S_0 generation represent one population and different F_1/S_0's denote different populations, while the seed produced after selfing an individual plant/clone of a population makes one family and different plants make different families of a population.

2.2. Flow cytometry for ploidy analysis

The analysis was performed as described by Kardile et al. [19]. Observations were recorded on the relative amount of DNA by comparison of the G1 and G2 peaks of diploid samples using diploid (*S. chacoense*) and tetraploid cultivar (Kufri Girdhari) as external standards.

2.3. Pollen viability analysis

Pollen viability was examined in all the diploid accessions and inbreeding population progenies using a 2 % acetocarmine stain, visualized under the compound microscope. The representative field view at three different sites of the entire slide was used for analysis. The pollen grains that were turgid, round and stained red were considered viable.

2.4. Phenotyping for self-compatibility

The diploid clones obtained from TPS and in-vitro cultures were phenotyped for self-compatibility from 2018 to 2020 in the glasshouse (temp 25–27 °C day and 18–20 °C night; humidity 70–80 %) at ICAR-Central Potato Research Institute, Shimla. All tuber-forming plants in each generation were maintained and used for planting in the next season. Self-pollinations were done using fresh pollen collected from open flowers and self-compatibility was determined through fruit set [20]. We did ≥10 pollinations of individual flowers in a plant to determine self-compatible phenotype-the clones where the flowers did not show berry setting after repeated pollinations were considered self-incompatible.

2.5. Sample collection and preparation

The founder diploid clones (132) and S_0 (18) plants were raised in the pots using tubers for all 150 accessions. The different selfing generations (S_1, S_2 and S_3) plants were raised from selfed seed in trays and seedlings were transplanted in the pots. The plant materials were raised from March to September in the glasshouses in 2020 at ICAR-Central Potato Research Institute, Shimla, HP, India. The leaf samples were collected for genotyping and fresh leaves were used to punch two leaf discs of size ~5–6 mm diameter using a single-hole puncher and placed in 96 deep well plates. The plates were kept in the oven at 40 °C for 24 h to dry the samples and sealed with sealing mats. The dried leaf samples were used for automated high-throughput DNA isolation and PCR analysis as per the standard protocol of LGC genomics, UK [21].

2.6. KASP markers

Out of 18 haplotype-specific KASP markers designed from SNPs in the *Sli* candidate region on Chr 12 [15], we used a subset of 10 KASP assays. For homozygosity evaluation of the diploid clones in different generations, we designed 100 SNP KASP markers from PotSNPs [22]. Out of 384 SNPs identified by Anithakumari et al. [22], we initially selected 10 SNPs per chromosome (a total of 120 markers in the genome) based on their position on the genome through nucleotide BLAST in the potato genome website (http://spuddb.uga.edu/) using *Solanum tuberosum*, DM 1–3516 R44 v6.1 (DM) as the reference genome. The selected markers were evenly distributed over the 12 chromosomes, including the most distal telomeric and centromeric markers. The SNPs were selected with balanced allele frequency and informativeness. The marker sequences were selected to get at least 50bp sequences on both sides of the SNP for designing KASP markers using DM as the reference genome. The markers showing a mismatch in this 101 bp sequence with the DM were discarded and finally, 100 markers were selected for designing KASP assays (Supplementary Material Table S2).

2.7. Genotyping

DNA extractions, quality control and genotyping using KASP technology (LGC, UK) was done at INTERTEK Laboratory in Hyderabad, India, following standard procedures as detailed in Ref. [21] and https://biosearch-cdn.azureedge.net/assetsv6/KASP-anchoring-explaination.pdf. The marker data output in each case was further visualized using SNPViewer software (LGC Genomics, Beverly, MA, USA).

2.8. Evaluation of diploid clones

All founder clones (132) and selected S_0 (15), S_1 (20) and S_2 (25) generation lines were planted from tubers in net-house at CPRI, RS, Modipuram in the year 2020-21 winter season for tuber multiplication and visual observations on tuber traits, i.e., tuber shape, tuber size, tuber number, tuber skin colour and tuber flesh colour as per the DUS descriptors of potato (https://plantauthority.gov.in/sites/default/files/fpotato.pdf). The number of tubers planted for each clone varied from 2 to 5. Each clone was planted in a row with a row-to-row spacing of 40 cm and plant-to-plant spacing of 20 cm. The crop duration was 100 days and tubers were harvested manually using hand tools.

2.9. Statistical analysis

The marker genotype was associated with the self-compatible or self-incompatible phenotype and the Chi-square test for goodness of fit was used to test and validate the efficiency of 10 *Sli* KASP markers. Similarly, the homozygosity markers data for 99 SNP loci were used to calculate the per cent homozygosity of each diploid clone in the Excel worksheet.

3. Results

Flow cytometry-based ploidy analysis confirmed that all the founder clones were diploids (Fig. 1). Diploids (Fig. 1A) were clearly distinguished from tetraploids (Fig. 1B) based on the histograms in flow cytometer. Selfing was attempted but no berry setting was observed in these clones which indicated self-incompatibility. However, the *Sli* donor line and progenies of three F_1 populations (DM × M18, DM × M6, USW4 × M6) were fertile and self-compatible.

3.1. Inbred development through selfing

Twenty-one selected founder clones were crossed as female with *Sli* gene donor line, resulting in 15 successful crosses. The flowering in the founder clones was sparse in most cases and introgression of self-compatibility gene through crossing observed few seeds (30–100 TPS). TPS of these 15 crosses and 3 diploid populations (DM × M18, DM × M6, USW4 × M6) formed the S_0 generation. The seed of all these 18 families was grown, however, the plants of only five families (DM × M18, DM × M6, USW4 × M6, C13 × PI 654351, BS240-1 × PI 654351) flowered and showed berry setting on selfing. The plants in the S_0 generation were self-compatible, as confirmed through genotype and phenotype data (except a few plants which did not flower and could not be confirmed for phenotype). In the next generation, the S_1 seeds of five populations were grown and plants were observed for flowering and selfing. The plants of three populations viz., DM × M18, DM × M6, USW4 × M6 observed profuse flowering while the plants of two populations (C13 × PI654351, BS240-1 × PI654351) had sparse flowering. Berry setting on selfing was observed in the plants of previously mentioned three populations only, while the plants of other two populations failed to set seeds. Out of 62 plants, 54 % showed selfing and berry setting. The selfed seeds of these populations were further grown in S_2 and S_3 generation to achieve homozygosity. In S_2 generation, 23 % of plants observed selfing out of 95 plants belonging to three different populations (Table 1). The plants of the population USW4 × M6 failed to set selfed berries in the S_2 generation. Thus, selfing was attempted in 50 plants raised from 12 families of two populations (DM × M18, DM × M6) in the S_3 generation, which resulted in 11 % selfed plants. In the S_3 generation, flower drop was maximum in both the populations. The vegetative and floral parts of the plants were also reduced, and pollen shedding was poor in the S_3 generation.

3.2. Screening for self-compatibility using Sli KASP markers

The *Sli* gene KASP markers were used for screening of diploid clones to avoid time in transferring the *Sli* gene in self-incompatible (SI) clones and study the self-compatibility trait segregation. The allele calls of individual SNPs for all the 357 diploid clones of different selfing generations are presented in Table 2. Based on marker allele consistency of all the ten SNPs, 123 clones were homozygous for the favourable allele (self-compatibility), 109 were homozygous for the unfavourable allele (self-incompatibility), and 89 were heterozygous for the *Sli* gene genotype (Table S1). The allele call inconsistencies among markers were observed in rest 35 clones (Table S1). The results of KASP markers used in the study were consistent with an efficiency of >90 % based on allele analysis for all the 10 markers (Fig. 2a&b; Supplementary Material Fig. S1).

The KASP markers results of all the 10 SNPs were statistically in agreement with SC and SI phenotype as revealed through the Chi-
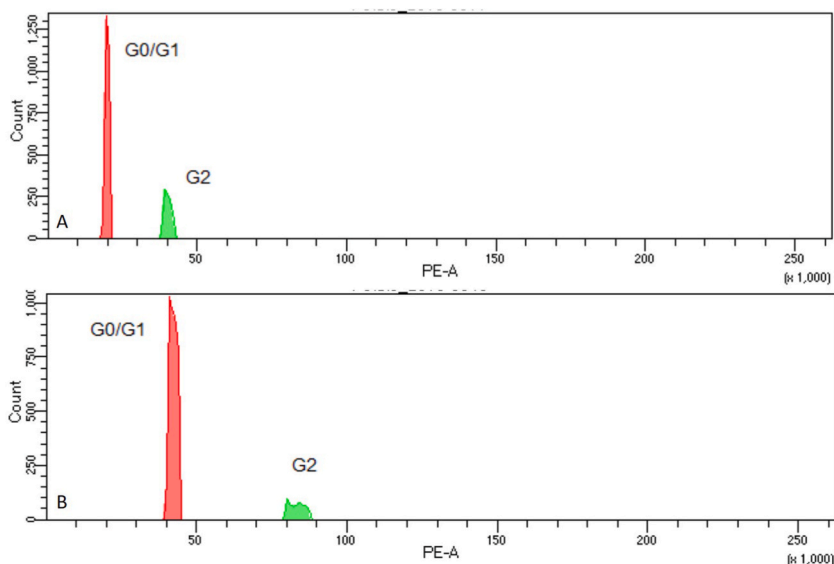

Fig. 1. Flow cytometry-based detection of diploids/dihaploids in potatoes based on PI stain in a flow cytometer A) Diploid B) Tetraploid. Histograms show the relative DNA content (linear scale) of nuclei in the G1 (red) and G2 (green) cell cycle phases. The X-axis represents the nuclear DNA content, while Y axis shows the cell count.

Table 1

Inbred development in potatoes at the diploid level.

Diploid clones	Generation	Accessions/Population	Families	Number of plants	Percent selfers[a]
Founder diploid clones	–	132	–	–	–
Sli introgressed lines	S_0	18	18	540	73
First generation selfing	S_1	5	15	62	54
Second generation selfing	S_2	3	14	95	23
Third generation selfing	S_3	2	12	50	11

  [a] recorded on plant basis in each generation; the number of flowers selfed in each plant varied from 10 to 76; All plants in an F_1/S_0 generation represent one population and different F_1/S_0's denotes different populations, while seed produced after selfing of an individual plant/clone of a population makes one family and different plants make different families of a population.

Table 2

KASP markers-based segregation of diploid clones into homozygous self-compatible (SC), heterozygous and homozygous self-incompatible (SI) genotypes.

S.No.	Marker ID[a]	SNP Position	Marker genotype		
			Homozygous SC	Heterozygous genotype	Homozygous SI
1.	snpST00297	ST4_03ch12_58961580	135	109	109
2.	snpST00298	ST4_03ch12_58962561	135	102	117
3.	snpST00299	ST4_03ch12_58974932	134	103	116
4.	snpST00300	ST4_03ch12_59002442	134	102	117
5.	snpST00301	ST4_03ch12_59019319	134	104	115
6.	snpST00302	ST4_03ch12_59023684	134	104	117
7.	snpST00303	ST4_03ch12_59155291	141	88	127
8.	snpST00304	ST4_03ch12_59184424	130	97	128
9.	snpST00305	ST4_03ch12_59211572	142	85	128
10.	snpST00306	ST4_03ch12_59271443	139	87	128

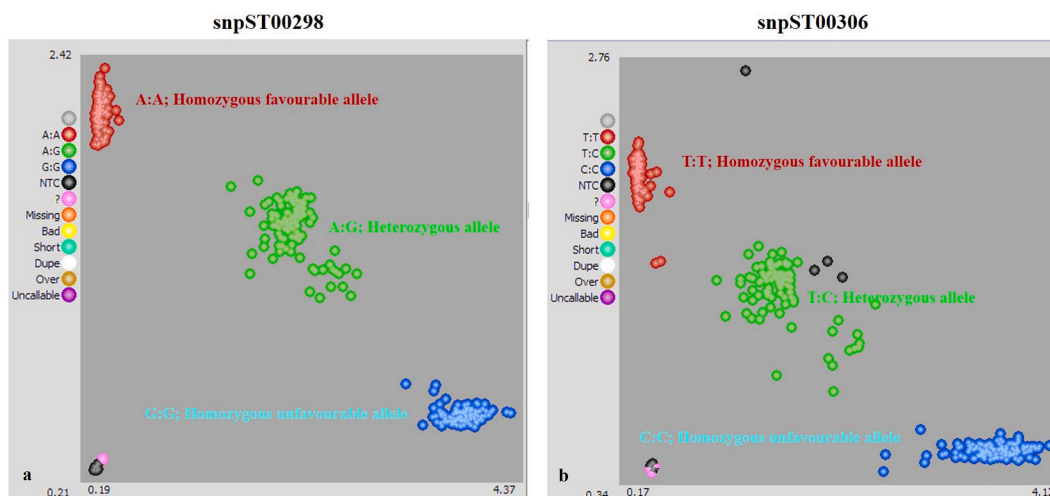 [a] Marker ID corresponds to Intertek SNP ID.

Fig. 2. Scatter plots for two kompetitive allele-specific PCR (KASP) marker assays for self-compatibility a) snpST00298 b) snpST00306. Red dots represent the favourable genotypes (self-compatible), while blue dots show the unfavourable genotypes (self-incompatible). Green dots represent the heterozygous genotypes.

squared test (Table 3). The phenotype data observed through the berry setting revealed that 130 diploid clones were SI and 2 were SC out of 132 founder diploid clones. However, the marker genotype data showed 109 SI homozygous and 2 heterozygous genotypes. Rest 21 clones showed discrepancies with respect to one or the other marker, hence not included in any of three categories of marker genotypes. Likewise, in the inbreeding generations of three diploid populations, all the selfers (Table 1) carried favourable allele for SC, proving the efficacy of *Sli* gene KASP markers for SC. The plants showing berry setting in selfing generations of three diploid populations had the *Sli* gene in favourable homozygous or heterozygous condition.

Table 3
Association of KASP marker genotypes with self-compatible phenotype through Chi-squared likelihood ratio test.

Marker ID	Df	Chi-square	P value	Critical value at p = 0.05
snpST00297	2	181.02	4.92E-40[a]	5.991
snpST00298		174.75	1.20E-38[a]	
snpST00299		183.24	1.62E-40[a]	
snpST00300		176.71	4.25E-39[a]	
snpST00301		172.03	4.41E-38[a]	
snpST00302		170.64	8.83E-38[a]	
snpST00303		185.89	4.31E-41[a]	
snpST00304		178.47	1.76E-39[a]	
snpST00305		195.33	3.84E-43[a]	
snpST00306		196.13	2.59E-43[a]	

[a] Significant at $P \geq 0.05$; means observed marker genotypes agree with the SC phenotype; non-significant differences suggest that there is no relationship between observed genotype through marker alleles and expected phenotype.

3.3. Pollen viability analysis

The pollen viability analysis of diploid clones showed varying degree of fertility with very high pollen viability in *Sli* gene donor (>70 %) and progenies of two populations (DM × M18, DM × M6 (>70 %), while other diploid clones had poor viability (10–40 %) to complete sterility (Fig. 3a–c). More than 50 % self-incompatible diploid clones had poor (10–40 %), while >80 % of plants of S_0 to S_2 generation had high pollen viability (>70 %) (Fig. 3d–h).

3.4. Homozygosity evaluation using SNP genotyping

Genotype data of 99 out of 100 KASP SNP markers was used for homozygosity evaluation. A marker (snpST00245) was excluded as it had more than 75 % missing genotype data. The homozygosity analysis of diploid clones in different selfing generations through 99 SNP KASP assays spread across all the 12 chromosomes in the potato genome based on inbreeding by state (IBS) showed that the homozygosity in the founder diploid clones, including dihaploids varied from 20.2 to 78.3 %. Most of the founder clones had >50 % heterozygous loci except seven clones (Table S3) The range of homozygosity in S_0, S_1, S_2 and S_3 generations was 36.1–80.4, 56.9–82.8, 59.5–85.4 and 73.7–87.8 %, respectively (Table 4; Table S3; Fig. S1). Three plants of *Sli* introgressed clones namely C13xPI654351, DMM6 1 and DMM6 6 in the S_0 generation showed >70 % homozygosity while four clones observed <50 % homozygosity. In S_1 generation, 6 clones had >80 % homozygous loci while 36 had >70 % homozygosity. Similarly, 16 clones in S2 generation and all the clones except two in S_3 generation observed >80 % homozygosity. The realized values of homozygosity were lower than the expected value for clones in all the generations (Table 4).

Population wise homozygosity analysis showed that plants in DM × M18 population had 46.5–63.2 % homozygosity, while DM × M6 plants observed 48.5–75.5 % and USW4 × M6 plants recorded 36.1–53.1 % homozygosity in S_0 generation. The homozygosity level of the plants of these three populations increased considerably to 61.9–82.8, 63.6–80.2 and 56.9–73.2 (S_1) and 67.4–85.4, 68.0–84.8 and 59.4–72.4 (S_2) in DM × M18, DM × M6 and USW4 × M6 populations, respectively. The S3 generation had all the plants of DM ×
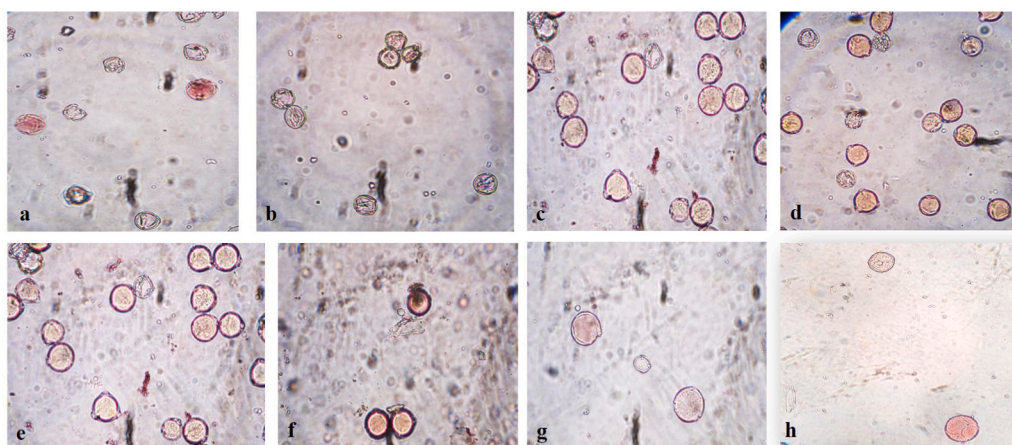

Fig. 3. Pollen viability analysis of plants in different generations a) Low pollen viability in a founder diploid clone b) Non-viable pollens in another founder diploid clone c) Viable pollen grains in DM × M18 population plants d) Pollen viability in S_0 generation plants e) S_1 generation plants pollen viability f) Viable pollen grains in S_2 generation plants g&h) pollen viability of two different S_3 generation plants.

Table 4
Homozygosity percentage in different diploid potato generations assessed through 99 KASP SNP markers.

Inbreeding Generation	N	Homozygosity (%)			Expected homozygosity (%)[a]		
		Min	Max	Average	Min	Max	Average
Founder diploid clones	69	20.2	78.3	59.1	–	–	–
Sli introgressed clones	18	36.1	80.4	58.1	–	–	–
First generation selfing	62	56.9	82.8	71.7	72.8	90.5	84.1
Second generation selfing	95	59.5	85.4	75.9	86.4	95.3	92.1
Third generation selfing	50	73.7	87.8	85.3	93.2	97.6	96.1

N- number of plants/diploid clones assayed.

[a] expected homozygosity worked out using the *Sli* introgressed lines homozygosity as base value.

M6 population along with one plant of DM × M18 population and the homozygosity level of DM × M6 population was 73.7–87.9 while the homozygosity of sole plant of DM × M18 population was 85.7 % (Table S3).

3.5. Elite Sli donors and diploid clones

In the inbreeding generations of two diploid populations (DM × M18 and DM × M6), we identified BS 49-1 and BS 48-6 as vigorous and highly fertile *Sli* donor lines, which had improved tuber size and number in comparison to the original *Sli* donor line (Fig. 4a–c). Both the lines were homozygous for the *Sli* gene genotype and had >80 % homozygosity for the genetic background, as revealed by 99 KASP SNP loci spread across all the 12 chromosomes in the potato genome. The field evaluation (net-house) of founder diploid clones for tuber traits identified 21 elite lines for incorporating self-compatibility and inbreeding (Table S1) (Fig. 5a–f). The lines were selected based on visual observation on tuber size, shape and number in the net house.

4. Discussion

To enhance genetic gain in potato breeding, there is a need to shift the potato from a tuber-propagated tetraploid crop into an inbred-line-based diploid seed crop. However, diploid potatoes and di-haploids are self-incompatible and inbreeding results in severe depression in potatoes. Efforts were made in this study to develop partial inbred lines of potato at the diploid level. Most potato clones at the diploid level show SI, therefore KASP markers of *Sli* gene were used for SC/SI detection at the genotype level and results were compared with the phenotype data. The standard procedure of inbreeding through selfing was followed to develop partial inbred lines.

To proceed the work, initially the founder diploid clones were confirmed for their ploidy using flow cytometry. Flow cytometry has been used as a high throughput and reliable method for ploidy estimation in potato [19,23]. The SC/SI of the diploid clones was determined through berry setting upon selfing which has been used as one of the methods earlier [15,16]. However, lack of berry setting upon selfing could be attributed to other reasons like gametic infertility, lack of gamete fusion, embryo abortion, etc. [15]. In the inbred development programme, the S_0 generation plants showed self-compatibility at the genotype level indicating that the *Sli* gene in pollen parent was in dominant homozygous condition in all the populations. The crosses of 15 elite founders with the *Sli* gene donor line performed poorly and failed to proceed beyond the S_1 generation due to poor flowering and no seed set on selfing. These observations revealed that these lines may carry deleterious mutations expressed in the first or second selfing generations [24,25]. It has been observed that deleterious recessive mutations are more easily exposed in diploids, and thus, ploidy reduction aggravates fertility problems [15]. Zhang et al. [24] identified 15 genomic regions with severe segregation distortion in the potato genome. They also found that most deleterious recessive alleles occur in regions of high recombination rates, which affect survival and vigour. Therefore, recombination breeding could also purge the deleterious alleles in these lines [24]. Moreover, the deleterious mutations are line-specific, and selecting vigorous and fertile plants requires raising many progenies of a cross. Advancement of only three populations (DM × M18, DM × M6, USW4 × M6) in S_1 and onwards generations further emphasized the importance of founder parents' selection in the inbred development programme. Reduction in vigour and fertility is a general rule in inbreeding heterozygous clones carrying deleterious alleles [26], which was evident in our study too. The progenies of three populations, advanced through selfing
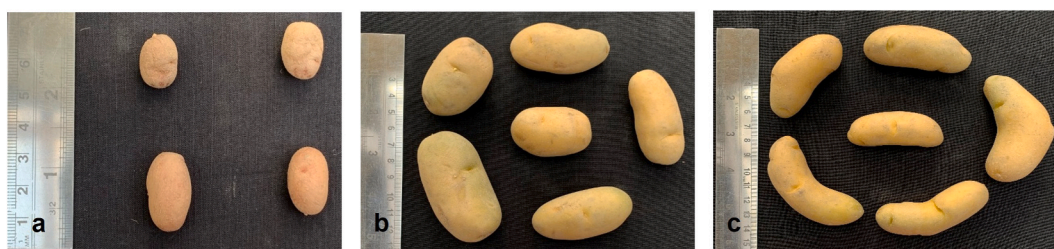

Fig. 4. Original *Sli* gene donor along with two newly identified vigorous highly fertile self-compatibility gene (*Sli*) donor lines for Indian conditions: a) PI 654351 b) BS 49-1 c) BS 48-6.
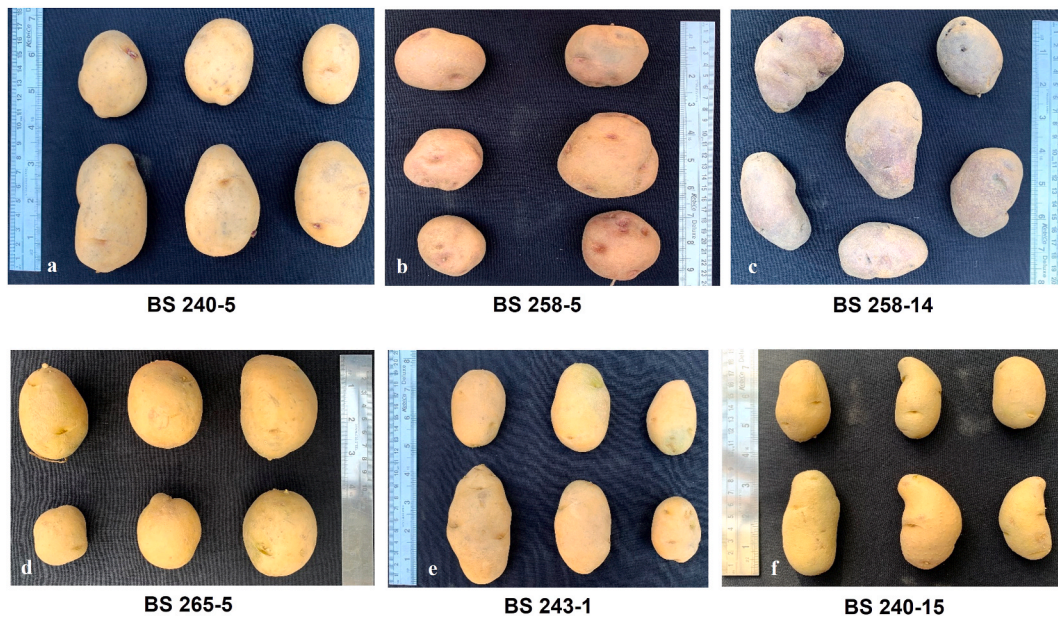
Fig. 5. Elite diploid clones selected for inbreeding based on tuber traits a) BS 240-5 b) BS 258-5 c) BS 258-14 d) BS 265-5 e) BS 243-1 f) BS 240-15.

showed reduced vigour and fertility in advanced generations. It indicated that recessive deleterious alleles with minor effects are not eliminated even after 2–3 generations of selfing [27]. Therefore, developing inbred lines requires a deep understanding of the potato genomes from an evolutionary perspective to effectively identify and purge major and minor effect deleterious mutations [4,25]. Moreover, selfing could not be achieved in all self-compatible clones, which could be attributed to other factors related to fertility reduction, such as selection against genes for fertility over the years of clonal propagation in potatoes [4].

Few inconsistencies in the *Sli* gene genotype in different diploid clones through different *Sli* gene KASP assays may be attributed to some other loci governing self-compatibility [28] or error in allele calling, i.e., wrong allele might have been called in by different markers [29]. The results also highlighted the need to find other genomic regions that are essential for SC and the role of the environment on the expression of genes associated with the SC reaction. We observed good association of Sli KASP markers genotype data with SC phenotype data. The results revealed that all the 10 KASP SNP markers of the *Sli* gene could be used for high throughput rapid screening of diploid clones for SC to develop inbred lines and identify new sources of SC in diploid potatoes. Some disparity between genotype data and phenotype could be partly attributed to the difficulty of precise phenotyping for SC, as observed earlier [16]. In an earlier similar study, Kaiser et al. [16] evaluated the contribution of *Sli* to SC in the diploid germplasm originating from North American breeding programmes using a subset of six KASP markers of the *Sli* gene and found similar results with some discrepancies. In a separate study, Ma et al. [14] inferred that the *Sli* gene can interact with a variety of allelic forms of the S-ribonucleases (S-RNases) that are specific to the pistil and as a result, serves as a general S-RNase inhibitor to confer self-compatibility in dihaploids of commercial tetraploid cultivars. These studies on the discovery of self-incompatibility inhibitor genes have opened new avenues for researchers to investigate the production of diploid inbred lines for DHTPS potato breeding programme [13,14].

High pollen viability in initial inbreeding generations (S_0– S_2) could be ascribed to the genome constitution of female parent of two populations (DM × M18 and DM × M6) and partly due to the genome contribution of the *Sli* gene donor male parent. DM has been observed to be a good female parent in previous studies [12,30]. The male parents viz., M6 and M18 are vigorous, male and female fertile, and highly homozygous [9] (https://www.ars.usda.gov/research/publications/publication/?seqNo115=339913). Jayakody et al. [31] also observed good pollen viability of elite diploid clones, including M6 and USW4. Zhang et al. [4] observed pollen sterility in some selfed progenies during inbreeding while we observed good pollen viability in S_3 generation plants (40–70 %), possibly due to the good fertility of parents involved in these diploid populations (Fig. 3).

The homozygosity assessment of plants in different inbreeding generations revealed increase in homozygosity in each selfing generation. The reduction rate in heterozygosity per generation differed from previous studies [32,33], probably due to different initial clones used in our study. The reduction in heterozygosity was lower than the expected values (Table 4), which is likely due to preferred heterotic effects on fecundity, the gametic or zygotic selection such as pollen competition, lethality and sterility of recessive homozygotes [3,23]. Homozygosity for these regions could lead to an intolerably high deleterious burden, thus resulting in substantially reduced fitness [25]. However, the parental lines require high genome homozygosity, adequate vigour and fertility, and a reasonable degree of genetic divergence to make a hybrid of sufficient heterosis and uniformity [4]. Although we observed plants (C13xPI654351) with high homozygosity (>80 %) in S_0 generation but they could not be propagated further through sexual reproduction. Similarly, the plants (DMM18 3-1 S1, DMM18 3–9 S1, DMM18 7–9 S1 and DMM6 2–9 S1) with high homozygosity (>80 %) in the first selfing generation could not be carried further due to random selection of plants based on vigour. A wide range of homozygosity varying from 20.2 to 78.3 % in the founder clones suggest they were highly heterogeneous. Extent inbred, allele frequencies and heterozygous

advantage could all be factors for this cryptic range of homozygosity/heterozygosity in the founder clones. Our results on homozygosity levels of plants in early selfing generations corroborate with the previous study by Lindhout et al. [3], where 6 plants with 100 % homozygosity were identified in the F_3 generation (equivalent to S_3 generation in our study) using 24 markers, while we observed 48 plants (out of 50 plants) with >80 % homozygosity in S_3 generation using 99 markers.

The founder diploid clones were of diverse backgrounds, including dihaploids of varieties, while S_0 - S_3 generation material was mostly derived from three F_1 hybrid populations, i.e., DM × M18, DM × M6 and USW4 × M6. A plant in founder diploid clones had minimum homozygous loci (20.2 %), while the level of minimum homozygosity of individual plants varied from 36.1 of the population USW4 × M6 in S_0 to 73.7 % in S_3 generation of the population DM × M6 (Table S3). The homozygosity level of the plants of the population DM × M6 was higher than the other two populations in the S_0 generation itself and increase in homozygosity of the plants was also higher in each selfing generation for this population, resulting in greater number of plants in S_3 generation. The plants of the population USW4 × M6 had high initial heterozygosity in comparison to the other two populations which resulted in slow increase in homozygosity during selfing and the plants could not be carried forward after two selfing generations. High minimum homozygosity in all the three populations in S_0 - S_3 generation could be attributed to the choice of marker i.e. KASP assay and their number (100 markers) may not be enough to scan the whole genome for homozygosity and some homozygous/heterozygous regions might not have been captured in advance selfing generations. Further, the KASP markers used for homozygosity assessment may not reflect the true picture due to their weakness of assay failure. However, the preliminary evaluation based on these loci explained the importance of homozygosity testing in initial inbreeding generations of diploid clones to develop highly homozygous, vigorous and fertile inbred lines. A positive correlation of homozygosity with fertility-related traits has been well-studied in diploid potatoes [26]. The initial selection of diploid clones with high homozygosity at most of the loci followed by continued selection of lines with high homozygosity in each selfing generation might purge the deleterious alleles as delineated in the genome design of hybrid potatoes by Ref. [4]. Van Lieshout et al. [34] observed ~80 % homozygosity in F_9 (similar to S_9) generation inbred lines; however, Hosaka and Sanetomo [33] showed complete homozygosity in S_{11} inbred lines using 22K potato V3 SNP genotyping for 21,027 SNP loci. Hosaka and Sanetomo [33] demonstrated that continued selfing using the *Sli* gene could create a completely homozygous diploid potato retaining self-fertility. A recent study showed that homozygosity for known desirable alleles could be achieved using haplotype analysis in the initial selfing generations [35]; however, whole genome sequencing is essential to demonstrate complete homozygosity in the advanced selfing generations [33].

An agronomically superior *Sli* donor line can speed up the development of vigorous diploid inbred lines with good fertility in selfing generations. Bradshaw [30] also highlighted that inbred lines must have acceptable levels of vigour and fertility for use in a diploid hybrid breeding programme. Therefore, the first priority should be identifying improved *Sli* donor and elite diploid lines in DHTPS breeding. A self-compatible *Sli* donor line, PI 654351, is the first self-compatible diploid clone used in inbred development in potatoes globally [33]. Afterwards, many lines have been derived from the same line in different genetic backgrounds [9], and new sources of self-compatibility have been identified in our study. A population of self-compatible diploid potatoes can be created using these elite lines through recurrent selection as demonstrated by Alsahlany et al. [20] to generate new diploid germplasm for self-compatibility and desired tuber traits.

5. Conclusions

DHTPS breeding is a new potato breeding methodology of variety propagation via true seeds, which aims to obtain quick genetic gain by fixing major genes for disease resistance or other key traits, and exploit F_1 heterosis. In this direction, we made an effort to identify diploid clones for inbred development and heterosis exploitation in diploid potatoes. The efforts successfully generated partial inbreds in the S_3 generation with 88 % homozygosity. We also validated 10 *Sli* gene KASP markers for high-throughput screening of diploid germplasm for self-compatibility. In addition, 99 SNP KASP markers distributed across the potato genome were designed and validated for homozygosity assessment in selfing generations. Phenotypic and genotypic evaluation of founder diploid clones identified 18 elite lines for inbred development and 2 *Sli* gene donors with improved tuber traits than the original *Sli* donor line in our conditions. These elite lines could be used in recurrent selection program for population improvement through desirable allele accumulation in each cycle. The results revealed that the use of KASP markers for self-compatibility and homozygosity assessment would help speed up the inbred development in DHTPS breeding. Although hybrid breeding is a well-known technology in major crops like maize, rice, brassica, millets etc., the potato is a different case due to the long history of clonal propagation with negative selection for fertility. Thus, all the steps in the DHTPS breeding program, like identification of diploid clones with acceptable vigour, fertility and homozygosity, F_1 hybrid combinations superior to existing cultivars for different segments, technologies to produce true potato seeds and established procedure for commercialization must be standardized to harness the potential of this technology.

Data availability statement

Data included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Salej Sood: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Vinay Bhardwaj:** Writing – review & editing, Funding acquisition. **Vikas Mangal:** Writing – review & editing, Investigation. **Hemant Kardile:** Methodology. **Bhawna Dipta:** Investigation. **Ashwani Kumar:** Investigation. **Baljeet Singh:** Investigation. **Sundaresha Siddappa:** Investigation. **Ashwani K. Sharma:** Writing – review & editing. **Dalamu:** Investigation. **Tanuja Buckseth:** Investigation. **Babita Chaudhary:** Investigation. **Vinod Kumar:** Writing – review & editing. **N.K. Pandey:** Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:I am working as associate editor in the Journal.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31507.

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