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PRSV Resistance Gene Action in Intergeneric F_2 Population of Carica Papaya and Vasconcellea Cauliflora

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Abstract

The current investigation was undertaken to develop PRSV (Papaya Ringspot Virus) resistance gene action in intergeneric F_2 populations. Successful crosses have been achieved between Carica papaya and wild Vasconcellea cauliflora, to break intergeneric hybridization barrier, various nutrients used. In the present study the F_2 populations were raised from the seeds obtained from three F_1 plants whose hybridity was confirmed using polymorphic ISSR markers. F_2 seedlings and five seedlings each of CO 7, Pusa Nanha, CP 50 and male parent Vasconcellea cauliflora were also sap inoculated for PRSV symptoms. Among the parent, only Vasconcellea cauliflora seedlings alone did not produce PRSV symptoms, whereas all the female parents, CO 7, Pusa Nanha and CP 50 plants developed typical PRSV symptoms. 10 F_2 seedlings out of 138 from CO 7 x Vasconcellea cauliflora (CO7V3), 45 F_2 seedlings out of 310 from Pusa Nanha x Vasconcellea cauliflora (PNV9) and 33 F_2 seedlings out of 235 of CP 50 x Vasconcellea cauliflora (CPV23) did not exhibit PRSV disease symptoms.

In the cross combination, CO 7 x Vasconcellea cauliflora (CO7V3), 10 out of 138 F_2 seedlings did not show any PRSV symptoms when subjected to serological test. All of them except the male parent recorded OD values less than 0.300. Similarly, in case of F_2 progenies involving Pusa Nanha x Vasconcellea cauliflora (PNV9) and CP 50 x Vasconcellea cauliflora (CPV23) all the F_2 progenies recorded OD values less than 0.300. However, susceptible female parents showed OD values more than 0.962. The F_2 populations of various combinations segregated for susceptibity and resistance in two different ratio. The cross combination CO 7 x Vasconcellea cauliflora (CO7V3) exhibited a 15:1 ratio explaining duplicate gene interaction and the crosses Pusa Nanha x Vasconcellea cauliflora (PNV9) and CP 50 x Vasconcellea cauliflora (CPV23) exhibited a 13:3 ratio explaining inhibitory gene interaction. The study revealed multi-genic inheritance of resistant to PRSV which uniquely exhibit inter-genic interactions.

Keywords: Carica papaya; Vasconcellea cauliflora; intergeneric F_2 populations; papaya ringspot virus (PRSV); IISR markers

Introduction

Papaya, also called papaw or pawpaw, is an edible melon-like fruit of a tropical softwood tree (Carica papaya) of the family Caricaceae. Papaya is considered one of the most economically important and nutritious fruits, being a rich source of antioxidant nutrients such as carotenes, vitamin C and flavonoids; the B vitamins folate and pantothenic acid; the minerals potassium and magnesium; and fiber. In addition, papaya is the source of the digestive enzyme papain, which is an industrial ingredient used in brewing, meat tenderizing, pharmaceuticals, beauty products and cosmetics.

It is believed to have originated in Central-America with South-Mexico and Costa Rica as origin (De Candolle, 1884). It was introduced to India during 16th century by Portuguese travellers. At present, it is cultivated throughout the world. India is the largest producer of papaya in the world has an area of about 106, 000 ha with an annual production of about 4196 million tonnes (NHB, 2011). Other leading producers are Brazil, Mexico, Nigeria, Indonesia, China, Peru, Thailand and Philippines. In India, it is commercially cultivated in Andhra Pradesh, Gujarat, Maharashtra, Karnataka, West Bengal, Assam, Orissa, Madhya Pradesh, Manipur, Tamil Nadu, Bihar and certain extent in Kerala.

Trees infected with PRSV develop a wide range of symptoms which include ring spot on fruit, mosaic and chlorosis of leaf lamina, water-soaked oily streaks on the stem and petiole and mottling and distortion of young leaves. Production and productivity are affected due to decreasing photosynthetic capacity of plant, which subsequently display stunted growth, deformed and inedible fruit, and leading to mortality due to this virus. When plants are infected at seedling stage or within two months after planting, the trees will not produce mature fruits. If trees are infected at later stage, fruit production is reduced along with poor quality of fruits because of ringspot on fruit with low sugar concentration [1].

The PRSV disease is the biggest constraint to papaya production in the growing areas.

Existing management opts for this malady includes cultural practices, cross-protection and planting of tolerant cultivars [2]. None of these has been very successful and the development of virus resistant cultivars through conventional breeding is the only reliable tool for long term control of the disease. None of the Carica papaya cultivars has natural resistance to PRSV-P. Several related wild species of Carica have been reported as resistant to PRSV-P. However, several wild Carica species such as C. cauliflora, C. pubescens and C. quercifolia are resistant to PRSV [3]. Conventional interspecific hybridization of Carica papaya with other species has been difficult because of interspecific reproductive barriers [4]. In Australia, interspecific hybrids between C. papaya and Vasconcellea cauliflora were produced via interspecific hybridization and embryo rescue technique (Magdalita et al., 1996) [5] reported that Papaya ringspot virus (PRSV) seriously limits papaya (Carica papaya L.) production in tropical and subtropical areas throughout the world. Coat protein (CP) - transgenic papaya lines resistant to PRSV isolates in the sequence-homology-dependent manner have been developed in the U.S.A. and Taiwan. A previous investigation revealed that genetic divergence among Hainan isolates of PRSV has allowed the virus to overcome the CP-mediated transgenic resistance. In this study, we designed a comprehensive RNAi strategy targeting the conserved domain of the PRSV CP gene to develop a broader-spectrum transgenic resistance to the Hainan PRSV isolates. We used an optimized particle bombardment transformation system to produce RNAi-CP-transgenic papaya lines. Southern blot analysis and Droplet Digital PCR revealed that line 474 contained a single transgene insert. Challenging this line with different viruses (PRSV I, II and III subgroup) under greenhouse conditions validated the transgenic resistance of line 474 to the Hainan isolates. Northern blot analysis detected the siRNAs products in virus-free transgenic papaya tissue culture seedlings. The siRNAs also accumulated in PRSV infected transgenic papaya lines. Our results indicated that this transgenic papaya line has a useful application against PRSV in the major growing area of Hainan, China.

Breakdown of transgenic PRSV resistance, which depends on sequence homology between the transgene and attacking virus strain, is a major concern facing papaya cultivation, since genetically distinct strains of PRSV have been identified throughout the world [6]. In Hawaii, due to geographical isolation and the relative homology of PRSV strain existing there, the transgenic

resistance to PRSV introduced in papaya cultivars Rainbow and SunUp in 1998 is still effective against local strains [1].

[7] produced intergeneric hybrids in Carica papaya and Vasconcellea cauliflora using sucrose 5 % as an agent to break reproductive barrier. [8] produced intergeneric hybrids in Carica papaya and Vasconcellea cauliflora using sucrose 5 % (T1), sucrose 5 % + boron 0.5 % (T2) and sucrose 5 % + $CaCl_2$ 0.5 % (T3) as an agents to improve the fruit set and seed set percentage. Hence, present investigation was carried out to transfer the resistance gene to the popular cultivars of papaya. The study revealed multi-genic inheritance of resistant to PRSV which uniquely exhibit inter-genic interactions.

Materials And Methods

Raising F2 Generations

F_2 population was raised from selected F_1 combinations confirmed through molecular markers [7,8].

Screening of F2 Progenies and Parents Through Artificial Inoculation Against PRSV Under Glass House Conditions

Intergeneric hybrid seedlings along with parents were raised and artificially inoculated with PRSV under glass house conditions for screening. Observation for PRSV was done 27 days after inoculation. One gram of infected leaves was ground in a pre-chilled mortar and pestle using 1 ml of 0.1M chilled sodium phosphate buffer (pH 7.2) containing β-mercaptoethanol and 0.01 M EDTA. The sap was rub inoculated using the pestle or glass rod on the young leaves of seedlings at 3 leaves stage previously dusted with Carborundum powder 600 meshes. After 5 minutes, the excess sap was washed off by distilled water. The disease incidence and intensity score was given using the scale developed by [9]

DAS ELISA of Hybrids and Parents

Apparently virus free plants from different hybrid combinations and parents were subjected for ELISA confirmation using PRSV specific antibody. Antibody for PRSV and their positive samples were provided from DSMZ, Braunschweing, Germany.DAS-ELISA was performed for the detection of PRSV by following the manufacturer's instructions (DSMZ Gmbh, Braunschweig, Germany).

Inheritance of susceptible and resistant plants in F_2 population

Chi Square ratio was worked out for susceptible and resistant plants for testing goodness of fit.

$\chi^2 = (O - E)^2 / E$ with (n-1) df

Where, *O* - is the Observed Frequency in each category

E - is the Expected Frequency in the corresponding category sum of

df - is the "degree of freedom" (n-1)

The test of significance of chi square (χ^2) was made referring to the table for chi square at 1 degrees of freedom at P= 0.05 and 0.01 level of significance.

Results and Discussion

Raising of F_2 Generations

Intergeneric hybridization of Carica papaya with other genus in the same family of Vasconcellea cauliflora was started with the aim to transfer the desirable genes for PRSV resistance. Accordingly, a programme was evolved to cross Carica papaya with Vasconcellea cauliflora to incorporate the resistant gene from the later into the cultivars of papaya.

Out of 99 F_1 hybrids taken to the main field, cross combinations involving CO 7 x Vasconcellea cauliflora (6 F_1 hybrid seedlings), Pusa Nanha x Vasconcellea cauliflora (23 F_1 hybrid seedlings) and CP 50 x Vasconcellea cauliflora (70 F_1 hybrid seedlings), 18 hybrid progenies were confirmed for hybridity through molecular markers [9]. Among the 18 hybrid progenies the cross combinations viz., CO 7 x Vasconcellea cauliflora (CO7V3), Pusa Nanha x Vasconcellea cauliflora (PNV9) and CP 50 x Vasconcellea cauliflora (CPV23) were advanced to F_2 generations. Seeds from these cross combinations were used for raising seedlings which served as F_2 population.

Screening of F_2 Progenies and Parents Through Artificial Inoculation Against PRSV Under Glass House Conditions

A total of 138 F_2 seedlings from CO 7 x Vasconcellea cauliflora (CO7V3), 310 F_2 seedlings from Pusa Nanha x Vasconcellea cauliflora (PNV9) and 235 F_2 seedlings from CP 50 x Vasconcellea cauliflora (CPV23) were raised and sap inoculated for screening of resistance to PRSV. Five seedlings each of CO 7, Pusa Nanha, CP 50 and Vasconcellea cauliflora were also sap inoculated for PRSV symptoms. Among the male parent, only Vasconcellea cauliflora seedlings alone did not produce PRSV symptoms, whereas all the female parents, CO 7, Pusa Nanha and CP 50 plants developed typical PRSV symptoms.

It was observed that only 10 F_2 seedlings out of 138 from CO 7 x Vasconcellea cauliflora (CO7V3), 45 F_2 seedlings out of 310 from Pusa Nanha x Vasconcellea cauliflora (PNV9) and 33 F_2 seedlings out of 235 of CP 50 x Vasconcellea cauliflora (CPV23) did not exhibit PRSV disease symptoms **(Figure 1, Table1).**

Figure 1: Field view of Intergeneric hybrids

Parents / Hybrids	Number of plants inoculated	Number of plants without symptom 27 days after inoculation
Parents		
CO 7	48	0
Pusa Nanha	47	0
CP 50	48	0
Vasconcellea cauliflora	5	5
Hybrids		
CO 7 x *Vasconcellea cauliflora* (CO7V3)	138	10
Pusa Nanha x *Vasconcellea cauliflora* (PNV9)	310	45
CP 50 x *Vasconcellea cauliflora* (CPV 23)	235	33

Data not statistically analyzed

Table 1: Reaction of parents and F_2 progenies against artificial inoculation PRSV under glass house conditions

All the F2 populations exhibited variation within the parental ranges which explained the resistance might be of oligogenic inheritance and not polygenic.

In a perennial crop like papaya, field screening for diseases is very difficult since, it requires a larger area for planting. Hence, screening in glass houses in the nursery stage proved quick and rapid method. Regarding the female parents, all were found to exhibit the virus symptoms uniformly after sap inoculation. Symptom free F2 hybrids were transplanted in the main field for further evaluation. The failures of PRSV symptoms to develop on the manually inoculated hybrid plants indicate the incorporation of genes resistant to PRSV (Figure 1). Further, the wild genus V. cauliflora was found to be completely resistant to the strain PRSV prevalent in Coimbatore area of Tamil Nadu, India [10].

DAS ELISA of Hybrids and Parents

Serological test is more sensitive and convenient than back-inoculation tests when large numbers of plants have to be screened (Miller and Martin, 1988). Segregation can be studied only in the F_2 populations, which will throw light on the gene action **(Figure 2)**.

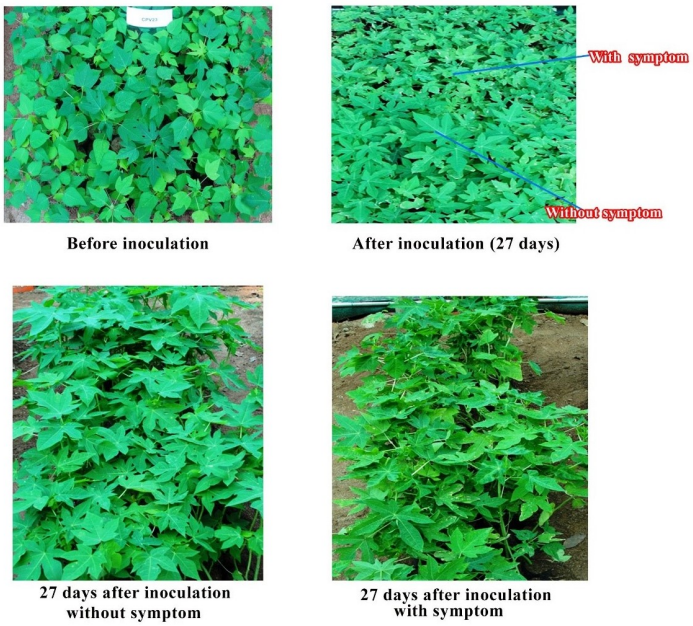

Figure 2: Confirmation of PRSV resistance in F2 seedlings

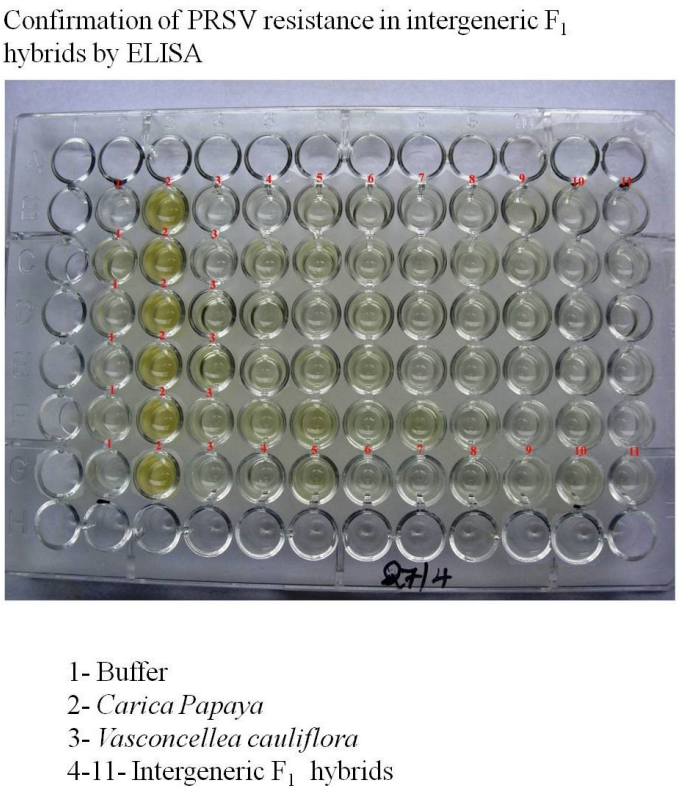

Figure 3: Confirmation of PRSV resistance in in intergeneric F1 hybrids by ELISA

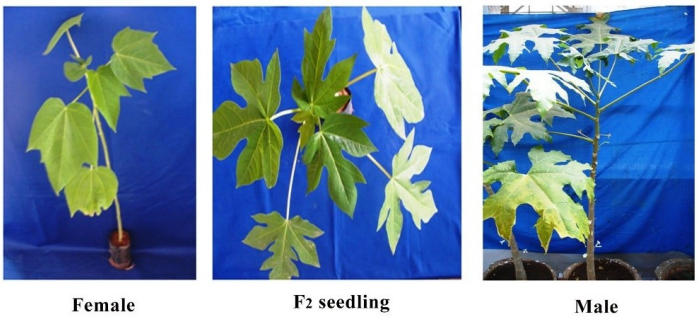

Figure 4: Segregation in F2 population

In the present study, in the cross combination, CO 7 x Vasconcellea cauliflora (CO7V3), 10 out of 138 F_2 seedlings did not show any PRSV symptoms when subjected to serological test. All of them except the male parent recorded OD values less than 0.300. Similarly, in case of F_2 progenies involving Pusa Nanha x Vasconcellea cauliflora (PNV9) all the F_2 progenies recorded OD values less than 0.300. Similarly, in the case of CP 50 x Vasconcellea cauliflora (CPV23) all the F_2 progenies recorded OD values less than 0.300. However, susceptible parents showed OD values more than 0.962 **(Table 2, 3, 4).**

Sl.No	Parentsand their hybrids	OD value at 405nm
1.	*Vasconcelleacauliflora*	0.216
2.	CO 7	0.972
3.	Buffer	0.102
4.	CO7V1	0.266
5.	CO7V2	0.259
6.	**CO7V3**	**0.243**
7.	CO7V4	0.261
8.	**CO7V5**	**0.245**
9.	**CO7V6**	**0.247**

CO 7V (CO 7 x Vasconcellea cauliflora)

Table 2: ELISA titre value for parentsand F1 population involving CO7 (apparently free from PRSV after inoculation)

Sl.No	Parentsand their hybrids	OD value at 405nm	Sl.No	Parentsand their hybrids	OD value at 405nm
1.	*Vasconcelleacauliflora*	0.216	14.	**PNV11**	**0.220**
2.	PusaNanha	0.952	15.	PNV12	0.266
3.	Buffer	0.102	16.	**PNV13**	**0.223**
4.	**PNV1**	**0.219**	17.	PNV14	0.268
5.	PNV2	0.278	18.	PNV15	0.284
6.	**PNV3**	**0.218**	19.	PNV16	0.286
7.	PNV4	0.275	20.	PNV17	0.285
8.	PNV5	0.251	21.	PNV18	0.286
9.	**PNV6**	**0.220**	22.	PNV19	0.275
10.	PNV7	0.278	23.	PNV20	0.280
11.	**PNV8**	**0.222**	24.	**PNV21**	**0.224**
12.	**PNV9**	**0.218**	25.	PNV22	0.270
13.	PNV10	0.287	26.	PNV23	0.274

PNV (PusaNanha x Vasconcelleacauliflora)

Table 3: ELISA titre value for parentsand F1 population involving PusaNanha (apparently free from PRSV after inoculation)

Sl.No	Parents and their hybrids	OD value at 405nm	Sl.No	Parents and their hybrids	OD value at 405nm	Sl.No	Parents and their hybrids	OD value at 405nm
1.	*Vasconcelleacauliflora*	0.216	26.	**CPV23**	**0.218**	51.	CPV48	0.286
2.	CP 50	0.942	27.	CPV24	0.285	52.	CPV49	0.289
3.	Buffer	0.102	28.	CPV25	0.279	53.	CPV50	0.279
4.	**CPV1**	**0.222**	29.	**CPV26**	**0.226**	54.	CPV51	0.277
5.	CPV2	0.285	30.	CPV27	0.282	55.	CPV52	0.279
6.	CPV3	0.286	31.	CPV28	0.284	56.	CPV53	0.288
7.	CPV4	0.292	32.	CPV29	0.296	57.	CPV54	0.299
8.	CPV5	0.294	33.	CPV30	0.292	58.	CPV55	0.269
9.	CPV6	0.277	34.	**CPV31**	**0.221**	59.	**CPV56**	**0.219**
10.	CPV7	0.278	35.	CPV32	0.281	60.	CP V57	0.297
11.	CPV8	0.287	36.	CPV33	0.286	61.	CPV58	0.295
12.	CPV9	0.282	37.	CPV34	0.284	62.	CPV59	0.294
13.	CPV10	0.285	38.	CPV35	0.285	63.	CP V60	0.279
14.	CPV11	0.284	39.	CPV36	0.280	64.	CP V61	0.286
15.	**CPV12**	**0.232**	40.	CPV37	0.283	65.	CPV62	0.287
16.	CPV13	0.285	41.	CPV38	0.284	66.	CP V63	0.299
17.	CPV14	0.295	42.	**CPV39**	**0.220**	67.	CP V64	0.298
18.	CPV15	0.292	43.	CPV40	0.287	68.	CP V65	0.295
19.	CPV16	0.290	44.	CPV41	0.284	69.	CP V66	0.294
20.	CPV17	0.284	45.	CPV42	0.296	70.	CP V67	0.289
21.	CPV18	0.282	46.	CPV43	0.298	71.	CP V68	0.287
22.	CPV19	0.275	47.	CPV44	0.296	72.	CP V69	0.285
23.	CPV20	0.289	48.	CPV45	0.298	73.	CP V70	0.296
24.	CPV21	0.292	49.	CPV46	0.289			
25.	CPV22	0.294	50.	CPV47	0.295			

CPV (CP50 x Vasconcellea cauliflora)

Table 4: ELISA titre value for parents and F1 population involving CP 50 (apparently free from PRSV after inoculation)

Frequency of Susceptible / Resistant Plants in F_2 Populations

Chi-square (χ^2) test of goodness of fit was employed to arrive the segregation ratio in F_2. [11] studied the segregation of susceptible to resistant phenotypes in the F_2 and reported that single recessive gene or a group of tightly linked genes at a single locus was responsible for PRSV-P resistance in V. cundinamarcensis and V. parviflora. However, in this study, none of the crosses produced 3:1 ratio and exhibited ratio explaining different di-genic interaction models. There might be couple of reasons for obtaining single locus inheritance ratio as explained followingly. If there were common alleles in other loci (or) only one locus between the parents is different (allelic) and the segregation would be recorded as a single gene inheritance. Moreover, study of small population in the F_2 generations might be another reason for recording it as a single gene inheritance.

In the present study, segregation ratio of 15:1 for susceptible to resistant was recorded in CO 7 x Vasconcellea cauliflora hybrid namely CO7V3 **(Table 5).**

Hybrids	Total numberof F_2 plantsobserved	Frequency		*Chi-square value*	
		Susceptible	Resistant	15:1	13:3
CO 7 x *Vasconcellea cauliflora* (CO7V3)	138	128	10	0.23*	-
Pusa Nanha x *Vasconcellea cauliflora* (PNV9)	310	265	45	-	2.27 *
CP 50 x *Vasconcellea cauliflora* (CPV23)	235	202	33	-	3.41*

*ns- non significant

Table 5: Frequency of susceptible / resistant plants in F_2 populations

The results indicated the presence of two dominant genes contributing the resistance for PRSV. Resistant to PRSV trait was observed to be governed by two recessive genes and the susceptibility was observed to be governed by two dominant gene interaction with the expected ratio of 15:1 explaining duplicate gene interaction determined by two completely dominant genes. These dominant genes produce the same phenotype susceptibility (S_1S_2) whether they are alone or together; the contrasting phenotype resistant was produced only when both the genes were in homozygous recessive state. The possible gene designations are furnished in **Table 6.**

Parents			F_2 generations	
Name	Genotype	Reaction	Genotype	Reaction
CO 7	$S_1S_1S_2S_2$	Susceptible	S_1-S_1-	Susceptible (9)
Vasconcellea cauliflora	$s_1s_1s_2s_2$	Resistant	S_1-s_2s_2	Susceptible (3)
			$s_1s_1S_2$-	Susceptible (3)
			$s_1s_1s_2s_2$	Resistant (1)
Pusa Nanha and CP 50	$S_3S_3s_4s_4$	Susceptible	S_3-S_4-	Susceptible (9)
Vasconcellea cauliflora	$s_3s_3S_4S_4$	Resistant	S_3-s_4s_4	Susceptible (3)
			$s_3s_3S_4$-	Resistant (3)
			$s_3s_3s_4s_4$	Susceptible (1)

Table 6: Genotype designations for the susceptible / resistant plants

Segregation ratio of 13:3 for susceptible to resistant phenotypes in Pusa Nanha x Vasconcellea cauliflora (PNV9) and CP 50 x Vasconcellea cauliflora (CPV23) were observed. The character, susceptibility to PRSV was observed to be governed by two dominant genes with inhibitory gene action (13:3) but the resistance to PRSV was governed by recessive gene. In inhibitory gene action, one of the two completely dominant genes produces the concerned phenotype or the character susceptibility while its recessive allele in homozygous state produces the contrasting phenotype. The second dominant gene has no effect of its own on the character in question; however, it has the ability to stop the expression of the dominant allele of the first gene. As a result, when the two dominant genes are present together, they produce the same phenotype as that produced by the recessive homozygote of the first gene. The recessive allele of the second gene does not affect the development of the character in anyway. Thus in inhibitory gene action, one dominant gene is capable of producing a character only if its expression is not prevented by another dominant gene known as inhibitory gene action and the F_2 ratio is modified to 13:3 ratio in this case. The possible genotype designations are presented in **Table 6**. In the present study, in the three crosses we have observed the genetic ratio which

explained two different kinds of gene interaction. This is possible in the sense that the resistance to PRSV is governed by multiple loci which have independent and different types of interaction. The genetics of the trait, resistance to PRSV is explained with respect to individual cross and the two parents might be possessing different genes governing resistance mechanisms.

In the present study the three F_2 populations were raised from the seeds obtained from F_1 plants whose hybridity was confirmed using polymorphic ISSR markers [8]. The F_2 populations of various combinations segregated for susceptibity and resistance in two different ratio. The cross-combination CO 7 x Vasconcellea cauliflora (CO7V3) exhibited a 15:1 ratio explaining duplicate gene interaction and the crosses Pusa Nanha x Vasconcellea cauliflora (PNV9) and CP 50 x Vasconcellea cauliflora (CPV23) exhibited a 13:3 ratio explaining inhibitory gene interaction. The study revealed multi-genic inheritance of resistant to PRSV which uniquely exhibit inter-genic interactions.

Conclusion

Based on the disease intensity score, reaction to the PRSV and performance, the cross combinations viz., CO 7 x Vasconcellea cauliflora (CO7V3), Pusa Nanha x Vasconcellea cauliflora (PNV9) and CP 50 x Vasconcellea cauliflora (CPV23) were advanced to F_2 generations. The F_2 populations of various combinations segregated for susceptibility and resistance in two different ratio. The cross combination CO 7 x Vasconcellea cauliflora (CO7V3) exhibited a 15:1 ratio explaining duplicate gene interaction and the crosses Pusa Nanha x Vasconcellea cauliflora (PNV9) and CP 50 x Vasconcellea cauliflora (CPV23) exhibited a 13:3 ratio explaining inhibitory gene interaction. The study revealed multi-genic inheritance of resistant to PRSV which uniquely exhibit inter-genic interactions.

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