Unraveling Genetics of Semi-Determinancy and Identification of Markers for Indeterminate Stem Growth Habit in Chickpea (*Cicer Arietinum* L.)

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Research

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Abstract

Background

Chickpea (*Cicer arietinum* L.) is predominantly an indeterminate plant and tends to generate vegetative growth when the ambient is conducive for soil moisture, temperature and certain other environmental conditions. The semi-determinate (SDT) types are comparatively early, resistant to lodging and found to be similar in their yield potential to indeterminate (IDT) lines. Indeterminate types and semi-determinate genotypes are found to be similar during early stage, which makes it difficult to distinguish between them. Thus, there is a need to identify molecular marker linked either to indeterminate or semi-determinate plant type. The present study was carried out to study the genetics of semi-determinacy and identify molecular markers linked to stem growth habit.

Methods

The study was undertaken in the cross involving BG 362(IDT) X BG 3078-1(SDT). All F_1 plants were indeterminate, which indicates that indeterminate stem type is dominant over semi-determinate. In further advancement to F_2 generation, F_2 plants are segregated in the ratio of 3(Indeterminate): 1(Semi-determinate) that indicates that the IDT and SDT parents which are involved in the cross differed for a single gene. The segregation pattern observed in F_2 is confirmed in F_3 generation. The parental polymorphic survey was undertaken for molecular analysis using total of 246 SSR markers, out of which 41 polymorphic markers were found to distinguish the parents and were utilized for bulked segregant analysis (BSA).

Results

The segregation pattern in F_2 indicates that the IDT(Indeterminate) and SDT(Semi-determinate) parents which are involved in the cross differed for single gene. The segregation pattern of F_2 and F_3 derived from the cross BG 362 (IDT) x BG 3078-1 (SDT) confirmed the genotypic structure of the newly found SDT genotype BG 3078-1 as *dt1dt1Dt2Dt2*. Three SSR markers *TA42*, *Ca_GPSSR00560* and *H3DO5* were found to be putatively linked to *Dt1* locus regulating IDT stem growth habit.

Conclusion

Our results indicate that the SSR markers identified for *Dt1* locus helps to differentiate stem growth habit of chickpea in its early growth stage itself and can be efficiently utilized in Marker Assisted Selection (MAS) for changed plant type in chickpea.

Background

Chickpea (*Cicer arietinum* L.) is an annual legume crop that is included in the *Leguminosae* family, *Papilionaceae* sub-family, *Cicereae* Alef. tribe and *Cicer* L. genus. It is a true diploid (2 n = 2 x = 16) self-pollinated and its estimated size of genome is 738 Mb having 28,269 genes [18]. Morphologically, flowering plants are categorized as determinate (DT), semi-determinate (SDT) and indeterminate (IDT) based on vegetative or reproductive phase of terminal meristems. Indeterminate types are characterized by vegetative buds at terminal meristems and stem apices, which regulate development of new nodes with leaves and produces inflorescence in auxiliary meristem and therefore, keeps on growing in length of stem, flower and produces pods till temperature and humidity allows [2, 16]. The plants with semi-determinate growth habits are similar to indeterminate but terminal meristems are terminated with floral buds. In determinate types, the terminal meristems transformed from a vegetative state to a reproductive phase, leads to the development of a terminal flower and as a consequence, the vegetative growth stops to bloom or persist for a short

duration [1, 2]. Therefore, the stem growth habit plays a significant role in determining plant architecture, which is of major agronomic importance and specifies plant adaptability to crop cultivation as well as seed yield potential [14]. The modification of plant architecture improves crop adaptation to various environments and enhances the yield including its stability [6].

Chickpea is predominantly an indeterminate type and tends to generate vegetative phase, when the temperature, soil moisture and certain other environmental conditions are conducive [21]. Due to its indeterminacy, excessive water induces vegetative development, that serves as a competitive sink for pod formation, while reduces the fruit set [8]. The indeterminate growth instigates competition between vegetative and reproductive phase for assimilation partitioning. This promotes to a low and unstable harvest index leading to lower and fluctuating seed yield. It causes considerably long cycles and late maturation, due to the prolonged vegetative phase [6]. Chickpea is primarily grown under marginal conditions in rainfed areas predominated by lack of moisture and fertility. Therefore, alteration of plant architecture is required to get a progress in productivity of chickpea and stabilization of production.

The performance of the crop is adversely affected by the high fertility and irrigation. In their yield potential, the semi-determinate types are found to be similar to that of indeterminate genotypes and they are found to be relatively early and resistant to lodging. During early vegetative growth stages, the semi-determinate forms are similar in their stem growth habit to that of indeterminate chickpeas and therefore, difficult to distinguish indeterminate or semi-determinate chickpeas in breeding populations. Thus, identification of a molecular marker linked to indeterminate or semi-determinate stem growth helps to differentiate plant type for selection in seedling stage itself and elucidation of genetics of semi-determinacy facilitate breeding to improve the plant type of existing cultivars from indeterminate to determinate / semi-determinate stem growth with better responsiveness to high input environments is necessary to achieve a major advance in its productivity.

Results

Genetics of semi-determinate stem growth

A cross between parents of indeterminate (BG 362) and semi-determinate (BG 3078-1) (Fig. 1) were examined for the heritage of semi-determinate vegetation. All the F_1 plants were indeterminate. The F_2 plants of the cross BG 362 x BG 3078-1were segregated into 138 indeterminates and 45 semi-determinates (Table 1). These data matched well with the ratio of 3 indeterminate: 1 semi-determinate stem elongation pattern in BG 362 x BG3078-1 (χ^2= 0.029, P = 0.9 – 0.5).

The segregation pattern found in F_2 has been confirmed by the analyzing the breeding behaviour of 172 F_3 families derived from BG 362 x BG3078-1 and specifics of segregating and non-segregating progenies are presented in Table 2. The F_3 segregation pattern indicate that all of the 43 semi-determinate individuals identified in F_2 bred true in F_3 and the result is in good fit as per predicted proportion of 0 segregating: 1 non-segregating plant (χ^2=1.00, P=1.00). Out of the 129 F_2 indeterminate plant progenies 96 were segregated into indeterminate and semi-determinate plants and 33 did not segregate, respectively. The proportion of non segregating and segregating progenies recorded in F_3 of indeterminate plants are also in good fit as per the anticipated proportion of 2 segregating: 1 non-segregating (χ^2=3.48, P = 0.1 – 0.05).

Identification of molecular markers linked to stem growth habit

Parental polymorphic survey

Parental polymorphic survey has been conducted between two parents contrasting for stem growth habit say, BG 362 (IDT) used as parent 1 (P_1) and BG 3078-1 (SDT) used as parent 2 (P_2) using 246 chickpea SSR markers. Among them 41 SSR markers showed polymorphism between two parents involved in the cross. The gel pictures of polymorphic SSR markers between two parents are shown in the Fig. 2a, 2b and 2c.

Table 1 Segregation of stem growth habit in F_2 of a chickpea cross involving indeterminate and semi-determinate parents

Parents & Cross	Total plants	Observed		Expected		Ratio tested	χ^2 value	P value
		IDT	**SDT**	**IDT**	**SDT**			
BG362 (P-1)	1	1	0					
BG 3078-1 (P-2)	1	0	1					
F_1 (BG 362 x BG 3078-1)	1	1	0					
F_2 (BG 362 x BG 3078-1)	183	138	45	137	46	3:1	0.029	0.9-0.5

IDT- Indeterminate; SDT- Semi-determinate

Table 2 Segregation of stem growth habit in F_3 of a chickpea cross (BG 362 x BG 3078-1) involving indeterminate and semi determinate parents

Phenotypic class	**No. of progenies**	**Observed**		**Expected**		**Ratio Tested**	**χ^2value**	**P value**
		Segregating	Non-segregating	Segregating	Non-segregating			
IDT	129	96	33	86	43	2:1	3.48	0.1-0.05
SDT	43	0	43	0	43	0:1	0.00	1.00

IDT- Indeterminate; SDT- Semi-determinate

Bulked segregant analysis

Bulked segregant analysis was carried out using 41 SSR markers those showed polymorphism between parents involved in the cross. Bulks are constituted by pooling DNA from 20 plants showing extreme indeterminate type and semi-determinate type separately. Polymorphic markers for the parents of IDT and SDT and the bulks of IDT and SDT were believed to be putatively linked to the locus *Dt1*. Among 41 polymorphic SSR markers, 3 SSR markers were found to be polymorphic between two bulks of DNA along with parents (Fig. 3a, 3b and 3c). Three SSR markers *Ca_GpSSR00560, TA42* and *H3DO5* co-segregated with the indeterminate stem growth habit and are identified as being putatively linked to *Dt1* locus in chickpea.

Discussion

Genetics of semi-determinate stem growth habit

The two non-allelic genes regulate stem growth habit in chickpea which is designated as *Dt1/dt1*, *Dt2/dt2* with *Dt1* epistatic to *Dt2* as well as *dt2* [5]. The *Dt1* allele develops indeterminate growth habit, whether it is present in homozygous (*Dt1Dt1Dt2_* and *Dt1Dt1dt2dt2*) or in heterozygous state (*Dt1dt1Dt2_* and *Dt1dt1dt2dt2*). The *Dt2* allele present either in homozygous (*dt1dt1Dt2Dt2*) or in heterozygous (*dt1dt1Dt2dt2*) state produces semi-determinate type subject to non presence of *Dt1*. A determinate phenotype was generated by the availability of recessive alleles (*dt1dt1dt2dt2*) in homozygous condition at both loci. In the current study, an indeterminate (BG 362) x semi-determinate (BG 3078-1) cross was studied to know the genetics of a semi-determinate (SDT) stem growth habit. BG 362 is an indeterminate parent and based on the earlier study [5] it is assumed that it carries *Dt1* and *Dt2* alleles in homozygous condition at both the loci and its genotype is designated as *Dt1Dt1Dt2Dt2*. The genetic control and the inheritance of semi-determinacy of BG 3078-1 genotype are dissected in this study.

The F_1 plants obtained from the cross made between BG 362 (IDT) X BG 3078-1 (SDT) were indeterminate suggesting that gene (s) regulating the IDT stem growth habit was dominant over the SDT stem growth habit. The dominance of the IDT stem has also been reported earlier in chickpea [5, 17]. The dominant nature of the IDT stem growth was also noticed in *Glycine max* [1], *Vicia faba* [3] and *Cajanus cajan* [7, 20]. The F_2 plants derived from BG 362 (IDT) x BG 3078-1 (SDT) cross segregated into 138 IDT: 45 SDT (Table 1). These observed segregation patterns are in good fit as per expected proportion of 3 IDT: 1 SDT stem types (χ^2 value= 0.029, P= 0.9 – 0.5) and indicates that the IDT and SDT parents which are involved in the cross differed for a single gene. The segregation pattern observed in F_2 of this cross is according to the expected ratio as predicted in the previous study [5].The genotype of IDT parent BG 362 was assumed as *Dt1Dt1Dt2Dt2* on the basis of previous study [5]. Hence, the genotype of the SDT genotype BG 3078-1 is assigned as *dt1dt1Dt2Dt2*.

A study of the breeding behavior of 172 F_3 progenies of BG 362 (IDT) x BG3078-1(SDT) confirmed the segregation pattern as documented in F_2 (Table 2). The pattern of segregation in F_3 progenies confirmed that all 43 SDT plants selected in 43 F_2 bred true SDT type in F_3 generation and the result is in perfect fit with the predicted 0 segregating: 1 non-segregating ratio (χ^2 value=1.00, P= 1.00). Out of the 129 indeterminate plant progenies 33 were non-segregating and 96 were segregated into IDT and SDT plants. The proportion of non-segregating and segregating progenies observed in F_3 of indeterminate plants is in good fit with the predicted ratio of 2 segregating: 1 non-segregating (χ^2=3.48, P = 0.1 – 0.05). On the basis of F_2 phenotypic ratio of 3 IDT: 1 SDT, 1/3 (*Dt1Dt1Dt2Dt2*) of the IDT plants were predicted to be non-segregating in F_3, whereas 2/3 (*Dt1dt1Dt2Dt2*) of the IDT plants are anticipated to be segregated into IDT and SDT. All plants of the SDT (*dt1dt1Dt2Dt2*) are considered to be non-segregating in F_3 and bred true to SDT type. The segregation pattern in F_3 found for stem types IDT and SDT thus supported the segregation pattern found in F_2. These findings obtained in the present study are similar to the inheritance study made on the stem growth habit recorded in pigeonpea [7]. The segregation pattern of F_2 and F_3 derived from the cross BG 362 (IDT) x BG 3078-1 (SDT) also confirmed the genotypic structure of the newly found SDT genotype BG 3078-1 as *dt1dt1Dt2Dt2*. Similar results and conclusions were also reported in previous studies done in chickpea [4].

Identification of molecular markers linked to stem growth habit

Parental polymorphism was carried out between two parents using 246 SSR markers which are distributed evenly on all 16 chromosomes of chickpea. Out of 246 SSR markers, 41 markers were found to be polymorphic. Bulked segregant analysis was conducted to identify putatively linked molecular marker for *Dt1* locus using polymorphic markers

distinguishing two parents which differ in stem growth habit. IDT and SDT bulks are prepared separately by mixing equal amount of DNA from 20 individual indeterminate and 20 semi-determinate F_2 plants respectively. 41 polymorphic SSR markers were used for BSA, among them 3 markers *i.e.,Ca_GpSSR00560, TA42* and *H3DO5* showed polymorphism between indeterminate and semi-determinate bulks along with two parents. These 3 markers are considered as co-segregating with *Dt1* locus. The marker, *Ca_GpSSR00560* was reported to be located on linkage group 4 [13], *TA42* was mapped on linkage group 7 [12] and *H3DO5* was mapped on linkage group 1 [9]. Out of the 3 identified linked markers, the marker *TA42*, has already been reported to be linked with *Dt1* locus in chickpea [4]. Since, the other two markers, *Ca_GpSSR00560* and *H3DO5* are located on linkage group 4 and linkage group 1 respectively, thus further detailed study is required to confirm their association with the stem growth habit in chickpea. Thus, our study validates that the association of *TA42* with the stem growth habit and can be used in marker assisted selection. Similarly, closely linked markers *TA34* for stem elongation in soybean [19] and *CcLG03* for IDT growth habit locus (*Dt1*) in pigeonpea have also been identified / mapped [15, 18]. Thus, these three markers *Ca_GpSSR00560, TA42* and *H3DO5* may be considered as putatively linked to *Dt1* locus in chickpea.

Methods

Plant materials

The study comprised of using a cross involving BG 362(IDT) and BG 3078-1(SDT) in an un-replicated design in the experimental field of Division of Genetics, IARI, New Delhi. The F_1s were planted during post rainy season of 2017-18 and selfed to obtain F_2 generation seeds. F_2 seeds were grown during rabi 2018-19 to obtain F_2 mapping population. Twenty seeds of each of the parents were sown in each row of 4 m length and 183 F_2 seeds were sown in 15 rows of 4 m length with a maximum of 13 plants per row. The crop was provided with dosage of fertilizer of 20 kg/ha N and 40 kg/ha P_2O_5 as basal fertilizer. The pod borer (*Helicoverpa armigera*) was effectively controlled by spraying 0.2% Spinosad at thirty, forty-five and sixty days after sowing (DAS). During the rabi 2019-20, F_3 generation was grown to validate the heritage pattern observed for elongating stems in F_2. Each progeny in F_3 consisted of 15-20 plants. The crop husbandry as well as protection strategies remained the same as those of preceding season.

Genetics of stem growth habit

F_1 and F_2 plants along with parental genotypes were screened for the trait stem growth habit for inheritance studies. Observations on stem elongation during flowering along with maximum pod formation stage were recorded on 5 plants of the parents, F_1 plants as well as all individual plants of the F_2 population. Two different stem elongation patterns could be seen in F_2 of IDT x SDT cross. All F_2 plants with elongated flowering offshoots that ended with the vegetative elongation phase are categorized as indeterminate, plants like those of indeterminate varieties with continuous flowering offshoots but ending with a flower bud or fully opened flower are termed as semi-determinate [5]. The expected values were calculated on the basis of Mendelian ratio, corresponding to the observed values for indeterminate: semi-determinate plants. To assess the goodness of fit, the deviations of these were analyzed through the chi-square (χ^2) test.

During the rabi 2019-20, the inheritance pattern observed for elongating stems in F_2 was validated in F_3. For stem growth habit, both indeterminate and semi-determinate plants phenotyped in F_2 were identified and their offspring were examined along with their corresponding forefathers. At the stage of maximum flowering and pod formation, every other offspring was observed for elongation of stem on individual plant basis and classified them as non-segregating *vs* segregating for stem growth habit.

Isolation of genomic DNA

The young and tender leaf samples from both the parents as well as F_2 individual plants were collected and DNA was extracted using the procedure of CTAB method [11] with minor alteration. The DNA was purified using 3 µl (10µg/µl) of RNase and incubated for 30 minutes at 37°C. Purified DNA was quantified on agarose gel of 0.8% concentration along with Hind III-cut λ DNA as standard. The concentration of DNA in an individual sample was identified on the basis of the intensity of the bands as compared with the λ DNA ladder. DNA samples were diluted with TE buffer to prepare working solution with the 25 ng/µl concentration followed by storing at 4°C.

PCR amplification

PCR for the specific SSR marker analysis was done in 10 µl reaction volume. The reaction mixture of 10 µl was made by adding 1 µl of 25ng/µl template DNA, 1 µl of forward primer, 1 µl of reverse primer, 5 µl master mix and 2 µl nuclease free water was added to make final volume. All the primers were amplified using touchdown PCR Thermocycler from “Applied Bio System” model “Veriti”. The amplification was conducted for 5 minutes with initial denaturation at 94^{o}C followed by a two-step 'touch-down' process. The first step had 18 cycles: Denaturation for 30 seconds at 94^{o}C, annealing for 1 minute at 52-65^{o}C and extension for 1 minute at 72^{o}C. The second step was set for 20 cycles: denaturation for 30 seconds at 94^{o}C, annealing for 1 minute at 55^{o}C and extension for 1 minute at 72^{o}C.

Gel electrophoresis and visualization of amplicons

Amplified PCR products were separated on 3 percent agarose gel. Using 1.0X TBE buffer, the amplified products were separated on a horizontal electrophoresis platform for 3-4 hrs at 120 V. The gels were stained with ethidium bromide (10mg/ml) and visualized using Gel Documentation (AlphaImager 2200, Alpha Innotech Corporation, USA) system. For each marker loci, amplicons were graded as alleles. Manual scoring of the alleles was performed and their sizes (in bp) were determined by comparison with the DNA ladder of 100 bp.

Parental polymorphic survey and bulked segregant analysis

Parental polymorphism was examined using 246 SSR markers between BG 362 and BG 3078-1. The 41 parental polymorphic markers were used for the study of bulked segregant analysis (BSA) as described by Michelmore *et al*., (1991). Bulked segregant analysis on stem growth habit [11] was conducted to recognize the molecular markers that were putatively associated with stem growth habit. The BSA was done for two bulks of plants showing extreme phenotypes of indeterminate forms and semi-determinate type from the individuals in F_2. Twenty F_2 individuals with indeterminate and 20 F_2 individuals identified as semi-determinate forms were used separately for bulk productions. Two DNA bulks, namely indeterminate (B1) and semi-determinate (B2), were given equal quantities of DNA from indeterminate and semi-determinate individuals respectively. The indeterminate and semi-determinate bulks were screened along with parents using 41 polymorphic SSR markers which had revealed parental polymorphism. Amplified products were run on 3% agarose gel. The bands for respective BSA polymorphic markers were verified for consistency by repeating the reactions twice.

Conclusions

Our data revealed that the F_2 and F_3 segregation in BG 362 (IDT) x BG 3078-1 (SDT) confirmed the genotype of the new found SDT line BG 3078-1 as *dt1dt1Dt2Dt2*. We have identified *TA42, H3DO5* and *Ca_GpSSR00560* SSR markers as putatively linked to *Dt1* locus in chickpea. Our study validates the association of *TA42* with the stem growth habit and can be used in marker assisted selection. Since, the other two markers, *Ca_GpSSR00560* and *H3DO5* are located on LG4

and LG1 respectively different than LG 7 as reported for *TA42*, therefore, further detailed investigation is required to confirm their association with the stem growth habit in chickpea. Further investigation on mapping of *Dt1* and *Dt2* locus are required for locating the exact genomic region involved in the inheritance of stem growth habit in chickpea. The markers identified for *Dt1* locus helps to differentiate stem growth habit of chickpea in its early growth stage itself and can be efficiently utilized in Marker Assisted Selection (MAS) for changed plant type in chickpea.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All the data generated or analyzed during this study are included in this published article.

Competing interest

The authors declare that they have no competing interests.

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Author's contribution

VH provided the experimental material as F_1 seed. RK and VH designed the experiment. Ambika, RK, VH and RKS performed field experimentations and data collection. Ambika, MSN, CB, RK and RKS executed the lab experiments. Ambika, RK, VH, and ST analyzed the data and interpreted the results. Ambika and RK contributed to the writing of manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Stem growth habit of BG 362 (IDT) and BG 3078-1 (SDT) at early stage

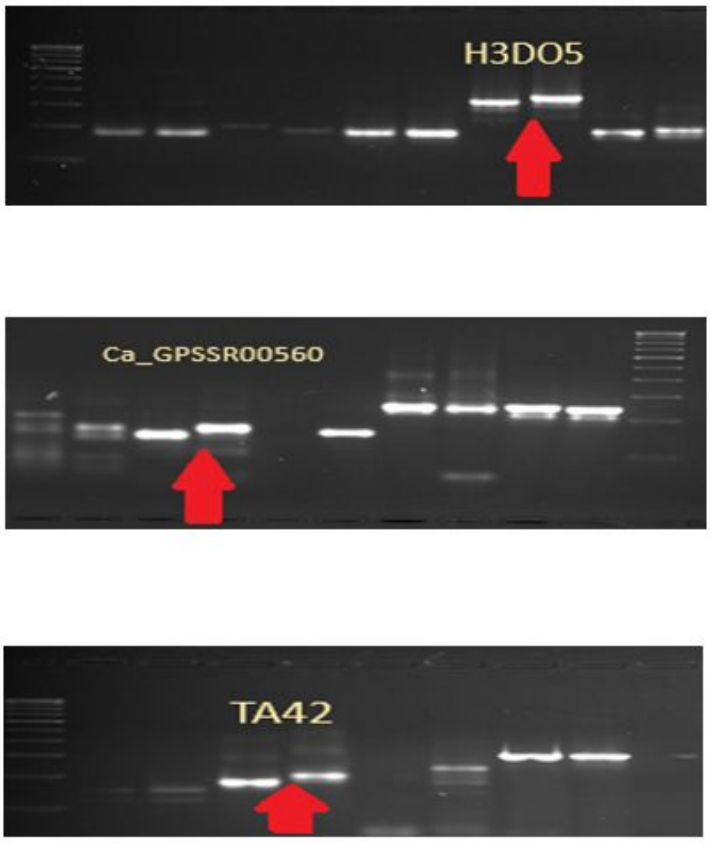

Figure 2

2a Parental polymorphism of H3DO5 between BG 362(IDT) and BG 3078-1(SDT) 2b Parental polymorphism of Ca_GPSSR00560 between BG 362(IDT) and BG 3078-1(SDT) 2c Parental polymorphism of TA42 between BG 362(IDT) and BG 3078-1(SDT)

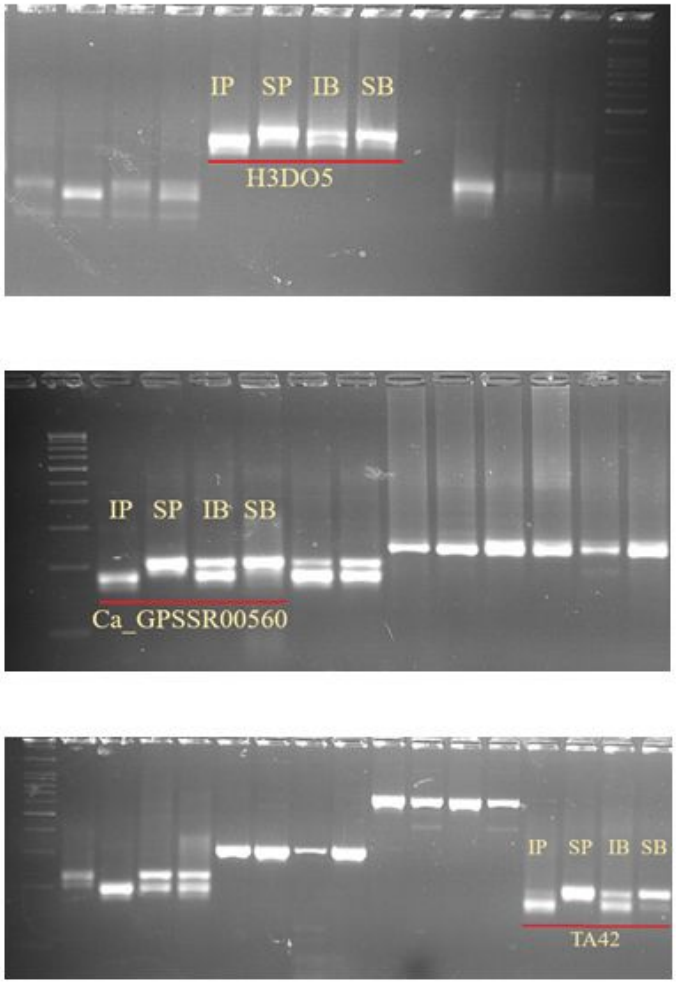

Figure 3

3a Bulked Segregant Analysis (BSA) of H3DO5 showing putatively linked marker 3b Bulked Segregant Analysis (BSA) of Ca_GPSSR00560 showing putatively linked marker 3c Bulked Segregant Analysis (BSA) of TA42 showing putatively linked marker IP=Indeterminate parent; SP=Semi-determinate parent; IB=Indeterminate bulk; SB=Semi-determinate bulk