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Application of *Cry1Ab/Ac Bt* strip for screening of resistance for *Maruca vitrata* in cowpea

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Maruca vitrata **is a significant constraint to cowpea production in most cowpea growing areas of sub-Saharan Africa. Yield losses caused by** ***M. vitrata*** **in these regions are estimated in millions of tons annually and the prevalence of** ***M. vitrata*** **infestation is steadily increasing. Recombinant DNA technology have led to development of some cowpea lines with** ***Maruca*** **resistance as well as other important agronomic traits but it is time-consuming and difficult to screen for the resistant trait especially in the segregating populations using conventional screening techniques, which will lead to delay in the development of** ***Maruca*** **resistant cowpea varieties. The use of allele-based selection tool will make it easier to select plant traits and reduce the time needed to develop new** ***Maruca*** **resistant cowpea varieties. In this study, the efficacy of using** ***Cry1Ab/Ac Bt*** **strip for detecting** ***Maruca*** **resistant transgene in transgenic cowpea was systematically investigated for the first time through field derived progenies. The results show that the** ***Cry1Ab/Ac Bt*** **strip was effective for detecting the presence of the resistant gene in cowpea genome.** ***Maruca*** **resistant plants were successfully screened from the segregating cowpea plants and the genetics of the gene was monitored. The** ***Cry1Ab/Ac Bt*** **strip was found to be suitable for genetic analysis of the** ***Maruca*** **resistant transgene in cowpea. This study has demonstrated the precision of using** ***Cry1Ab/Ac Bt*** **strips as a screening tool of transgenic lines containing** ***Cry1Ab*** **gene, this has an importance in the hybridization programme where genotypes having** ***cry*** **gene can be distinguished at seedling stage at lesser time, with the potential of putting the breeding process on a fast track and increase the efficiency of breeding activities.**

Key words: *Bacillus thuriengiensis*, *Cry1Ab/Ac Bt* strips, transgenic cowpea, *Maruca vitrata*.

INTRODUCTION

Cowpea *(Vigna unguiculata L. Walp)* is considered the most important food grain legume in the dry savannas of tropical Africa (NGICA, 2002). It is the most important indigenous African legume for both home use and as a cash crop and especially important for the Sahel because of its drought tolerance (Kushwaha et al., 2004). It is rich in quality protein and has energy content almost equivalent to that of cereal grains, it is a good source of quality fodder for livestock and also provides cash income (Davis et al., 1991). Nearly 200 million people in Africa consume the crop (AATF, 2010; NGICA, 2002). Cowpea is consumed in many forms; the young leaves, green pods, and green seeds are used as vegetables, dry seeds are used in various food preparations, the haulms are fed to livestock as nutritious supplement to cereal fodder and being a fast growing crop, cowpea curbs erosion by covering the ground, fixes atmospheric nitrogen, and its decaying residues contribute to soil fertility (Singh et al., 2002).

The overall productivity of its existing traditional geno-

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types are low due to their prominent susceptibility to insect pests (Darshana et al., 2007) and among the most damaging insects are aphids, flower thrips, cowpea pod borer, pod sucking bugs and the cowpea weevils (Darshana et al., 2007). The cowpea pod borer *(Maruca vitrata)* is a serious lepidopteran pest that inflicts severe damage to cowpea on farmers' fields (Figure 3). In severe infestations, yield losses of between 70-80% have been reported (AATF, 2010). Control through spraying with insecticide has not been fully adopted by farmers due to the prohibitive costs, causing resource-poor farmers to opt for cheaper but more toxic alternatives that impact their health (AATF, 2010).

Breeding for insect resistance with the aid of phenotypic selection is time consuming, laborious and relatively expensive (Xu and Crouch, 2008). In addition, most crops have a high level of heterozygosity that makes visual selection difficult but selection based on allele composition will avoid this problem (Ibitoye and Akin-Idowu, 2010). Ability to select breeding progeny early at the seedling stage is another advantage of using allele-based selection tools (Ibitoye and Akin-Idowu, 2010). The number of plants that are needed to be maintained in a crop breeding programme can be reduced by eliminating progenies that do not carry the desirable allele at the seedling stage, saving space, time, labor and other resources (Ibitoye and Akin-Idowu, 2010). The present study was designed and conducted in order to understand the efficacy of using *Cry1Ab/Ac Bt* strips for detecting *Maruca* resistant transgene in transgenic cowpea through field derived progenies.

MATERIALS AND METHODS

The Research was conducted under the confined field trial site (CFT) between July, 2011 to August, 2012 at the Institute for Agricultural Research (IAR), Samaru-Zaria, Nigeria. Two genetically engineered cowpea lines: Transgenic cowpea line TCL-709 and TCL-711, and three non-transformed cowpea genotypes: IT97K-499-35, IT93K-693-2 and IT86D-1010, were used in this study. Data were collected as scores of *Cry1Ab Bt* strip test.

Cry1Ab inheritance studies

To establish the potency of *Cry1Ab*/*Ac Bt* strips as a screening tool for *Maruca* resistant transgene, the inheritance of *Cry1Ab* gene was monitored with the aid of *Bt* strips in filial generations.

Development of the genetic population

The transgenic cowpea lines TCL-709 and TCL-711 along with three non-transgenic genotypes: IT97K-499-35, IT93K-693-2 and IT86D-1010 (the original parent of the transformed lines having the same genetic architecture except the *Cry1Ab* gene) were crossed using biparental mating as described by Sharma (2006) to generate F_1 population. Some F_1 seeds were advanced to second filial generation (F_2) populations by self pollination. The following six combinations of crosses were produced: IT97K-499-35 x TCL-709, IT97K-499-35 x TCL-711, IT93K-693-2 x TCL-709, IT93K-693-2 x TCL-711, IT86D-1010 x TCL-709 and IT86D-1010 x TCL-711.

Field evlauation

The parents, F_1 and F_2 generations were evaluated under field conditions during the 2012 cowpea growing season at CFT Samaru-Zaria between June to August, 2012. The trial was planted using randomized complete block design with three replications. The plant to plant and row to row spacing was kept at 30 by 75 cm, respectively. The plot size was 3 x 5 m for all entries except F_2 plants which were 6 x 5 m. No insecticidal spray against lepidopteran insects was applied.

Test procedure

The screening was carried out with the aid of *Cry1Ab/Ac Bt* strips to check for the presence of *Cry1Ab* gene in the genetic populations (P_1, P_2, F_1 and F_2) of transgenic cowpea. Detection of *Cry1Ab* proteins on cowpea involved assaying plant leaves for expression of the *Cry1Ab* gene. A quick *Bt* strip test was used to confirm the expression of the *Cry1Ab* protein in cowpea transgenic lines. This was achieved by placing leaf discs in test tubes containing buffer and then slowly inserting *Bt* strips into the buffer. Then, formation of a single line in the test tube proved that the test was working while the appearance of a second lower line showed that *Cry1Ab* protein was present (Envirologix, 2008). Figures 1 and 2 illustrate a typical type of *Cry1Ab Bt* strip test. In these figures, the appearance of two lines on the test membrane indicates the presence of the *Cry1Ab Bt gene*, while the appearance of only the top (control) line indicates a negative response.

Cry1Ab gene screening in F_1 generation

The plants were screened with the aid of *Cry1Ab/Ac Bt* strips and the transfer of *Cry1Ab* gene from a transgenic cowpea plant to a non- transgenic cowpea plant was checked. The number of positive and negative plants indicating presence and absence of the transgene respectively, were taken to infer the behaviour of the transgene whether dominant or recessive and establish the efficacy of the *Cry1Ab/Ac Bt* strips.

Cry1Ab gene screening in Segregating Generations

Adequate sample size was taken from each F_2 family and analyzed with the aid of *Cry1Ab/Ac Bt* strips. Since the gene is expected to segregate in F_2 generations, the plants were clearly classified as *Cry1Ab*-positive or *Cry1Ab*-negative regarding the *Cry1Ab* expression where *Cry1Ab* positive plants indicates resistance to *M. vitrata* while *Cry1Ab* negative plants indicates susceptibility to *M. vitrata*. Envirologix (2008) procedures for *Cry1Ab*/Ac *Bt* strip test was carefully followed. The data was subjected to Chi-square goodness of fit test against the Mendelian ratio 3:1 for the F_2 generations (Kiani et al., 2009).

Statistical analysis

Data recorded for the genetic segregation of *Cry1Ab* transgene were analyzed with the help of Chi-square (X^2) goodness of fit test, to determine whether the observed data conforms to the expected Mendelian 3:1 ratios for F_2 segregating populations of each cross. The following formula was used using a *Proc Frequency* for a chi-square test of goodness of fit by Mcdonald (2009):

$$\chi^2 = \sum \frac{(O-E)^2}{E}$$

Figure 1. *Cry1Ab/1Ac Bt* strips showing positive, negative and invalid result (Envirologix, 2008).

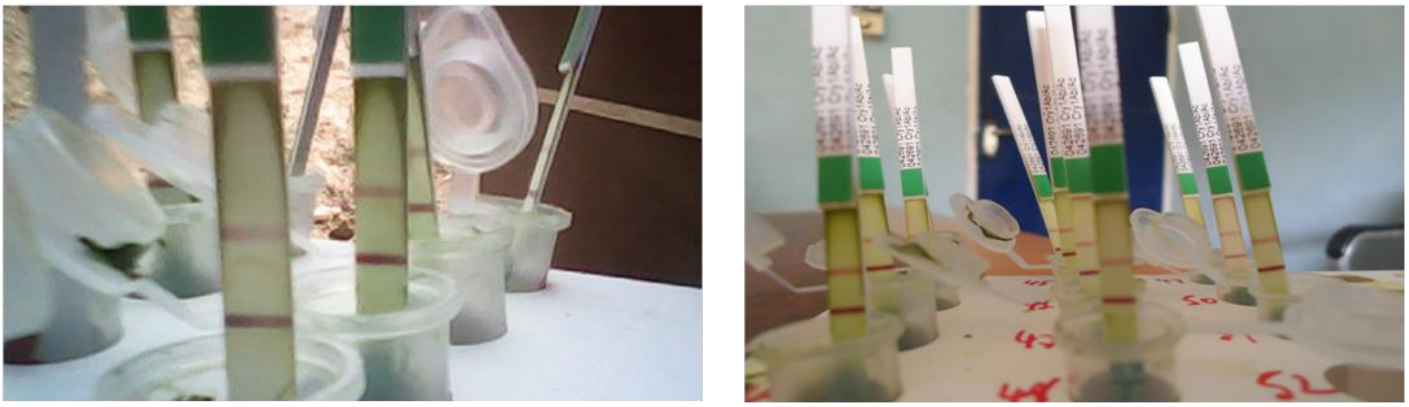

Figure 2. *Cry1Ab/Ac Bt* strips in test tubes showing positive results.

Figure 3. Showing larvae and adult *Maruca vitrata* (legume pod borer) pest.

Table 1. Detection of *Cry1Ab* gene in Parents and F_1 populations of transgenic cowpea.

Genotype	Number of plants tested	Positive	Negative	Expected ratio
TCL-709	50	50	0	1:0
TCL-711	50	50	0	1:0
IT97K-499-35	25	0	25	0:1
IT93K-693-2	25	0	25	0:1
IT86D-1010	25	0	25	0:1
IT86D-1010 x TCL-709	23	23	0	1:0
IT86D-1010 x TCL-711	23	23	0	1:0
IT97K-499-35 x TCL-709	30	30	0	1:0
IT97K-499-35 x TCL-711	28	28	0	1:0
IT93K- 693-2 x TCL-709	25	25	0	1:0
IT93K-693-2 x TCL-711	23	23	0	1:0

Positive, *Cry1Ab* gene is present, that is, resistant to *M. vitrata*; negative, *Cry1Ab* is absent, that is, susceptible to *M. vitrata*.

Table 2. Detection of *Cry1Ab* gene in F_2 populations of transgenic cowpea crosses.

Cross (female x male)	No. of plants tested	Positive	Negative	Expected ratio	Chi-square	DF	Probability
IT86D-1010 x TCL-709	105	81	24	3:1	0.26	1	0.61^{ns}
IT86D-1010 x TCL-711	81	61	20	3:1	0.004	1	0.95^{ns}
IT97K-499-35 x TCL-709	89	65	24	3:1	0.18	1	0.67^{ns}
IT97K-499-35 x TCL-711	111	85	26	3:1	0.15	1	0.70^{ns}
IT93k- 693-2 x TCL-709	75	58	17	3:1	0.22	1	0.64^{ns}
IT93k- 693-2 x TCL-711	131	101	30	3:1	0.31	1	0.58^{ns}

Positive, *Cry1Ab* gene is present, that is, resistant to *M. vitrata*; negative, *Cry1Ab* is absent, that is, susceptible to *M. vitrata*; ns, not significant at p=0.05.

Where, O, Observed value; E, expected value; $\sum$, summation.

RESULTS

Screening for *Cry1Ab* transgene in F_1 generations

The results of the six set of F_1 plants analyzed with the aid of *Cry1Ab Bt* strips to study the efficacy of *Bt* strips for detecting the transgene's presence through transmission and expression of the transgene are given in Table 1. It was found that all the F_1 plants were positive to *Cry1Ab Bt* strip test. It thus means that the gene was successfully transferred from *Bt* lines to non-*Bt* lines and the *Cry1Ab Bt* strips were potent as detecting tool for the target gene.

Screening for *Cry1Ab* transgene in segregating populations

As shown in Table 2, it reveals that the Mendelian segregation ratios (3:1) existed in all the six cross combinations for the F_2. The F_2 populations of these crosses segregated into plants with positive and negative *Cry1Ab* gene indicating the presence and absence of the *Maruca* resistant gene, respectively, with a good fit to the Mendelian ratio of 3:1 with non significant Chi-square values (X^2) for F_2 plants of the following crosses; IT97K-499-35 x TCL-709 (X^2 = 0.18 ; P = 0.67), IT97K-499-35 x TCL-711 (X^2 = 0.15, P = 0.70), IT93K-693-2 x TCL-709 (X^2 = 0.22; P = 0.64), IT93K-693-2 x TCL-711 (X^2 = 0.31 ; P = 0.58), IT86D-1010 x TCL-709 (X^2 = 0.26 ; P = 0.61), IT86D-1010 x TCL-711 (X^2 = 0.0041; P = 0.95) (Table 2). This has demonstrated the potency of the *Bt* strips for detecting the presence of the transgene in the segregating populations of transgenic cowpea crosses. The strip screening clearly grouped the F_1 plants as resistant plants just like the transgenic parents and the segregating progenies of F_2 were seen clearly behaving as hypothesized into 3:1 Mendelian test ratio.

DISCUSSION

Detection of *Cry1Ab* with *Bt* strips

The genetic segregation and pattern of inheritance of *Cry1Ab* gene in the genetically modified cowpea were monitored in six crosses of cowpea involving transgenic and non-transgenic lines. In the present study, the segre-

gation of *Cry1Ab* gene was found to be in Mendelian fashion in all the six cowpea crosses, the results indicate that the resistant trait was controlled by a single dominant gene in the crosses that were examined. The transgenic lines carried the dominant gene while the recessive allele resides in the susceptible genotypes. In the F_1 generation studies, the *Cry1Ab* gene was found to be successfully transferred from transgenic to non-transgenic and it was dominant. These results are in agreement with earlier research works on genetically modified *Bt* crops with *Cry1Ab* transgene: *Cry1Ab* transgene is inherited as single dominant gene, in *Bt* corn where the *Cry1Ab* conferred resistance to stem borer (*Ostrinia nubilalis*) (Murenga et al*.,* 2012), in *Bt* Rice containing resistant gene to striped stem borer (*Chilo suppressalis*) (Kiani et al., 2009; Wang et al*.,* 2012), in crosses of transgenic Rojolele Rice (Sulistyowati et al*.,* 2008) and in *Bt* Cotton where Khan (2008) and Zhang et al. (2000) studied the inheritance and segregation of foreign *Bt* (*Bacillus thuringiensis* toxin) and tfdA genes. The ability to obtain 3:1 segregation in F_2 generations using the *Cry1Ab Bt* strips means that these tests could be employed for wide-scale studies in the field to enhance cowpea breeding for resistance to *M. vitrata*.

The results obtained here indicate that it is possible to use this technology to select for *Maruca* resistant genotypes in cowpea. Similar results have been reported in other crops (corn, soybean, cotton and canola) using *Bt* strips technology to select plants carrying *Cry1Ab* transgene (Stave, 2002; USDA/GIPSA 2006) and had proven to be effective in detecting the presence of the transgene in these crops. *Cry1Ab Bt* strip tests for genetically engineered crops are currently being used on a large scale in the United States to manage the sale and distribution of grains that are genetically transformed (Stave, 2002). In several of these applications, it is important to get a result rapidly in the field, and in these situations strip tests are particularly useful.

Using the *Cry1Ab Bt* strips, the screening were done at seedling stage with good precision, this saves time and resources. The use of *Cry1Ab Bt* strips as a screening tool of transgenic lines containing *Cry1Ab* gene is strongly recommended, this has an importance in the hybridization programme where genotypes having the transgene can be distinguished at seedling stage at lesser time. The benefits of this technology have important implications for improving the efficiency of the characterization of cowpea genotypes for resistance to *Maruca* in the laboratory, especially when working in remote areas and in developing countries where access to laboratory facilities, chemicals, and equipment for polymerase chain reaction (PCR) procedures are limiting. The *Cry1Ab Bt* strip test was found to be the most suitable in order to rapidly analyze large number of plants in lesser time and to differentiate between the two groups. Elite and promising plants can be faithfully screened and selected at seedling stage particularly during the development of backcross population, aimed towards development of transgenic cowpea varieties. Results obtained from *Bt* strips sampled materials were effective and reproducible in our hands from the six F_2 populations used. The studies described here that the *Bt* strips screening offers a simple, sensitive and specific tool appropriate for identifying *Maruca* resistant transgene. We conclude that the application of this technology has the potential to significantly enhance the *Maruca* resistant cowpea breeding program, and the efficiency of breeders to speed-up the process of developing and deploying *Maruca* resistant cowpea varieties to farmers. This study demonstrates that *Bt* strip is an effective, economic and sensitive method for sampling and identifying resistant cowpea plants using leaf tissues.

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