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FIELD PERFORMANCE OF TRANSGENIC SUGARCANE PLANTS CARRYING GENES FOR RESISTANCE TO SCMV

by
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SUMMARY

The field resistance of transgenic sugarcane plants to sugarcane mosaic potyvirus was successfully demonstrated, and a number of transgenic lines are available for consideration for agronomic evaluation. Some of the transgenic lines yielded significantly more tonnes sugar per hectare in this trial, but a firm conclusion about the overall performance of the transgenic lines compared to the parental clone can not be concluded due to the limitations of this trial. These plants contain the coat protein gene of sugarcane mosaic virus and prove that pathogen-derived resistance can be engineered into a genetically complex monocot. The precise molecular basis of the resistance appears to be RNA mediated. More research is necessary to prove this as a number of the resistant lines do not exhibit the usual RNA profiles of transgenic plants from other species which are virus resistant. A second pathogen-derived resistance gene, based on the virus replicase gene, is also capable of conferring virus resistance in sugarcane.

Analysis of sugar and syrup produced from transgenic cane has revealed that no genes, native or transgenic, survive the laboratory production process. There is every confidence that this result would also be found with mill produced sugar, when the opportunity to mill transgenic sugarcane eventuates. There is now good scientific evidence to contribute to the debate that sugar manufactured from transgenic sugarcane plants is indistinguishable or substantially equivalent to sugar produced from non-transgenic plants.

1.0 BACKGROUND

The concept of pathogen-derived resistance (PDR) was first conceptualised by Sanford and Johnstone in 1985. Previous observations on the infection of a bacteria by a bacterial virus (a phage) suggested that if the host bacteria contained part of the phage genome, it was immune to subsequent infection by that phage. Sanford and Johnstone proposed that this situation may also function in the interactions between higher organisms and their pathogens, with part of the virus genome added to the genome of the host conferring resistance to the virus. In 1986, the work of Powell-Abel *et al*. provided the first demonstration of this hypothesis with plants transformed with the coat protein gene of tobacco mosaic virus (TMV) resistant to subsequent infection by TMV. Since, this time there have been numerous reports of pathogen-derived resistance genes conferring resistance in a wide range of virus-host combinations (Grumet, 1994). In 1995, 'Freedom II' a transgenic squash with resistance to zucchini yellow mosaic virus was released commercially in the USA (Shah *et al*. 1995).

In the 1980s an epidemic of sugarcane mosaic resulted in substantial yield losses of up to 40% in the Isis region of southern Queensland. SCMV is one of the most important viral pathogens of sugarcane worldwide, although fortunately in Australia its effects are usually restricted to localised epidemics. The cultivars best suited to the dry red soils of the Isis region such as Q95 and Q137 were relatively susceptible to SCMV: resistant cultivars such as H56-752 were not agronomically suited to this region. The lack of any agronomically suitable, SCMV-resistant plants in the breeding/selection programs to replace Q95 and Q137 and the demonstration by Powell-Abel *et al*. (1986) that pathogen-derived genes could be used to confer resistance provided a requirement and a strategy to develop SCMV-resistant sugarcane via genetic engineering. The coat protein (CP) gene of SCMV was selected as the region of the viral genome to introduce into sugarcane for

two principal reasons; firstly CP genes had been used to successfully develop virus-resistant transgenic plants to a wide range of viruses (Lomonossoff 1995) and, secondly, the CP gene is relatively easy to copy (clone) from the viral genome. In this virus, the coat protein is the structural component of the virus particle: it 'coats' the viral genome, with the particle being comprised of repeating subunits of the CP. The CP of SCMV was cloned (Frenkel *et al.* 1991) and developed into a construct suitable for expression of the gene in sugarcane (Smith *et al*. 1992).

The coat protein gene of SCMV has been developed into a PDR gene and transformed into sugarcane. Transgenic sugarcane plants of clones Q95, Q124, Q137, Q153 and Q155 have been co-transformed with the neomycin phosphotransferase (NPTII) gene and the SCMV coat protein (CP) gene driven by the Emu promoter. Selected, regenerated plants were grown in the PC2 glasshouse. These plants were screened by PCR and southern hybridisation for the presence of the transgene, but no gene expression (ie protein production) from these transgenes was demonstrated. However, approximately 20% of these plants were resistant via the RNA-mediated mechanism to inoculation challenge by SCMV in glasshouse trials.

There is genetic variation between the coat protein regions of SCMV isolates within Australia and the transgene sequence used to confer resistance to SCMV. This result, an outcome of project QUT1S, has implications for the long term exploitation of transgenic plants for virus control. A further outcome of QUT1S was that variation in the replicase region was substantially less than in the replicase region and this region. As variation between the sequence of the transgene and that of the infecting virus has resulted in the breakdown of transgene-mediated resistance, the replicase sequence offered the opportunity to develop a potentially more robust transgene for resistance to SCMV. Transgene resistance mediated by viral replicase genes has been demonstrated in a number of host-virus combinations (Lomonossoff and Davis 1992).

2.0 OBJECTIVES

- Field test transgenic plants carrying genes for resistance to SCMV at the BSES Pathology Farm.

- Produce further transgenic plants carrying other replicase genes for resistance to SCMV, characterise, and test in glasshouse trials.

3.0 OUTCOMES

- Field resistance of transgenic sugarcane to SCMV demonstrated.
- Transgenic plants containing a second pathogen derived resistance gene ('replicase') demonstrated to be resistant to SCMV in glasshouse trials.
- Laboratory sugar and syrup produced from juice extracted from transgenic plants contains no native or transgenic DNA when assayed by a sensitive and specific PCR-based test.
- Four of the mosaic resistant transgenic lines yielded significantly more tonnes sugar per hectare compared to the parental clone. However, a firm conclusion about the overall performance of the transgenic lines can not be concluded due to the limitations of this trial.

4.0 METHODOLOGY, RESULTS AND DISCUSSION

4.1 Coat protein transgenics

Production

Embryogenic callus of cultivars Q95 and Q155 was produced essentially as described by Franks and Birch (1991), on MS (Murasige and Skoog 1962) media supplemented with 3 mg/L of the plant growth regulator 2,4-dichlorophenoxy acetic acid (2,4-D). Callus was maintained in the dark at 27°C. The plasmid constructs used were based on either the Emu (Last *et al*. 1991) of Ubi (Christensen and Quail 1996) plant promoters, with the Nos termination sequence (Figure 1). The CP gene, described by Smith *et al*. (1992), was constructed into plasmids, designated pECPN, pUCPN (Emu or Ubi coat protein) and pUPCN (inverse origination of the coat protein gene (antisense)), were co-transformed into callus with an antibiotic selectable marker gene designated pEKN or pUKN. The plasmids pEKN and pUKN contain the Emu or Ubi promoter respectively, the neomycin phosphotransferase gene and a Nos termination sequence and results in transformed cells being resistant to the antibiotic geneticin. This permits selection of transformed regions of callus from non-transformed regions. The co-transformation frequency is 70-90%, so that most antibiotic resistance cells also contain the construct encoding the gene of interest. Following bombardment the callus recovered for three to four days and was then placed on a MS-2,4D media supplemented with 60 mg/L geneticin. Selection occurred over a six to eight week period, and then the antibiotic resistant callus pieces were transferred to a MS media supplemented with geneticin and placed in the light to initiate shoot regeneration. When the shoots were approximately 10 cm tall and had a well-established root system they were transferred to pots in a containment glasshouse. Plants were analysed for the presence of the transgenes by the polymerase chain reaction (PCR) using a modification of the system described by Smith and Gambley (1993). Southern, northern and western blots were performed essentially as described by Sambrook *et al.* (1989).

Molecular analysis

None of the transgenic lines appeared to express the viral coat protein from the translatable constructs. The level of steady state transgene-specific RNA in the resistant lines was variable, ranging from high to low. However, this range of RNA levels was also observed in susceptible lines (Figure 1). Thus no prediction, based on the RNA level could be made about whether a transgenic line would be resistant

Field trials

A detailed proposal to GMAC was prepared describing the proposed field trials and permission to proceed with the trial was received on 26 June 1997.

Replicated random block design trials containing mosaic rating standards, tissue-culture control plants of the parent clone and transgenic lines were planted at the BSES Pathology Farm, Eight Mile Plains. When the plants were ten weeks old they were mechanically challenged with an SCMV inoculum. Plants were then inspected at regular intervals and rated for resistance to mosaic.

Field trial 1

Objective: To test field resistance and yield of Q155 transgenic lines which had been rated in previous glasshouse trials.

- Planted October 1997.
- Inoculated December 1997.
- Resistance rating April 1998.
- Seven standards (Q63, Q78, Q82, CP29, NCO310, Pindar and Trojan).
- Q155 parent clone.
- Q155 tissue culture clone.
- 62 transgenic lines tested.

Fifteen test lines showed no symptoms and were considered to be immune (I) to mosaic (Table 1). Four of these lines (385, 386, 387 and 389) had the CP gene in the antisense (PC) orientation. Two lines 312 and 314, which had not been transformed with a coat protein construct, also showed an immune response in this trial, possibly due to somaclonal variation induced during tissue culture. Many of the plants tested in this trial contained a β-glucuronidase construct pEGUS originally inserted to assist in laboratory analysis of these plants. The presence of this gene is not believed to have any influence on these trials.

This successful trial indicated that resistance ratings obtained in the glasshouse were reproducible in the field. Fifteen of the transgenic lines were immune to viral infection. This type of resistance has been noted previously in lines in the glasshouse trial (Joyce *et al.* 1998) and has been reported in numerous other transgenic host-virus combinations (eg Smith *et al.* 1995). The immune plants displayed no symptoms of viral infection and

contained no virus after analysis by ELISA. Four of the resistant lines contained the antisense construct pUPCN whilst the rest contained pUCPN indicating that both antisense and sense pathogen-derived genes can confer virus resistance in sugarcane. The tissue culture control plants and the Q155 parent clone plants were SCMV susceptible. Interestingly, no recovery resistance phenotypes were observed during this field trial, although some of the resistant lines showed this phenotype in the glasshouse trials (Joyce *et al.* 1998). All transgenic lines susceptible to virus infection in the glasshouse remained susceptible to SCMV in the field trial establishing the glasshouse assay as a reliable assay to identify resistant from susceptible transgenic plants.

Analysis of yield of plants in field trial 1

After field trial 1 had been established it was proposed that a set of measurements on brix, pol, impurities, and yield be collected and analysed to provide some information on the overall agronomic performance of the transgenic lines. This data was collected from an infected pathology trial and hence must be interpreted with some caution.

Stalks were harvested and weighed, and then juice was expressed in a small mill and analysed for brix, pol and purity. Fibre was not measured, but was set at 13 for the subsequent calculations. CCS which is calculated using brix, pol, purity and fibre was calculated and 1.5 units subtracted for expressed juice from a small mill. Lines in the trial were compared for yield, CCS and tonnes sugar per hectare (TSH). As there were unequal number of replicates, Proc GLM within the statistical program SAS was used to calculate Least Squares Adjusted Means. The means of each character are compared pair-wise across the lines resulting in a 70x70 matrix of probability values. As there were too many pair-wise comparisons to present all of them, a small subset of the data is presented in Table 2. This table contains the probability (p) values for two sets of comparisons: (1) the resistant lines and the Q155 parental line; and (2) the resistant lines and Q155 tissue culture control. Probability values followed by a *, ** or *** are significant at .05, .01 and .001% levels respectively. There was no significant difference between the parental clone and tissue culture controls (p=0.2528), suggesting that somaclonal variation due to tissue culture is minimised in this genotype. Four resistant transgenic lines, 314, 343, 346 and 354 yielded significantly more tonnes sugar per hectare than the parental or tissue culture plants. However, a number of non-resistant transgenic lines also out-performed the control plants in this trial (data not shown) so it is difficult to reach a clear conclusion. More research, with the trial established as a yield trial rather than a pathology trial, should address this issue.

Field trial 2

Objective: To test further transgenic varieties for resistance to SCMV.

- Planted January 1998.
- Inoculated March 1998 (with Brisbane/Isis mixed isolate SCMV).
- Inspected August 1998.
- Seven standards (Q63, Q78, Q82, CP29, NCO310, Pindar and Trojan).
- Q124 and Q155 tissue culture clones.
- 61 transgenic lines.

Transgenic lines of Q95, Q124, Q137, Q153 and Q155 transformed with both pECP and pUCP were planted.

Unfortunately, this trial was not a success as none of the standards developed any symptoms after inoculation. The reasons for this failure are uncertain. The trial could not be repeated due to land and test material constraints.

Field trial 3

Objective: To test various transgenic lines against the Isis3 isolate on SCMV. Isis3 had been identified previously in QUT1S (Handley *et al.* 1998) as having sufficient nucleotide differences in the coat protein to the coat protein sequence used to transform the plants, that it may break the resistance conferred by the transgene sequence.

- Planted March 1998.
- Six standards (Q68, Q78, Q82, NCO310, Pindar and Trojan).
- Q124 and Q155 parent clones.
- Q124 and Q155 tissue culture clones.
- 25 transgenic lines (mixture of immune, recovery and susceptible).

Just after this trial was planted, Brisbane experienced a long period of wet weather. Germination of setts in this trial was so poor, that the trial was stopped. The trial was re-established as field trial 4.

Field trial 4

Objective: As per field trial 3. Test of performance of transgenic lines against SCMV-Isis3 isolate.

- Planted October 1998.
- Inoculated December 1998.
- Inspected March 1999.
- Eight standards (Q63, Q68, Q78, Q82, CP29, NCO310, Pindar and Trojan).
- Q155 tissue culture clone.
- Q155 parent clones.
- 19 transgenic lines.

This trial was also unsuccessful as the SCMV-Isis3 inoculum, whilst infectious to maize, did not infect the trial. The reason for this is uncertain as SCMV inoculums which infect maize are almost always infectious in the sugarcane test plants. This result was most disappointing especially after the first attempt at this trial failed due to poor germination.

Discussion

Four field trials were established during this project to test:

1. The reproducibility of glasshouse resistance ratings in the field;
2. Further transgenics of different cultivars carrying both Emu- and Ubi-based constructs; and
3. Whether variation between the sequence of the transgene and the coat protein of another isolate could break the resistance conferred by the transgene.

Problems with establishing and infecting the field trials were encountered, so that only the first question can be answered. Yes, the resistance found in the glasshouse trials is reproduced in the field. Interestingly, in the glasshouse trials we could identify three different types of resistance. There were ten lines which showed no symptoms of SCMV infection, contained no virus as analysed by the ELISA, and inoculum prepared from these lines was not infectious to the maize indicator plants. These plants are 'immune' to infection by SCMV. Immunity is one of the phenotypes described in other virus-transgenic plant systems (eg Smith *et al.* 1995) and is the most obvious form of transgene-mediated resistance. The second resistant phenotype observed was 'recovery'. This phenotype is also reported in other virus-transgenic plant systems and is typified by symptoms on the leaves that had emerged at the time of inoculation that decrease in intensity, until the newly emerging leaves have no symptoms. This phenotype was not observed in the field trials, but this may have been due to the difficulties of observing the recovery phenotype in a field crop like sugarcane. The second stage of recovery is immunity, so that these plants are effectively immune to SCMV infection in the field.

The third phenotype observed in the glasshouse trials was 'atypical'. An atypical phenotype has also been occasionally noted in other systems such as transgenic tobacco-tobacco etch virus (Dougherty *et al.* 1994). On the sugarcane plant lines displaying this phenotype there were small flecks, on the leaf blade, at the leaf margin or on the midrib. ELISA readings on these plants indicated that there was no virus present in these plants. Back-inoculation experiments confirmed that these plants did not contain virus. No atypical symptoms were seen in the field trial, and as this is a resistance phenotype this was also scored as immunity in the trial.

The apparent failure of the inoculum to infect two of the trials is of concern, especially as the inoculums were infectious to test maize plants. As this stage no hypothesis can be proposed to explain the failure to infect in these trials.

4.2 Replicase transgenics

Production

Production and initial analysis of sugarcane plants containing gene constructs based on the sugarcane mosaic virus replicase gene was essentially the same as described above for the coat protein transgenics. The gene constructs used for co-transformation with pUKN were based on sequence obtained by analysis of the replicase region of Australian isolates of SCMV (Handley *et al.* 1996). These plasmids, pURTN and pURUN (Ubi replicase translatable and Ubi replicase untranslatable) were designed so that the RNA transcribed

from the DNA was either capable of being translated into the replicase protein, or would be incapable of translation due to the presence of stop codons near the start of the gene.

Glasshouse trial

Objective: To test transgenic plants transformed with Emu and Ubi replicase constructs for resistance to SCMV.

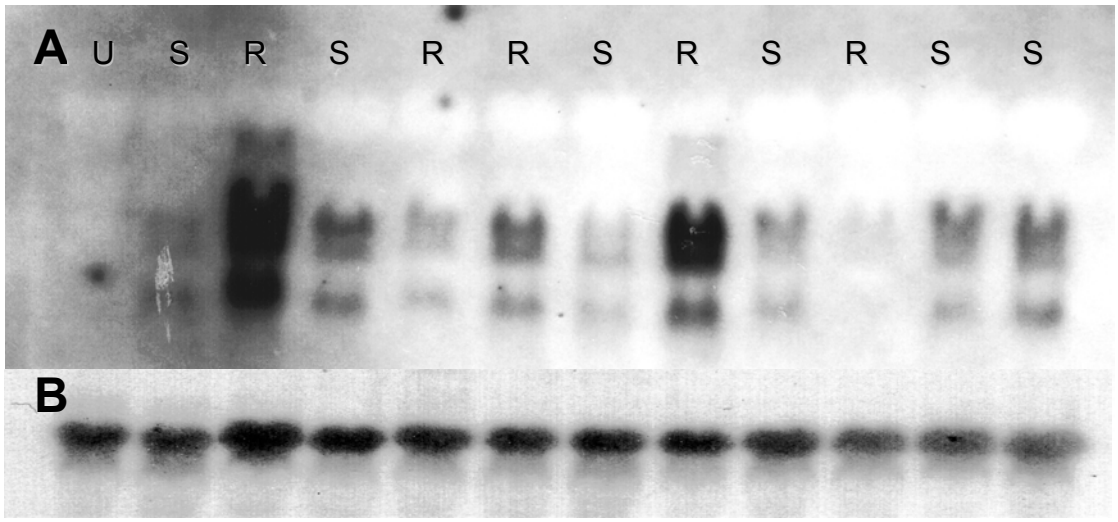
- Plants established in PC2 glasshouse in January 1999.
- Inoculated February 1999.
- Inspected April 1999.
- No standards available.
- Q124 tissue culture clones.
- Q124 tissue culture clone (NPTII positive).
- 34 Q124 pURTN plants.
- 16 Q124 pURUN plants.
- 16 Q155 pURUN plants.

Plants were inoculated with the Pathology Farm isolate of SCMV. Q124 (tc#2) and Q124 (NPTII only) were inoculated with the phosphate buffer alone as negative controls. Ten weeks after inoculation, two translated Q124 plants (TR25 and TR52), one Q124 untranslated plant (UR43) and one untranslated Q155 plant (UR39) still exhibited no visible symptoms (Table 3). TR25 showed the characteristics of a recovery phenotype. The other lines appeared to be immune.

Discussion

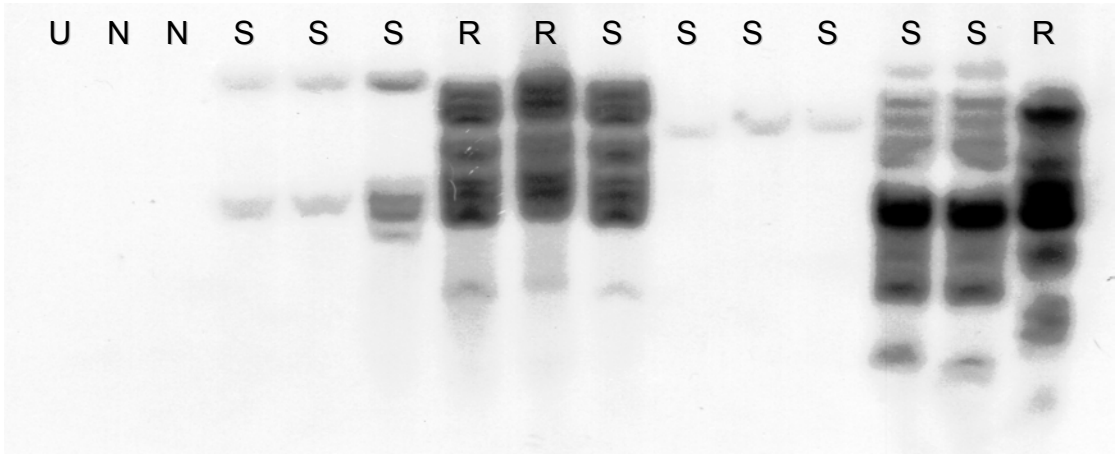
This trial established that resistance to SCMV could be conferred via both translatable and untranslatable replicase constructs. The number of plants showing resistance in this large trial was disappointing, as early glasshouse trials of coat protein had an approximately 20% success rate.

Figure 1. Northern blot of message RNA extracted from transgenic plants.



Panel A: RNA extracted from plants probed with CP specific probe. U, unshot control; R, resistant; S, susceptible. Panel B: Same RNA probed with ribosomal RNA probe as loading control (to confirm that the differences in RNA levels seen in Panel A are due to differences in the amount of CP specific RNA, rather than the total amount of RNA loaded).

Figure 2. Southern blot of genomic DNA extracted from transgenic plants.



DNA extracted from plants, restricted, and probed with CP specific probe. U, unshot control; N, NPTII control; R, resistant; S, susceptible.

Southern blot analysis of transgene copy number indicated a wide range of copies of the coat protein construct were present in the individual lines (Figure 2). The resistant lines all had multiple copies of the CP gene, but a number of susceptible plants also had high copy numbers. All the plants with a low copy number of the transgene were susceptible, a finding that concurs with reports from other transgenic host-virus systems (eg Smith *et al.* 1995). The predictive value of Southern blots is similar to that of northerns, probably a consequence of the complex aneuploid polyploid genome of sugarcane.

Table 1: Field resistance of Q155 transgenic lines to sugarcane mosaic virus.

No.	Plant No.	Constructs	Rating	Resistance status
1	303	pUKN	4	
2	305	pUKN	7	
3	307	pUKN	5	
4	308	pUKN	3	
5	312	pUKN	1	Immune
6	313	pUKN	6	
7	314	pUKN	1	Immune
8	315	pUKN, pUCP	7	
9	316	pUKN, pUCP	1	Immune
10	323	pUKN, pUCP	9	
11	325	pUKN, pUCP	8	
12	327	pUKN, pUCP	6	
13	330	pUKN, pUCP	9	
14	332	pUKN, pUCP	5	
15	333	pUKN, pUCP, pEGUS	3	
16	334	pUKN, pUCP, pEGUS	3	
17	341	pUKN, pUCP, pEGUS	1	Immune
18	342	pUKN, pUCP, pEGUS	7	
19	343	pUKN, pUCP, pEGUS	1	Immune
20	344	pUKN, pUCP, pEGUS	6	
21	345	pUKN, pUCP, pEGUS	4	
22	346	pUKN, pUCP, pEGUS	1	Immune
23	347	pUKN, pUCP, pEGUS	3	
24	349	pUKN, pUCP, pEGUS	5	
25	351	pUKN, pUCP, pEGUS	1	Immune
26	352	pUKN, pUCP, pEGUS	1	Immune
27	353	pUKN, pUCP, pEGUS	6	
28	354	pUKN, pUCP, pEGUS	1	Immune
29	355	pUKN, pUCP, pEGUS	3	
30	356	pUKN, pUCP, pEGUS	1	Immune
31	357	pUKN, pUCP, pEGUS	4	
32	361	pUKN, pUCP, pEGUS	1	Immune
33	364	pUKN, pUCP, pEGUS	5	
34	365	pUKN, pUCP, pEGUS	1	Immune
35	368	pUKN, pUCP, pEGUS	6	
36	369	pUKN, pUCP, pEGUS	6	
37	370	pUKN, pUCP, pEGUS	3	
38	371	pUKN, pUCP, pEGUS	9	
39	373	pUKN, pUCP, pEGUS	9	
40	374	pUKN, pUCP, pEGUS	7	
41	375	pUKN, pUCP, pEGUS	8	
42	376	pUKN, pUCP, pEGUS	8	
43	377	pUKN, pUCP, pEGUS	9	
44	378	pUKN, pUCP, pEGUS	8	
45	379	pUKN, pUCP, pEGUS	9	
46	380	pUKN, pUCP, pEGUS	9	
47	381	pUKN, pUCP, pEGUS	9	
48	382	pUKN, pUCP, pEGUS	9	
49	383	pUKN, pUCP, pEGUS	9	
50	384	pUKN, pUCP, pEGUS	6	
51	385	pEKN, pUPC	1	Immune
52	386	pEKN, pUPC	1	Immune
53	387	pEKN, pUPC	1	Immune
54	388	pEKN, pUPC	5	

No.	Plant No.	Constructs	Rating	Resistance status
55	389	pEKN, pUPC	1	Immune
56	390	pEKN, pUPC	-	
57	391	pEKN, pUPC	4	
58	340a	pUKN, pUCP, pEGUS	1	Immune
59	350a	pUKN, pUCP, pEGUS	8	
60	353a	pUKN, pUCP, pEGUS	9	
61	384a	pEKN, pUPC	9	
62	389a	pEKN, pUPC	4	
63	Q155tc		9	
64	Q155pc		7	6
65	Q63		3	3
66	Q78		9	9
67	Q82		1	1
68	CP29		5	6
69	NCO310		1	3
70	Pindar		1	2
71	Trojan		8	7

Table 2: Probability matrix of resistant transgenic lines vs Q155 parental clone and tissue culture control for total sugar hectare.

Line	Q155 pc	Significance	Q155 tc	Significance
312	0.0429	*	0.3176	
314	0.0007	***	0.0098	**
316	0.0200	*	0.1793	
340a	0.2158		0.9151	
341	0.2559		0.9933	
343	0.0001	***	0.0002	***
346	0.0001	***	0.0001	***
351	0.3178		0.9622	
352	0.5929		0.5765	
354	0.0003	***	0.0046	**
356	0.1995		0.8748	
361	0.1014		0.5754	
365	0.0872		0.5210	
385	0.0533		0.3710	
386	0.0772		0.4799	
387	0.0740		0.4663	
389	0.6425		0.6803	
Q155 pc	---------		0.2528	
Q155 tc	0.2528		---------	

TSH total sugar hectare
pc parental clone
tc tissue culture control
* $p<0.05$
** $p<0.01$
*** $p<0.001$

Table 3: Appearance of symptoms on transgenic plants transformed with Emu and Ubi replicase constructs for resistance to SCMV

Plant ID No	Construct	Variety	Presence of replicase gene	Observations of symptoms		
				29/1/99	03/11/99	04/08/99
TR1	pUKN/pURTN	Q124	+	-	-	+
TR2	pUKN/pURTN	Q124	+	-	+	+
TR3	pUKN/pURTN	Q124	+	-	+	+
TR4	pUKN/pURTN	Q124	+	-	+	+
TR5	pUKN/pURTN	Q124	+	-	+	+
TR6	pUKN/pURTN	Q124	+	-	+	+
TR7	pUKN/pURTN	Q124	+	-	+	+
TR8	pUKN/pURTN	Q124	+	-	+	+
TR9	pUKN/pURTN	Q124	+	-	+	+
TR10	pUKN/pURTN	Q124	+	-	+	+
TR11	pUKN/pURTN	Q124	+	-	+	+
TR12	pUKN/pURTN	Q124	+	-	+	+
TR13	pUKN/pURTN	Q124	+	-	+	+
TR14	pUKN/pURTN	Q124	+	-	+	+
TR15	pUKN/pURTN	Q124	+	-	+	+
TR16	pUKN/pURTN	Q124		-	+	+
TR17	pUKN/pURTN	Q124	+	-	-	+
TR18	pUKN/pURTN	Q124	+	-	+	+
TR19	pUKN/pURTN	Q124	+	-	+	+
TR20	pUKN/pURTN	Q124	+	-	+	+
TR21	pUKN/pURTN	Q124		-	+	+
TR22	pUKN/pURTN	Q124		-	+	+
TR23	pUKN/pURTN	Q124	+	-	+	+
TR24	pUKN/pURTN	Q124		-	+	+
TR25	pUKN/pURTN	Q124	+	-	+	-
TR26	pUKN/pURTN	Q124	+	-	-	+
TR27	pUKN/pURTN	Q124		-	+	+
TR28	pUKN/pURTN	Q124		-	+	+
TR29	pUKN/pURTN	Q124		-	+	+
TR30	pUKN/pURTN	Q124		-	+	+
TR31	pUKN/pURTN	Q124		-	+	+
TR32	pUKN/pURTN	Q124		-	+	+
TR33	pUKN/pURTN	Q124		-	+	+
TR34	pUKN/pURTN	Q124	+	-	+	+
TR35	pUKN/pURTN	Q124	+	-	+	+
TR36	pUKN/pURTN	Q124	+	-	+	+
TR37	pUKN/pURTN	Q124	+	-	+	+
TR38	pUKN/pURTN	Q124	+	-	+	+
TR39	pUKN/pURTN	Q124	+	-	+	+
TR40	pUKN/pURTN	Q124	+	-	+	+
TR41	pUKN/pURTN	Q124	+	-	+	+
TR42	pUKN/pURTN	Q124		-	+	+
TR43	pUKN/pURTN	Q124	+	-	+	+
TR44	pUKN/pURTN	Q124		-	+	+
TR45	pUKN/pURTN	Q124		-	+	+
TR46	pUKN/pURTN	Q124	+	-	+	+
TR47	pUKN/pURTN	Q124	+	-	+	+
TR48	pUKN/pURTN	Q124		-	+	+
TR49	pUKN/pURTN	Q124		-	+	+
TR50	pUKN/pURTN	Q124		-	+	+
TR51	pUKN/pURTN	Q124		-	+	+
TR52	pUKN/pURTN	Q124	+	-	-	-
Q124#1	Control	Q124		-	+	+
Q124#2	Control	Q124		-	-	-
UR1	pUKN/pURUN	Q155		-	+	+

Plant ID No	Construct	Variety	Presence of replicase gene	Observations of symptoms		
				29/1/99	03/11/99	04/08/99
UR2	pUKN/pURUN	Q155	+	-	+	+
UR3	pUKN/pURUN	Q155		-	+	+
UR4	pUKN/pURUN	Q155		-	+	+
UR5	pUKN/pURUN	Q155	+	-	+	+
UR6	pUKN/pURUN	Q155	+	-	+	+
UR7	pUKN/pURUN	Q155	+	-	+	+
UR8	pUKN/pURUN	Q155	+	-	+	+
UR9	pUKN/pURUN	Q155		-	+	+
UR10	pUKN/pURUN	Q155	+	-	+	+
UR11	pUKN/pURUN	Q155	+	-	+	+
UR12	pUKN/pURUN	Q155	+	-	+	+
UR13	pUKN/pURUN	Q155		-	+	+
UR14	pUKN/pURUN	Q155		-	+	+
UR15	pUKN/pURUN	Q155	+	-	+	+
UR16	pUKN/pURUN	Q155		-	+	+
UR17	pUKN/pURUN	Q155		-	+	+
UR18	pUKN/pURUN	Q155		-	+	+
UR19	pUKN/pURUN	Q155		-	+	+
UR20	pUKN/pURUN	Q155		-	+	+
UR21	pUKN/pURUN	Q155		-	+	+
UR22	pUKN/pURUN	Q155		-	+	+
UR23	pUKN/pURUN	Q155		-	+	+
UR24	pUKN/pURUN	Q155		-	+	+
UR25	pUKN/pURUN	Q155		-	+	+
UR26	pUKN/pURUN	Q155		-	+	+
UR27	pUKN/pURUN	Q155	+	-	+	+
UR28	pUKN/pURUN	Q155		-	+	+
UR29	pUKN/pURUN	Q155		-	+	+
UR30	pUKN/pURUN	Q155		-	+	+
UR31	pUKN/pURUN	Q155		-	+	+
UR32	pUKN/pURUN	Q155		-	+	+
UR33	pUKN/pURUN	Q155		-	+	+
UR34	pUKN/pURUN	Q155		-	+	+
UR35	pUKN/pURUN	Q155	+	-	+	+
UR36	pUKN/pURUN	Q155	+	-	+	+
UR37	pUKN/pURUN	Q155	+	-	+	+
UR38	pUKN/pURUN	Q155	+	-	+	+
UR39	pUKN/pURUN	Q155	+	-	-	-
UR40	pUKN/pURUN	Q155	+	-	+	+
UR41	pUKN/pURUN	Q124	+	-	+	+
UR42	pUKN/pURUN	Q124	+	-	+	+
UR43	pUKN/pURUN	Q124	+	-	-	-
UR44	pUKN/pURUN	Q124	+	-	+	+
UR45	pUKN/pURUN	Q124	+	-	-	+
UR46	pUKN/pURUN	Q124	+	-	+	+
UR47	pUKN/pURUN	Q124	+	-	+	+
UR48	pUKN/pURUN	Q124	+	-	+	+
UR49	pUKN/pURUN	Q124	+	-	+	+
UR50	pUKN/pURUN	Q124	+	-	+	+
UR51	pUKN/pURUN	Q124		-	+	+
UR52	pUKN/pURUN	Q124	+	-	+	+
UR53	pUKN/pURUN	Q124	+	-	+	+
UR54	pUKN/pURUN	Q124	+	-	+	+
UR55	pUKN/pURUN	Q124		-	+	+
UR56	pUKN/pURUN	Q124	+	-	+	+
UR57	pUKN/pURUN	Q124		-	+	+
UR58	pUKN/pURUN	Q124	+	-	+	+
UR59	pUKN/pURUN	Q124	+	-	+	+
UR60	pUKN/pURUN	Q124		-	+	+
UR61	pUKN/pURUN	Q124		-	+	+
UR62	pUKN/pURUN	Q124		-	+	+
UR63	pUKN/pURUN	Q124		-	+	+
UR64	pUKN/pURUN	Q124		-	+	+

4.3 Production and analysis of sugar from transgenic plants

Production

Transgenic sugarcane mosaic virus resistant plants were harvested and juice was extracted. Crystallisation of sugar in the BSES David North Plant Research Centre laboratory involved liming the raw cane juice, clarifying it and concentrating the clarified juice to produce sugar crystals. Aliquots were removed during the purification process of leaf, fibre, juice, filtrate, syrup and crystals and were analysed for the presence of the two transgenes (NPTII gene, and SCMV-CP) using the highly sensitive and specific polymerase chain reaction (PCR) technique. A PCR test for the native sugarcane acetolactate synthase (ALS) gene was also performed on the sugar to test for the presence of native sugarcane DNA. Samples from the various stages of crystallisation were tested. Samples were 'spiked' (had DNA added back into the sample) to prove that negative results were not the result of inhibition of the PCR, rather than absence of the DNA target.

Results

No transgene or native DNA could be detected in the filtrate, syrup or crystals (Table 4). The PCR tests were not inhibited by impurities in the samples analysed, confirming that failure to detect the genes was because they were not present.

Table 4: Presence of transgene and native DNA during sugar production.

Sample	NPTII transgene	SCMV-CP transgene	ALS native gene
Leaf	+	+	+
Fibre	+	+	+
Juice	+	+	+
Juice spiked	+	+	+
Filtrate	-	-	-
Filtrate spiked	+	+	+
Syrup	-	-	-
Syrup spiked	+	+	+
Crystals	-	-	-
Crystals spiked	+	+	+

Discussion

No transgene DNA is present in the crystallised sugar. We could not detect the native sugarcane acetolactate synthase (ALS) gene in the crystallised sugar, suggesting that all DNA is removed or destroyed during the production of sugar. This is an important outcome, and establishes that the hypothesis that DNA would not survive the sugar purification process (alkaline hydrolysis and boiling) is valid. Whilst these results are on laboratory-produced sugar, there is every confidence that this outcome will be reproduced on mill-produced sugar when the opportunity to mill transgenic sugarcane eventuates. This sugar was produced for the purpose of scientific experimentation only. Klein *et al.* (1998) also reported that nucleic acids (and proteins) are eliminated during the sugar manufacturing process of conventional and transgenic beets. There is now good scientific evidence for contribution to the debate that sugar manufactured from transgenic plants (cane or beet) is indistinguishable or substantially equivalent to sugar produced from non-transgenic plants.

5.0 CONCLUDING DISCUSSION

We successfully achieved the two principal aims of this research, namely the demonstration of field resistance of transgenic sugarcane to sugarcane mosaic virus and the demonstration that plants containing constructs based on the viral replicase gene confer resistance against the virus. We experienced a number of difficulties in establishing the field trials including some unseasonal wet periods during sett germination, resulting in the loss of the trials.

Molecular data obtained from selected transgenic lines indicated that selection of lines to test could not be performed in the laboratory based on the molecular profiles of RNA and DNA. The precise mechanism by which transgene-mediated resistance acts in plants is unknown. Initial reports (eg Powell-Abel *et al.* 1986) suggested that the resistance was protein-mediated. However, there is a growing number of reports supporting the hypothesis that resistance is mediated at the RNA level (eg Dougherty *et al.* 1994, Smith *et al*. 1994). The two coat protein constructs were designed to be translatable, but no coat protein was detected in any of the transgenic lines assayed prior to inoculation. It appears likely that the mode of transgene-mediated resistance to SCMV in sugarcane is enacted at the RNA level. Resistance mediated at the RNA level has been reported to be very nucleotide sequence specific. Small differences in sequence between the RNA mediating the resistance and the RNA of the infecting virus can result in a breakdown of the transgenic resistance (Wilson 1993). There is a difference of approximately 3% between the sequence mediating the transgenic resistance and the sequence of the Pathology Farm isolate of SCMV (Handley *et al*. 1998). This variation has not affected the mediation of resistance by the transgene sequence, and we remain confident that this transgene will mediate resistance against any of the Australian isolates of SCMV. Unfortunately the trial to test this hypothesis was unsuccessful for reasons that remain unclear.

In response to the growing debate about genetically modified foods and transgene flow in purification systems we were presented with an ideal opportunity to test out hypothesis that genes, whether transgenic or native, would not survive the sugar production system. Our hypothesis was correct on the laboratory purified sugar produced from the transgenic mosaic resistant plants and this data was presented at the 1999 ASSCT Conference (Taylor *et al*. 1999). This result is being used to contribute to the ongoing debate on genetically engineered foods.

6.0 RECOMMENDATIONS

The plants produced and evaluated in this project are being used in Laurelea Pickering's current PhD study on the molecular basis of virus resistance in sugarcane. Virus resistant transgenic plants are available if mosaic should become a serious problem. These plants would require further testing especially of yield and agronomic performance but this is not warranted at this time. With the increasing levels of Fiji disease and other potential problems (smut, orange rust) influencing industry practices we recommend that:

- The expertise and knowledge gained in this project be used to enhance the efforts to develop Fiji disease resistant transgenics

- Resources be directed to developing resistance genes for other serious threats to the industry such as smut and Pachymetra.

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