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Short communication. Partial resistance of a cotton mutant to Cotton leaf curl Burewala virus

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Abstract

Cotton leaf curl disease (CLCuD), caused by Cotton leaf curl Burewala virus (CLCuBV), is a major constraint for a successful production of cotton in Pakistan. All the available cotton cultivars are susceptible to this virus. Breeding for resistance to CLCuBV is the best tactic to reduce economic losses caused by this virus. In the absence of a resistant source the present investigations were carried out to find out CLCuBV-resistant genotypes by mutagenization. NIAB-111 (female parent) was crossed separately with two male parents, NIAB-999 and CIM-499. The resulting F_1 seeds were gamma irradiated. Resistance to CLCuBV was evaluated by visual symptom scoring in the field conditions and in nethouse/greenhouse using whitefly and graft inoculations. Out of 20 mutants tested in M_5 generation, one, mutant M-112-59/22 showed partial resistance to CLCuBV, as concluded from its low severity index (SI) of 2.3 and percent disease index (PDI) of 20. M-112-59/22 consistently expressed resistance to CLCuBV in the normal cotton growing season, while it showed a more moderate resistance response when sown late in the field, or under greenhouse conditions following inoculation by whiteflies (SI = 3.3) or graft inoculation (SI= 3.4). The yield per plant of M-112-59/22 was higher than its parents with desirable fiber characteristics even under conducive disease development conditions. These results show that mutant M-112-59/22 is a CLCuBV partially resistant source when yield, fiber quality and response to virus infection are collectively taken into consideration.

Additional key words: begomovirus; CLCuBV; CLCuD; *Gossypium hirsutum*; partially resistant mutant.

Resumen

Comunicación corta. Resistencia parcial de un mutante de algodón al virus Burewala de la hoja rizada del algodón

La enfermedad de la hoja rizada del algodón (CLCuD), causada por el virus Burewala de la hoja rizada del algodón (CLCuBV), es un obstáculo importante para la producción de algodón en Paquistán, ya que todos los cultivares de esta planta son susceptibles a este virus. La mejor táctica para reducir las pérdidas económicas causadas por este virus es la mejora genética para resistencia a CLCuBV. Este trabajo se llevó a cabo para buscar genotipos resistentes a CLCuBV por mutagenización. NIAB-111 (progenitor femenino) se cruzó por separado con dos progenitores masculinos, NIAB-999 y CIM-499. Las semillas F_1 resultantes fueron tratadas con radiación gamma y se evaluó la resistencia a CLCuBV por visualización de los síntomas en campo y mediante inoculaciones con mosca blanca y por injerto en invernadero. Se analizaron 20 mutantes en la generación M_5 y uno de ellos, M-112-59/22, mostró resistencia parcial a CLCuBV, como se deduce de su bajo índice de severidad (SI = 2,3) y bajo porcentaje de incidencia de enfermedad (PDI = 20). M-112-59/22 expresó consistentemente resistencia a CLCuBV durante la temporada normal del algodón, mientras que mostró una resistencia más moderada cuando se sembró tardíamente o en condiciones de invernadero tras inoculaciones mediante moscas blancas (SI = 3,3) o injertos (SI = 3,4). El rendimiento por planta de M-112-59/22 fue mayor que los parentales, con características de fibra adecuadas, incluso en condiciones propicias al desarrollo de la enfermedad. Estos resultados mostraron que M-112-59/22 es una fuente parcialmente resistente al CLCuBV cuando se tiene en cuenta en conjunto el rendimiento, la calidad de la fibra y la respuesta a la infección por el virus.

Palabras clave adicionales: begomovirus; CLCuBV; CLCuD; *Gossypium hirsutum;* mutante parcialmente resistente.

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Abbreviations used: AAP (acquisition access period), CLCuBV (Cotton leaf curl Burewala virus), CLCuD (cotton leaf curl disease), CLCuKV (Cotton leaf curl Kokhran virus), CLCuMV (Cotton leaf curl Multan virus), DPI (days post inoculation), GOT (ginning out-turn percentage), IAP (inoculation access period), NIAB (Nuclear Institute for Agriculture and Biology), PDI (percent disease index), SI (severity index).

Since 1988, cotton (*Gossypium hirsutum* L.) in Pakistan is under constant threat of cotton leaf curl disease (CLCuD). A combination of distinct whitefly (*Bemisia tabaci* Genn.) transmitted begomoviruses has been shown to be associated with CLCuD (Briddon, 2003). Begomoviruses associated with CLCuD are characterized by twined isometric particles, 18-20 nm in diameter and 30 nm long, containing a circular single stranded DNA molecule. About seven species of begomoviruses have been reported so far and five of these are in Pakistan and one each in India and Sudan (Amin *et al*., 2006; Sharma and Rishi, 2007), all requiring a recently identified symptom modulating single stranded betasatellite component. An additional, satellite-like component, alfasatellite, is invariably found in infected plants, although it is not required for disease development (Briddon, 2003). Recently, a recombinant begomovirus derived from Cotton leaf curl Multan virus (CLCuMV) and Cotton leaf curl Kokhran virus (CLCuKV) has been associated with the resistance breaking strain of CLCuD present in Pakistan since 2001. Moreover, recent sequence analysis of viruses associated with the resistance breaking complex in this country revealed that only this recombinant begomovirus type is prevalent, in contrast to the situation before the appearance of the resistance breaking strain (Amin *et al*., 2006; Amrao *et al*., 2007). This recombinant virus was named as Cotton leaf curl Burewala virus (CLCuBV), which is now prevalent in cotton-growing areas of Pakistan.

Attempts to manage CLCuD by the control of inoculum reservoirs and vector populations have been ineffective particularly during high inocula pressures. Breeding resistant varieties is one of the best ways to combat CLCuD. Since its outbreak in Pakistan, there have been considerable efforts to develop CLCuD-resistant varieties using conventional and mutation breeding (Ali, 1997; Awan *et al*., 1998; Akhtar *et al*., 2000; 2002a,b; 2004). Studies showed that disease was efficiently managed by developing resistant cotton varieties, but the recent emergence of resistance-breaking strain of virus breakdown the resistance and all previously resistant varieties become susceptible (Mahmood *et al*., 2003; Akhtar *et al*., 2008, 2009, 2010). Therefore, we planned this study to search for cotton plants resistant to this new recombinant virus, CLCuBV, by mutagenization.

Allotetraploid *Gossypium hirsutum* L. NIAB-111 (female parent) was crossed with NIAB-999 (male parent) and CIM-499 (male parent) to produce M-112 and M-219 respectively. F_1 seeds were gamma irradiated and M_1 was sown during 2002 crop season. The parents were selected on the basis of their resistant response to CLCuMV (Akhtar *et al*., 2008), high yield, heat tolerance and good boll size. Resistant mutants from M_2 were selected on the basis of their field response. From M_3 to M_7 generations, selections were made according to their degree of resistance to CLCuD. However, M_5 to M_7 generations were evaluated under the present study.

Field assessments were made between 2006 to 2008 growing season at Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad (hot spot for CLCuBV), Pakistan. Seeds were sown in field on 18th and 28th of May in 2006 and 2007 respectively. During 2008 seeds were sown on 20th May and 9th June with normal plant to plant and row to row distance. Normal agronomic practices were followed to keep the crop in good conditions. However no plant protection measures were applied against whiteflies in order to have the maximum inoculum pressure during the experiment. Data for CLCuBV were recorded following the rating system described in Table 1 to calculate severity index (SI), percent disease index (PDI) and response of genotypes. Individual symptomatic plant ratings (following Table 1) for each genotype were added and divided by the number of infected plants to calculate the corresponding SI. The PDI was calculated using the following formula:

$$\text{PDI} = \frac{\text{Sum of all disease ratings}}{\text{Total plants observed}} \times \frac{100}{6^*}$$

* = maximum disease severity rating

The sum of all disease ratings was obtained by multiplying the disease severity rating with the corresponding number of plants.

The inoculum of CLCuBV for graft inoculation and whitefly transmission was taken from naturally infected cotton plants in experimental area of NIAB Faisalabad and maintained in CIM-499 (positive control for CLCuBV) by grafting in the glasshouse.

Five to six weeks old plants grown in glasshouse, 10 of each test entry were graft-inoculated with CLCuBV following the method described by Akhtar *et al*. (2002c). Plants were observed daily for symptom development. Data were recorded on the percentage of successful grafts, percentage of disease transmission, mean latent period (average time required for disease symptom appearance after grafting), and average SI after 90 days of grafting using the rating scale described in Table 1.

Table 1. Disease scale for rating of cotton leaf curl disease (CLCuD)

Symptoms	Disease severity	% Disease index	Disease response
Complete absence of symptoms and virus can not be detected in plant tissues using molecular techniques.	0	0	Immune
Complete absence of symptoms but virus can be detected in plant tissues using molecular techniques. OR Thickening of few small scattered veins or only presence of leaf enations on one or few leaves of a plant observed after careful observations.	1	0.1-10	Highly resistant
Thickening of small group of veins, no leaf curling, no reduction in leaf size and boll setting.	2	10.1-20	Resistant
Thickening of all veins, minor leaf curling & deformity of internode with minor reduction in leaf size but no reduction in boll setting.	3	20.1-30	Moderately resistant
Severe vein thickening, moderate leaf curling followed by minor deformity of internodes and minor reduction in leaf size and boll setting.	4	30.1-40	Moderately susceptible
Severe vein thickening, moderate leaf curling & deformity of internodes with moderate reduction in leaf size and boll setting followed by moderate stunting.	5	40.1-50	Susceptible
Severe vein thickening, leaf curling, reduction in leaf size, deformed internodes and stunting of the plant with no or few boll setting.	6	> 50	Highly susceptible

For whitefly transmission, adult whiteflies collected from cotton fields were provided a 72-h acquisition access period (AAP) on CLCuBV-infected cotton plants. A total of 10 potted cotton plants (5-6 weeks old) per test entry (5 plants per cage) were inoculated using 100 potentially viruliferous whiteflies per plant. After 72-h inoculation access period (IAP) plants were sprayed with an insecticide to kill whiteflies. The inoculated plants were transferred into a net-house under insect free conditions. This experiment was conducted during the normal cotton growing season to provide natural environmental conditions. Plants were observed daily for symptom development. Data were recorded on the percentage of disease transmission, mean latent period, and average SI after 90 days of transmission using the rating scale described in Table 1.

For CLCuBV identification, total DNA was extracted from young symptomatic leaves of 10 symptomatic cotton plants per experiment using the CTAB method (Doyle and Doyle, 1987). PCR reactions were conducted using CLCuBV-specific primers: 5'-GTGACTCGAGTCTTCGTACGTGTACTAGACG-3' and 5;- GTCGCCATGGGAGATCAATTTACCTATTGGG-3'. PCR was performed following the method described by Briddon *et al*. (2002).

At maturity 10 randomly selected plants of each cotton genotype were hand-picked from the field. The seed cotton for each sample was weighted, cleaned and then ginning of seed cotton was made by the standard miniature roller gin. The lint obtained was weighed and the ginning out-turn (GOT) percentage was calculated by the formula [(lint weight/seed weight.) × 100], according to the standard technique of ASTM Committee (1997). Yield components consisting of number of bolls per plant and average boll weight was also determined from these plants. Fiber length was measured by high volume instrumentation (HVI 900), and fiber fineness with the help of Sheffield micronaire instrument, according to standard tests methods (ASTM, 1997).

Twenty mutant cotton plants, one female parent and two male parents listed in Table 2, developed CLCuD symptoms with great variation for average SI and PDI under field conditions during 2006. Natural spread of CLCuBV was confirmed by the visual symptoms and through PCR analysis. Interestingly, only one mutant, M-112-59/22 was resistant showing the lowest average SI value of 2.3 and PDI of 20 (Table 2). Another mutant, M-219-55/9, was moderately resistant with average SI of 2.6 and PDI of 27.18. Two mutants M-112-53/12 and M-112-4/7 were moderately susceptible, one mutant

Table 2. Field response of cotton mutants to CLCuBV infection from 2006 to 2008

Sowing date	Mutants/ varieties	No. of plants observed	No. of plants showing disease severity							Av. SI[a]	PDI[b]	DR[c]	No. of bolls/ plant	Av. boll wt. (g)	Av. yield/ plant (g)	GOT (%)	Fiber length (mm)	Fiber fineness (µg inch^{-1})
			0	1	2	3	4	5	6									
18-	M-112-25/11	41	0	0	0	1	0	40	0	5.0	82.54	HS	—	—	185.24	—	—	—
May-	M-112-53/12	43	10	1	1	30	1	0	0	2.9	37.60	MS	—	—	194.56	—	—	—
2006	M-112-58/13	47	6	0	0	32	0	9	0	3.4	50.01	HS	—	—	186.75	—	—	—
	M-112-7/1	46	2	10	9	6	9	9	1	2.8	48.20	S	—	—	189.53	—	—	—
	M-112-13/9	39	0	0	0	0	1	30	8	5.2	86.34	HS	—	—	102.61	—	—	—
	M-112-4/7	43	10	12	4	8	6	2	1	2.6	32.54	MS	—	—	190.00	—	—	—
	M-112-29/5	48	0	0	0	1	3	44	0	4.9	81.61	HS	—	—	167.89	—	—	—
	M-112-74/15	42	0	0	0	1	1	31	9	5.1	85.73	HS	—	—	177.86	—	—	—
	M-112-59/22	40	19	8	3	6	4	0	0	2.3	20.00	R	—	—	204.29	—	—	—
	M-112-17/3	45	5	1	2	4	5	26	2	4.5	66.31	HS	—	—	182.40	—	—	—
	M-219-97/2	46	0	0	0	0	0	11	35	5.8	98.17	HS	—	—	98.17	—	—	—
	M-219-232/3	46	0	0	0	0	1	7	38	5.8	96.76	HS	—	—	96.76	—	—	—
	M-219-157/8	47	0	4	0	0	6	25	12	4.8	81.54	HS	—	—	81.54	—	—	—
	M-219-102/11	46	0	0	0	0	0	25	21	5.5	90.96	HS	—	—	90.96	—	—	—
	M-219-85A/10	44	0	0	0	1	0	11	32	5.7	94.72	HS	—	—	94.72	—	—	—
	M-219-55/9	46	17	9	5	8	4	2	1	2.6	27.18	MR	—	—	27.18	—	—	—
	M-219-106/15	40	0	0	0	0	0	18	22	5.6	92.52	HS	—	—	92.52	—	—	—
	M-219-134/17	46	0	0	0	0	0	6	40	5.9	97.85	HS	—	—	97.85	—	—	—
	M-219-149/20	48	0	0	0	0	0	4	44	5.9	98.63	HS	—	—	98.63	—	—	—
	M-219-191/21	43	1	2	0	6	4	10	20	4.9	79.86	HS	—	—	79.86	—	—	—
	NIAB-111[d]	47	2	2	3	3	4	11	22	4.9	79.02	HS	—	—	179.62	—	—	—
	NIAB-999[e]	47	0	0	0	0	0	4	43	5.9	98.60	HS	—	—	53.29	—	—	—
	CIM-499[e]	47	1	0	0	0	0	9	37	5.8	94.70	HS	—	—	111.96	—	—	—
28-	M-112-59/22	1,102	1,047	15	22	10	8	0	0	2.2	1.83	HR	82	—	218.42	—	—	—
May-	NIAB-111[d]	110	49	6	1	3	19	22	20	4.5	48.95	S	49	—	173.21	—	—	—
2007	NIAB-999[e]	110	8	1	2	2	4	25	68	5.5	84.87	HS	27	—	63.08	—	—	—
20-	M-112-59/22	805	737	27	21	14	1	0	0	1.7	2.49	HR	84	3.70	220.00	38.50	28.63	3.9
May-	NIAB-111[d]	120	39	10	3	12	18	10	28	4.2	47.51	S	61	3.90	182.00	37.50	30.00	4.3
2008	NIAB-999[e]	110	13	11	8	4	3	7	64	4.9	71.23	HS	53	3.20	75.00	38.00	28.00	4.4
09-	M-112-59/22	189	11	86	70	18	4	0	0	1.7	26.10	MR	68	3.63	167.00	36.75	28.20	3.8
June-	NIAB-111[d]	40	0	0	0	0	4	9	27	5.6	92.93	HS	36	3.82	126.00	35.50	29.10	3.9
2008	NIAB-999[e]	42	0	0	0	0	2	3	37	5.8	97.24	HS	10	2.90	40.00	35.00	27.20	3.6

[a] Av. SI: average severity index. [b] PDI: percentage disease index. [c] DR: disease response; HR: highly resistant; R: resistant; MR: moderately resistant; MS: moderately susceptible; S: susceptible; HS: highly susceptible. [d] Female parent. [e] Male parent.

M-112-7/1 was susceptible, while the remaining mutants and varieties were highly susceptible with 50.01 to 98.63 PDI. During 2006, M-112-59/22 showed apart of the resistance to CLCuBV and high yield, *i.e.*, 204.29 g plant^{-1} (Table 2). Susceptible mutants produced severe symptoms starting as a minor vein thickening on young leaves, progressing to severe vein thickening, leaf curling, reduction in leaf size, deformed internodes, and culminating in severe stunting of plants with no or a few bolls.

On the basis of good performance during 2006, M-112-59/22 was evaluated in the field during 2007 and 2008 along with parents. During 2007, 1102 single plants of M-112-59/22 from 40 single plant progenies were raised. M-112-59/22 expressed resistant to highly resistant response (SI = 2.2, PDI = 1.83), while the parent NIAB-111 was susceptible (SI = 4.5, PDI = 48.95) and NIAB-999 was highly susceptible (SI = 5.5, PDI = 84.87) (Table 2). The average number of bolls per plant for M-112-59/22 was 82 with an average yield per plant of 218.42 g (Table 2).

During 2008 the response of M-112-59/22 was again compared with parents in the field at two different sowing dates (Table 2). M-112-59/22 was highly resistant

when planted during the normal sowing season, while the other varieties were highly susceptible to susceptible. In the late sown crop, all plants of male and female parents were infected, while 30% plants of M-112-59/22 were free from the disease 40-50 days after germination. When sown late, M-112-59/22 was moderately resistant with 26.10 PDI, while parents NIAB-111 and NIAB-999 were highly susceptible with PDI of 92.93 and 97.24, respectively. However, M-112-59/22 showed an average SI of 1.7 as compared to 5.6-5.8 for the parents under conducive conditions. At both sowing dates M-112-59/22 showed higher number of bolls (84 and 68) and higher average yield per plant (220 g and 167 g) than the parents. Average boll weight, GOT percentage, fiber length and fiber fineness of M-112-59/22 were very similar for both the sowing dates.

The response of M-112-59/22 to inoculation through grafting with CLCuBV was compared to that of NIAB-111 and NIAB-999. All the tested plants showed symptoms, indicating that the success of grafting and disease transmission was 100%. The data presented in Table 3 show high variations in disease severity of the cotton genotypes tested. M-112-59/22 showed attenuated symptoms varying between SI 3 to 4 with an average SI of 3.4, being ranked as moderately resistant. NIAB-111 was defined as susceptible with average SI of 5.3, while NIAB-999 was highly susceptible with average SIs of 6.0. The first disease symptoms started in the parent NIAB-999 as minute vein thickening after 9 days of inoculation (DPI) and culminating in severe vein thickening, leaf curling and severe stunting after 15 DPI. Concurrently, NIAB-111 showed more moderate symptoms but the symptoms appearing in M-112-59/22 were clearly milder. However, the latent period did not differ greatly as the disease started after 12, 11 and 9 DPI on M-112-59/22, NIAB-111 and NIAB-999, respectively.

Ten plants each from M-112-59/22, NIAB-999 and NIAB-111 were also compared for their response to

Table 3. Response of cotton mutant M-112-59/22 to CLCuBV inoculated through grafting in glasshouse or by whiteflies *Bemisia tabaci* under net-house conditions

	M-112-59/22 (Female parent)	**NIAB-111 (Male parent)**	**NIAB-999 (Male parent)**
After graft inoculation			
Latent period (days)	12	11	9
No of plants showing disease severity			
0	0	0	0
1	0	0	0
2	0	0	0
3	6	0	0
4	4	1	0
5	0	5	0
6	0	4	10
Average severity index	3.4	5.3	6.0
Disease response	Moderately resistant	Susceptible	Highly susceptible
After cage inoculation using vector whiteflies			
Latent period (days)	13	10	8
No of plants showing disease severity			
0	1	1	0
1	0	0	0
2	0	0	0
3	6	0	0
4	3	1	0
5	0	4	1
6	0	4	9
Average severity index	3.3	5.3	5.9
Disease response	Moderately resistant	Susceptible	Highly susceptible

whitefly-transmitted inoculation with CLCuBV. Data presented in Table 3 show that disease transmission was 100% in the case of NIAB-999 while it was 90% in NIAB-111 and M-112-59/22 with variable resistance/susceptibility levels. M-112-59/22 was scored as moderately resistant with 3.3 average SI, NIAB-111 was susceptible with 5.3 average SI and NIAB-999 was highly susceptible with 5.9 average SI. Disease development was delayed in M-112-59/22 with a latent period of 13 days as compared with NIAB-999 and NIAB-111, which exhibited disease after 8 and 10 DPI respectively.

Breeding of CLCuD-resistant cotton with high yield and appropriate fiber characteristics is the most important issue. Considering the increasing intensity of CLCuD during the past years, identification of CLCuD resistant cotton varieties after mutagenesis was initiated at NIAB, Faisalabad, Pakistan during 1995 (Awan *et al*., 1998). Mutation breeding is a complimentary approach to the conventional breeding which provides an opportunity to improve a crop cultivar for a particular trait without disrupting the genotype or to break undesirable linkage between existing genes (Awan *et al*., 1998). By this technique a number of CLCuMV resistant mutants were developed (Awan *et al*., 1998; Akhtar *et al*., 2000, 2002b, 2004). During 2001 a resistance breaking strain appeared and all the resistant germplasm became susceptible (Akhtar *et al*., 2002a, 2008, 2010; Mansoor *et al*., 2003).

The present study showed that resistance response of mutant M-112-59/22 was better than that of its parents when evaluated through different inoculation techniques. In the field tests M-112-59/22 showed a better resistance response and a higher production with acceptable fiber characteristics than the parents. The parents were susceptible to highly susceptible to CLCuBV over the last three seasons, suggesting that the inoculum level was appropriate to cause severe disease. Field screening is the most commonly used method for assaying CLCuD resistance. But this screening technique sometimes produces misleading results due to spatial and temporal variations in inoculum levels due to the effect of environmental conditions, vector host preference and possible host resistance to the vector (Rahman *et al*., 2005; Akhtar *et al*., 2010). These ambiguities were avoided by using graft inoculation and whitefly transmission in the greenhouse. M-112-59/22 showed similar level of severity with infection type range (ITR) of 3-4 using both transmission methods which supported the field findings. However, this mutant showed a higher average SI value in these tests as compared to the field, but still lower than the parents. The majority of plants of M-112-59/22 have shown an average SI of 3, which means that this mutant can produce good yield even under high disease pressure.

Using disease symptom development, yield and fiber quality parameters as the indicator of resistance M-112-59/22 is considered to be a CLCuBV partial resistant mutant that has medium boll size, early maturity, thin leaf, normal foliage, medium long fine fiber and medium short to medium sympodial (data not shown). It can be directly used in the field to reduce the losses caused by CLCuBV. The host reaction that reduces the same kind of epidemics has previously been reported by many workers for other viral pathosystems (Padgett *et al*., 1990; Buiel and Parlevliet, 1995; Dintinger *et al*., 2005; Delatte *et al*., 2006).

In conclusion it is important to note that the high level of resistance to CLCuBV was not observed in the mutants tested here. Due to the acceptable agronomic characteristics and partial resistance to CLCuBV, M-112-59/22 would be useful for managing the present epidemics of CLCuD and will be an essential tool in the hands of the breeders for the development and selection of new resistant genotypes.

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