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Inheritance and segregation of virus and herbicide resistance transgenes in sugarcane

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Abstract Transgenic sugarcane parents containing multiple copies of herbicide resistance (*bar*) and *Sorghum mosaic virus* (SrMV) resistance (*hut*) genes were crossed with non-transgenic sugarcane varieties. Segregation of the transgenes in the progeny was determined using Southern blot analysis; herbicide resistance and SrMV resistance were assessed using bioassays. The segregation data were used to infer linkage relationships between transgenes in the parent plants. In two of the parents, all transgene insertions were linked in one position in the genome, although some recombination between insertion events did occur. In the third parent, insertion had occurred in two independent, unlinked loci. Analysis of progeny of this parent indicated that rearrangement or mutation occurred in both loci, resulting in non-parental transgene DNA fragments in some progeny. Most transgenic progeny containing the *bar* gene showed resistance to herbicide. SrMV inoculation indicated that a fairly high proportion of the transgenic progeny showed susceptibility. As the post-transcriptional gene silencing mechanism responsible for the virus resistance phenotype may be reset during meiosis, phenotypic screening of older plants may be a more reliable indication of virus resistance than screening young seedlings. This is the first report of transgene segregation in sugarcane, and we have demonstrated that transgenic sugarcane parents showing stable inheritance of transgenes can be effectively used in breeding programs.

Keywords Herbicide resistance · Inheritance · Transgenes · Sugarcane · Virus resistance · Post-transcriptional gene silencing

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Introduction

The development of new sugarcane (*Saccharum* spp. hybrids) varieties is a lengthy and unpredictable process. Genetic transformation offers the potential to introduce some new, desirable characteristics into existing varieties, provided the transgenes are expressed in a stable manner. Using transgenic varieties as parents in breeding programs has some advantages over the *de novo* transformation of existing cultivars. Transformation of an existing cultivar requires several years of field evaluation to ensure that the introduced gene is stably expressed and that no other detrimental phenotypic effects are induced by the transformation process. In contrast, large numbers of transgenic progeny can be produced from crosses involving transgenic parents, and these can be evaluated during the normal stages of a selection program. No extra evaluation is required, which could save several years in the variety development program. This, however, is dependent on the stable transmission and expression of transgenes from parents to offspring and the breeding value of the parent genotypes.

Sugarcane has a complex polyploid-aneuploid genome which can result in meiotic instability and the production of aneuploid gametes (Burner and Legendre 1994). In selfs of elite sugarcane, Burner and Legandre observed from 3% to 11% of univalents in metaphase I microsporocytes of each clone, and up to 20% of micronuclei present at telophase II. As genetic transformation through particle bombardment could introduce additional sources of meiotic instability into an already unstable

Table 1 Transgenic parent genotypes used in crossing, and genotypes of their corresponding progeny. In each cross, the non-transgenic variety CP72-1210 was used as the male parent

Parent genotypes			
Female parent	CP72-1210#61	CP72-1210#323	CP65-357#120
Number of copies *hut*/*bar*	10/5	6/2	5/5
Cross	98-1258	98-615	98-613
Progeny genotypes			
Transgenic parental	20	25	21
Non-transgenic parental	27	24	11
Recombinants	11	20	32
Total progeny	58	69	64

system, examining the transmission of transgenes in sugarcane progeny is necessary to establish whether breeding with transgenic parents would be effective.

Reports from other crops indicate that the inheritance and expression of transgenes can be variable. Demeke et al. (1999) demonstrated that an Act1D-uidA::nptII cassette was inherited for five generations of selfing in wheat (*Triticum aestivum* L.), with no evidence of transgene rearrangement. In one cross, however, low B-glucuronidase (GUS) and neomycin phosphotransferase (NPTII) enzyme activity was related to methylation of the inherited transgene cassette. Kim et al. (1999) showed that the *bar* gene conferring herbicide resistance was stably transmitted to rice (*Oryza sativa* L.) progeny, but some seedlings containing multiple copies of the *bar* transgene were herbicide-sensitive due to silencing of the transgene. Also in rice, Chen et al. (1998) reported that co-expression of the *hph*, *uidA* and *bar* marker genes was stable over three generations and that gene silencing was observed only in some cases. Scott et al. (1998) observed both expected and distorted segregation ratios for a *uidA* transgene in the F_2 generation of white clover (*Trifolium repens* L.), where the segregation distortion was influenced by the genetic background of the inbred lines. The segregation distortion was due to a smaller than expected number of progeny carrying the transgene, which could be expected if the insertion event caused deleterious or lethal mutations. Irregular patterns of transgene silencing were reported by Pawlowski et al. (1998), for hexaploid wheat over several generations: silencing of *bar* and *uidA* transgenes was observed in 19 of 23 transgenic lines, which resulted in distorted segregation of phenotype in subsequent generations. This silencing and inheritance distortion was irregular and unpredictable but could be reversed in subsequent self-pollinated progeny. In squash (*Cucurbita* spp.) transgenic for different mosaic virus coat protein transgenes, Tricoli et al. (1995) observed that the transgenes were stably inherited after several generations of selfing but that the resistance phenotype depended on the form of the insert.

From these examples it is apparent that the transmission of transgenes from parents to progeny and the expression of the transgenes in the progeny, need to be well studied in order to effectively use transgenic parents for the development of new varieties. This is the first report of transgene segregation in sugarcane, a polyploid crop with a complex aneuploid genome.

Materials and methods

Parent plants

Transgenic sugarcane varieties containing an untranslatable *Sorghum mosaic virus* (SrMV) strain H coat protein gene (*Ubi-hut* construct) and a herbicide resistance gene (*Ubi-bar* construct) were produced as described by Ingelbrecht et al. (1999). In brief, callus of two varieties was co-transformed by particle bombardment and plants regenerated after selection on bialaphos-containing medium. Transgene integration patterns and copy number were determined from Southern analysis, and phenotype was measured after repeated inoculations with SrMV in the greenhouse. A tentative relationship between SrMV coat protein copy number and virus resistance was observed, with resistant plants tending to have intermediate coat protein gene copy numbers (4–10), and susceptible plants having either high (>10) or low (<4) copy numbers. Additional Southern analysis suggested that the mosaic virus coat protein transgenes in resistant plants were more highly methylated than those in susceptible plants, which suggests that the mosaic resistant phenotype is due to a post-transcriptional gene silencing (PTGS) mechanism (Ingelbrecht et al. 1999). Northern analysis and nuclear run-off assays confirmed that virus resistance was related to a PTGS mechanism (Ingelbrecht et al. 1999).

Three of the transgenic plants described in the above study, CP72-1210#61, CP72-1210#323 and CP65-357#120, were used as female parents in crosses during 1998. These plants were primary transformants (T0) that had undergone several cycles of vegetative propagation and had been phenotypically selected for general appearance and vigor. Their respective copy numbers for the *Ubi-hut* and *Ubi-bar* constructs are shown in Table 1.

Progeny

Crosses were made in isolation chambers at Canal Point, Florida by Dr. J Miller. Non-transgenic CP72-1210 was used as the male parent. Seedling progeny were planted to the field as single plants, spaced 45 cm apart (APHIS release notification 98-310-03N). The number of seedlings available for the three crosses is shown in Table 1.

Genomic DNA extractions and DNA gel-blot analysis

Young leaves were collected from each seedling, and total DNA was extracted as described by Tai and Tanksley (1990), with slight modifications. Twenty micrograms of DNA was digested with *Hin*dIII and electrophoresed overnight on 0.8% agarose gels, then transferred by downward alkaline blotting (Koetsier et al. 1993) to nylon membranes. Membranes were pre-hybridized overnight in Church's buffer (0.5 *M* Na_2 HPO_4, 0.5 *M* NaH_2 PO_4, 0.01 *M* EDTA, 7% SDS), then hybridized overnight at 65 °C with [^{32} P]-labelled DNA probes. Probes for the *bar* gene were prepared using a GIBCOBRL random priming kit, and probes for the SrMV coat protein gene were prepared using a GIBCOBRL (Gaithensburg, Md.) nick-translation kit. After washing using standard proce-

dures, the membranes were placed on film for 16–60 h, then developed and scored for the presence or absence of restriction enzyme fragments corresponding to the transgene insertions in the parent plants. In each cross, restriction fragments corresponding to transgene insertions were labeled H_1 to H_n and B_1 to B_n for *hut* and *bar*, respectively, with the lower subscripts referring to fragments with lower mobility.

Linkage analysis

Linkage relationships between transgenes were estimated using MAPMAKER 3.0 software (Lincoln et al. 1992). Each transgene insertion was treated as a dominant simplex (i.e. single copy) restriction fragment length polymorphism (RFLP) fragment which will segregate in a 1:1 ratio in the gametes, regardless of the type or level of ploidy (Sorrels 1992; Wu et al. 1992). This method has been used to create RFLP linkage maps in sugarcane (e.g. Grivet et al. 1996).

Herbicide resistance

Each seedling was vegetatively propagated by planting single-budded setts in small pots in a greenhouse. At four weeks of age, these plants (settlings) were sprayed with the herbicide Finale (119.8 g/l glufosinate ammonium, AgrEvo USA, Wilmington, Del.) at a rate of 45 ml Finale per liter. Damage was observed after 5 days, and genotypes were rated as resistant or susceptible. The original seedling plants growing in the field were also sprayed and subsequently rated as resistant or susceptible.

Sorghum mosaic virus resistance

Each seedling was vegetatively propagated by planting single-budded setts in small pots. SrMV was propagated on sorghum (*Sorghum bicolor*, Moench) var. Rio by inoculating sorghum seedlings with virus collected from field-infected sugarcane variety NCo310. Inoculum was prepared by homogenizing 10 g of symptomatic leaf tissue in 500 ml of 50 mM phosphate buffer, pH 7.2, 0.1% Na_2 SO_3 with 0.5% diatomaceous earth. The clones of each sugarcane seedling were then inoculated twice, 1 month apart, at the four-leaf and eight-leaf stage by rubbing inoculum prepared from sorghum on the leaf surface. SrMV symptoms were scored repeatedly over several months, and plants were rated as symptomatic or asymptomatic.

Results

Transgene segregation and linkage

The number of progeny in each cross having the respective parental transgene genotypes and the number of recombinants is shown in Table 1. Frequencies of the two parental genotypes were similar in crosses 98-1258 and 98-615, but cross 98-613 exhibited an excess of progeny with the transgenic parental genotype.

The segregation of each transgene insertion in all crosses was tested against a 1:1 segregation ratio hypothesis using a chi-square analysis. All *bar* and *hut* inserts for crosses 98-1258 and 98-615 showed a 1:1 segregation ratio, but in cross 98-613, there was an excess of progeny containing the *bar* and some *hut* inserts, indicating a departure from Mendelian inheritance (data not shown). Although the departure from a 1:1 ratio was statistically significant at the 5% level, a change in value of only one progeny would be sufficient to render the difference non-significant, indicating that sample size could be the cause of the result.

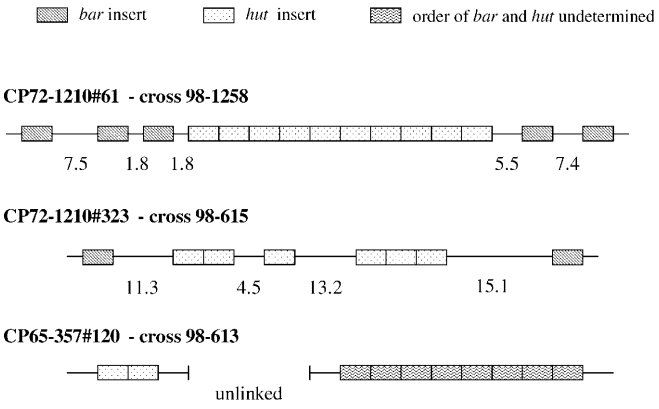

Fig. 1 Most likely arrangement of transgene inserts in three parent varieties, as deduced from progeny segregation data. *Numbers* refer to map units in centiMorgans (Haldane units). The relative order of five *bar* and three *hut* inserts in cross 98-613 could not be determined, as they segregated without recombination

In addition, two individuals from cross 98-613 showed re-arrangement of a *bar* insert, as shown by a increase in electrophoretic mobility of one of the parental bands, B_3, and one progeny showed a increase in mobility in *hut* insert H_4 (data not shown).

The transgene segregation data for each cross was analyzed using MAPMAKER 3.0 (Lincoln et al. 1992), treating each insertion event as a genetic locus. It should be noted that for each cross, the number of progeny available for study was relatively small, which will affect the estimated map distances and order of some transgene linkages. Results presented below reflect the most likely order of transgenes.

In cross 98-1258, the segregation data suggest that the insertion events are linked on the same chromosome, with all ten copies of the *hut* gene co-segregating without recombination, flanked by two *bar* inserts on one side, and three *bar* inserts on the other side. The arrangement of the transgenes and map distances (Haldane units) are shown in Fig. 1. An example of a DNA gel blot is shown in Fig. 2. Data from cross 98-615 showed that all insertion events in parent #323 were linked on the same chromosome, although some recombination did occur between transgenes, as shown in Fig. 3. In cross 98-613, segregation data indicated that transgene integration in parent #120 occurred in two unlinked locations, with H_1 and H_4 linked in one position, and the remainder of the inserts linked in a second location. The relative positions of the *bar* and *hut* inserts in the second location could not be established, as there was no recombination between them. In the two progeny with the higher mobility B_3 bands, the change in the B_3 insert was accompanied by the loss of the linked H_2 band, suggesting that the mutation event causing the change affected both transgenes simultaneously. This implies that H_2 and B_3 are likely to be adjacent to each other, at one end of the group of linked transgenes. One of the progeny also

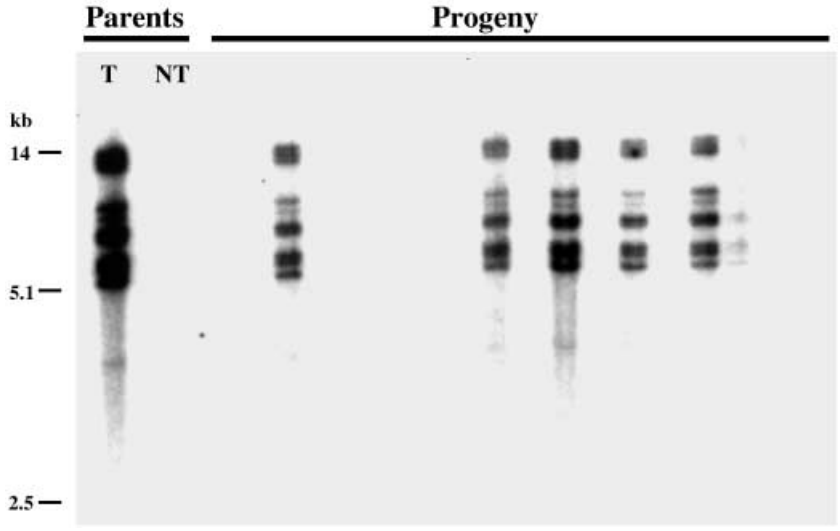

Fig. 2 DNA gel blot of parents and progeny of cross 98-1258 probed with *hut*. *T* = Transgenic parent CP72-1210#61, *NT* = non-transgenic CP72-1210. The progeny show segregation for all ten parental copies of *hut*, indicating linkage of *hut* inserts without recombination

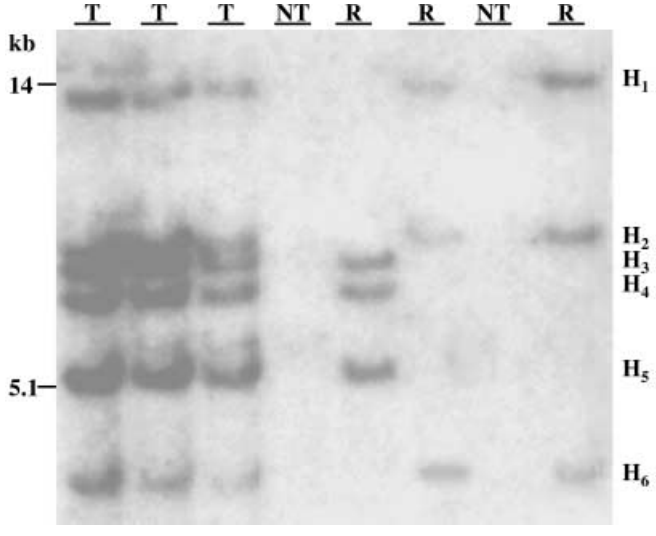

Fig. 3 DNA gel blot of progeny of cross 98-615 probed with hut. *T* = Progeny showing the transgenic parent CP72-1210#323 genotype, *NT* = progeny with the non-transgenic parental genotype, *R* = progeny showing recombination between linked transgene restriction fragments H_1, H_2 and H_6, and H_3, H_4 and H_5

showed a changed H_4 band. As H_1 and H_4 are not linked to the other inserts, this implies that rearrangement or mutation occurred at both transgene insertion positions.

Herbicide resistance

Herbicide resistance assays done in the greenhouse and by spraying plants in the field generally agreed. Some plants resistant in the greenhouse died in the field, but this could have been caused by other factors such as borer infestation, disease and stress. In these cases, the greenhouse results were used. The *bar* genotypes, numbers of progeny with each *bar* genotype and number of progeny susceptible to herbicide for each cross are shown in Table 2. The results were mostly as expected, with progeny lacking the transgenes showing herbicide susceptibility, while plants with at least one copy of the *bar* insert showing resistance. Parental controls reacted as expected, with the transgenic parent showing resistance and the non-transgenic parent susceptibility. Four plants without *bar* inserts showed resistance in both the greenhouse and field assays, possibly demonstrating some degree of tolerance to herbicide damage at the rates used. In crosses 98-1258 and 98-615, six progeny containing either one or two *bar* inserts showed herbicide damage, while plants with higher copy numbers of *bar* genes showed no effect of the herbicide treatment. In cross 98-613, all transgenic progeny contained the five parental *bar* inserts and showed no damage due to herbicide.

SrMV resistance

The inoculation of vegetatively propagated settlings of each progeny plant gave variable results. The *hut* genotypes, numbers of progeny with each genotype and number of plants showing mosaic symptoms for each cross is given in Table 3. Many plants that lacked the SrMV coat protein transgene showed no symptoms after two inoculations. Although it is possible that progeny could show endogenous resistance mechanisms unrelated to the presence of coat protein transgenes, it is likely that some plants escaped infection using the rubbing technique. Unfortunately no true control seedlings (from CP72-1210 × CP72-1210 and CP65-357 × CP72-1210 crosses) were available for inoculation to estimate the level of endogenous resistance. The non-transformed parent showed mosaic symptoms, while the transgenic parent did not. Some plants with parental *hut* transgene genotypes as well as recombinant *hut* genotypes did show strong mosaic symptoms. As the SrMV resistance mechanism in the parent plants is probably due to post-transcriptional gene silencing and DNA methylation

Table 2 Relationship between *bar* genotype in progeny and herbicide susceptibility

Number of *bar* Inserts	98–1258		98–615		98–613	
	Number of progeny	Number of progeny susceptible	Number of progeny	Number of progeny susceptible	Number of progeny	Number of progeny susceptible
0	27	26	28	25	23	23
1	5	1	13	3	–	–
2	3	0	28	2	–	–
3	1	0			–	–
4	2	0			–	–
5	20	0			41	0

Table 3 Relationship between *hut* genotype in progeny and SrMV symptoms

Number of *hut* inserts	98-1258		98-615		98-613	
	Number of progeny	Number of progeny symptomatic	Number of progeny	Number of progeny symptomatic	Number of progeny	Number of progeny symptomatic
0	35	10	29	9	11	4
2	–	–	–	–	13	3
3	–	–	7	1	17	6
4	–	–	3	1	2	1
5	–	–	–	–	21	4
6	–	–	30	9		
10	23	5				

(Ingelbrecht et al. 1999), the susceptibility shown by the transgenic progeny could be due to resetting of transgene PTGS during meiosis. This phenomenon has been reported in *Nicotiana benthamiana* (Guo et al. 1997).

Discussion

Transgene segregation and linkage

Segregation and linkage analysis of progeny proved to be a useful method in determining transgene organization in the parent plants. In two of the transgenic parents, all copies of the transgenes were linked, and in these crosses progeny plants containing the same transgene copy number and organization as the parents were recovered at a relatively high frequency. This implies that parent plants with a desirable transgene copy number could be selected and used effectively in a crossing program to produce transgenic progeny with the same transgene organization as the parents. Campbell et al. (2000) concluded that parents showing stable transmission of linked transgenes could be used in wheat breeding programs. Although this conclusion was not tested in this study, it suggests that crossing two transgenic parents containing different transgenes could be used as a strategy to pyramid multiple transgenes in progeny plants.

Analysis of cross 98-613 showed that parent CP65-357#120 had transgenes inserted at two loci. Under Mendelian inheritance, this would imply that progeny with the parental transgene genotype should be recovered at a lower frequency than if all the inserts were linked. This was not observed, as progeny with the transgenic parental genotype occurred with a higher than expected frequency, while progeny with the non-transgenic parental genotype occurred with a lower than expected frequency. Recombinant progeny were recovered with the expected frequency. Although the relatively small sample size (69 progeny) could account for this, meiotic instability may also be implicated. Three progeny exhibited transgene rearrangement, as shown by increased electrophoretic mobility of the detected transgenes, and this change affected both the insertion positions. In two cases, this was accompanied by the loss of the linked H_2 band. Chromosomal changes in these regions could affect meiotic behavior and result in non-Mendelian inheritance. Svitashev et al. (2000) have presented data suggesting that transgene integration following particle bombardment is accompanied by chromosome breakage and rearrangement. Aberrant transgene segregation ratios have also been observed in wheat (Campbell et al. 2000), clover (Scott et al. 1998) and rice (Zhang et al. 1998), although in each case the cause could not be determined. Parent plants giving rise to distorted segregation ratios could be discarded from crossing programs in favor of those parents showing effective transgene transmission.

Herbicide and SrMV resistance

Results from the herbicide bioassays showed that most transgenic progeny demonstrated herbicide resistance

(107 out of 113, Table 2.). Of the susceptible plants, four were recombinants that had only one copy of the *bar* gene, and two showed the parental genotype of two copies of *bar*. The high frequency of recovery of the desirable phenotype in the progeny indicates that breeding with transgenic parents would be effective in transmitting herbicide resistance. Progeny showing susceptibility can easily be eliminated from the population by spraying with herbicide.

The susceptible phenotype of the transgenic progeny is presumably due to gene silencing. As two of the susceptible progeny had the parental transgene genotype, this silencing is apparently not due to position effects (Gallo-Meagher and Irvine 1996; Matzke and Matzke 1998). Transgene silencing often occurs when multiple copies of the gene are present (see Finnegan and McElroy 1994 for review). This was not the case in our study, as plants with higher copy numbers of *bar* remained resistant. Other reports (Meins and Kunz 1994; Chareonpornwattana et al. 1999) have implicated RNA threshold models for gene silencing at the level of gene expression rather than at the transgene copy number level. The silencing phenomenon was not investigated further in this study, as the primary objective was to investigate the breeding potential of transgenic sugarcane.

Results from the SrMV inoculation highlighted the difficulties of the inoculation technique. In the sugarcane-breeding program at Canal Point, Florida, SrMV resistance is determined by inoculating 50 plants per variety in order to reduce errors and minimize escapes (J. Comstock, personal communication). Notwithstanding this, about 25% of the transgenic progeny showed symptoms of mosaic infection 2–10 weeks following inoculation. Post-transcriptional gene silencing was implicated in the SrMV resistance shown by the parent plants (Ingelbrecht et al. 1999). As PTGS can be reset during meiosis (Depicker et al. 1997; Stam et al. 1997; Matzke and Matzke 1998), it is possible that this mechanism is involved in the SrMV susceptibility shown by the sugarcane progeny. Guo and Garcia (1997) reported that the recovery phenotype following plum pox potyvirus (PPV) infection in transgenic *Nicotiana benthamiana* was not meiotically stable, and Guo et al. (1999) demonstrated that susceptibility to PPV in progeny of *N. benthamiana* correlated with resetting of transgene DNA methylation and restoration of transgene activity. It is possible that the susceptible sugarcane progeny from our study will show recovery from SrMV infection with time, following re-silencing of the *hut* transgenes.

From a sugarcane breeding perspective, it seems that screening progeny for phenotypes dependant on PTGS mechanisms, such as coat-protein mediated virus resistance, may be better done in later selection stages rather than in young seedlings. Screening young plants in which transgene silencing has been reset during meiosis may result in discarding valuable material that could later show the recovery phenotype. In addition, if inoculation of multiple replicate plants for each genotype is required for effective screening, the number of plants involved becomes too large to manage in a practical breeding program. Allowing one cycle of growth, harvest and regrowth (or vegetative replanting) may allow time for the re-silencing of genes reset during meiosis and the re-expression of the associated desirable phenotype.

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