THE SMUT-SUGARCANE INTERACTION AS A MODEL SYSTEM FOR THE INTEGRATION OF MARKER DISCOVERY AND GENE ISOLATION

BS HEINZE, LN THOKOANE, NJ WILLIAMS, JM BARNES AND RS RUTHERFORD

South African Sugar Association Experiment Station, Private Bag X02, Mount Edgecombe, 4300, South Africa

Abstract

Genes that are differentially expressed by resistant sugarcane varieties in response to challenge by pathogens or pests have the potential to be useful in Marker Assisted Selection (MAS) once a link with resistance has been confirmed. Methodologies and resources designed to maximise the discovery of genetic markers and to facilitate the isolation of full-length coding sequences of resistance genes are described. To test the potential of the authors' approach, the smut-sugarcane interaction was chosen for a preliminary study. Sequence analysis of genes differentially expressed in response to challenge by smut has identified putative receptors involved in the signalling of resistance mechanisms, transcription factors, and enzymes involved in phenylpropanoid-flavonoid metabolism. Fragments having homology with flavonoid pathway transcription factors X1 and P1 were utilised as RFLP probes in a marker discovery effort. A resultant multiple linear regression model included two polymorphisms and explained 26% of the variation in smut resistance ratings within a set of 78 phenotypically well-characterised sugarcane clones. The full-length coding sequence for transcription factor X1 is in the process of being isolated from a PCR amplified cDNA pool using a 'capture-enrichment' methodology. Two full-length thaumatin (PR5) antifungal protein-coding sequences have already been isolated and are available for use as transgenes.

Introduction

The sugarcane breeding and selection programme at the South African Sugar Association Experiment Station (SASEX) takes about 14 years from the time crosses are made to the commercial release of a new variety. This prolonged time period is due largely to the reliance of the selection process on phenotypic characters, which are influenced to greater or lesser extents by environmental conditions. In addition, many traits such as resistance to pests and pathogens can only be tested once sufficient clonal material has been built up several years after the cross is made.

It is generally recognised that molecular marker assisted selection (MAS) can be very beneficial to plant breeding programmes (Edwards and Page, 1994). If genetic markers can be found that are associated with pest and disease resistance traits in sugarcane, the breeding and selection programme would have a means to select at the genetic level, in the absence of environmental influence, when as little as one plant per clone is available. Accurate, early selection for traits would mean that the unnecessary and costly carriage of undesirable clonal genotypes to successive stages of the selection programme would be minimised.

As a means of attaining this goal, the work outlined here is designed to identify genes that are differentially expressed in response to challenge by the borer, *Eldana saccharina* Walker (Lepidoptera: Pyralidae), the fungal pathogen *Ustilago scitaminea* H&P Sydow causing sugarcane smut, and the viral pathogen sugarcane mosaic virus (SCMV). Differentially expressed genic fragments have the potential to be useful in MAS once a link with resistance has been confirmed, for example by Restriction Fragment Length Polymorphism (RFLP). In addition, full-length genic sequences can be isolated for use as transgenes in the genetic engineering programme.

Differentially expressed genes are likely to be induced following pathogen or pest challenge. Inducible components of pathogen resistance involve major (race specific) or minor (race non-specific) gene-for-gene interactions, e.g. in the latter case, specific receptor detection of non-race specific elicitors such as linear β-1,3 glucans from fungal cell walls and oligogalacturonides from plant cell walls (Klarzynski *et al*., 2000). Recently discovered receptor kinases possessing domains similar to plant lectins might be capable of detecting such elicitors (Swarup *et al*., 1996).

Insect pests such as *E. saccharina* cause extensive tissue damage and will induce wound-signalling pathways and wound response genes. However, herbivory is not exactly equivalent to mechanical wounding although wound responses are a part of the induced responses that accompany herbivory. Elicitors are known to be present in insect saliva and receptor mediated detection induces direct defences that interfere with feeding, growth and development, and fecundity (Walling, 2000). Insect herbivory also induces the production of an array of volatiles that creates an indirect mechanism of defence through the attraction of parasites and predators to infested plants (Pare *et al*., 1998).

Receptor mediated elicitor detection triggers a signal-transduction cascade leading to, among other things, the production of endogenous signalling compounds (Reymond and Farmer, 1998). Well-known signalling compounds which induce genes involved in resistance are salicylic acid (SA - pathogen response) and jasmonic acid (JA - wound and pathogen response) (Dürner

et al., 1997). Signalling compounds induce numerous genes encoding regulatory transcription factors (Eulgem *et al*., 2000), enzymes of secondary metabolism involved in the biosynthesis of plant antibiotic phytoalexins or structural defences such as lignin; and pathogenesis-related (PR) proteins which represent major quantitative changes in soluble protein during the defence response (Kitajima and Sato, 1999).

Given the importance of specific receptors, downstream regulatory elements and PR proteins in plant-pathogen/pest interactions, differentially expressed sequences homologous to known examples are the focus of the proposed research. These sequences can be employed as RFLP probes in the development of markers for resistance or susceptibility. Other objectives include gaining insight into resistance mechanisms, and the isolation of full-length sequences for use as potential transgenes. Appropriate methodologies have been selected with these objectives in mind.

The smut interaction with sugarcane was chosen for a 'test of potential' approach due to its apparent greater simplicity in terms of challenging sugarcane with the pathogen. Resistance to smut is thought to be determined by combinations of pre-formed bud structural characteristics (Waller, 1970), pre-formed bud phenylpropanoids and glycosyl-flavonoids (Lloyd and Naidoo, 1983) and two postulated 'physiological' (inducible) barriers, one of which is partially overcome by wounding the bud prior to inoculation (Dean, 1982). At present little is known about the actual nature of defences induced by smut infection and the same is true of induced defences against SCMV and *E. saccharina*. Preliminary results from one of two different methodologies for detecting sugarcane differential gene expression in response to smut are presented here. Results for the second methodology are presented separately by Thokoane and Rutherford (2001).

Methods and Resources

Single budded setts of resistant (N52/219) and susceptible (Co301) varieties were dipped in 0.01% Tween-20 with or without 10^8 teliospores ml^{-1}. After seven days RNA was extracted from breaking buds using TRIzol® (Life Technologies).

Since one of the stated goals is to isolate full-length coding sequences of candidate resistance genes, cDNA synthesis was performed using a Clontech SMART™ kit in order to generate as high a proportion of full-length products as possible (Ciavatta and Cairney, 2000). SMART cDNA can also serve as the starting material for detection of differential gene expression.

The chosen methodologies for detecting differential gene expression were Differential Display of cDNA-Amplified Fragment Length Polymorphism (AFLP) products (Bachem *et al*., 1996) and Suppression Subtractive Hybridisation (SSH) using a PCR Select™ kit (Clontech) (Diatchenko *et al*., 1998). Although both are PCR-based methods, cDNA-AFLP and SSH differ in some fundamental respects. In the cDNA-AFLP method, the cDNA population is subdivided and the resulting products are electrophoresed on a gel. This gel provides a visual Differential Display from which bands that represent a portion of all differentially expressed genes can be excised. These bands may be present in one state but absent in the other, or may simply differ in intensity. However, rare transcripts are not easily detected.

SSH is a subtractive hybridisation method in which differentially expressed sequences are selectively and, exponentially amplified. This functionally removes those genes present equally in both states, resulting in a pool of cDNAs that represents all of the differentially expressed genes. SSH normalises the abundance of high- and low-expression genes and greater than 1 000-fold enrichment of rare transcripts can be obtained. Furthermore, suppression PCR prevents both undesirable and biased amplification of small molecules, while enrichment of target molecules proceeds.

Cloning and sequencing of differentially expressed sequences was carried out as described by Thokoane and Rutherford (2001). Comparison to non-redundant nucleotide, protein and EST databases was by BLASTN and BLASTX at http://www.ncbi.nlm.nih.gov/blast/blast.cgi. Based on resultant homologies, differentially expressed sequences were assigned putative identities and functions.

For RFLP analysis, genomic DNA was extracted from a set of 78 phenotypically well-characterised clones. This set was selected from clones used as parents in the SASEX breeding programme. Given the current importance of *E. saccharina* in the industry, equal numbers of *E. saccharina* resistant, intermediate and susceptible clones were included. Clones are designated as resistant (ratings 1, 2 and 3), intermediate (4, 5 and 6), or susceptible (7, 8 and 9), based on comparisons with standard clones. Table 1 illustrates the resistance characteristics of the 78 selected clones.

Southern blotting and RFLP detection, using identified differentially expressed sequences as probes, were carried out according to standard protocols (Sambrook *et al*., 1989).

Differential gene expression can be analysed by Northern blotting (Sambrook *et al*., 1989). In the absence of sufficient RNA, virtual Northern blots were produced using the same SMART PCR-amplified tester and driver cDNA used for subtraction. According to Zhumabayeva *et al*. (2001), results are very similar to those provided by standard Northern analysis.

For the targeting of specific PR genes, degenerate primers were designed using CODEHOP methodology for conserved amino acid sequences found in PR protein examples from other

Table 1. Mean trait ratings for the set of 78 clones and inter-correlations between traits.

	Smut	*E. saccharina*	Mosaic virus (SCMV)
Mean rating	4.7	5.1	4.2
Inter-correlations	*E. saccharina*/smut	*E. saccharina*/mosaic	smut/mosaic
Pearson r	-0.396 ($p < 0.001$)	-0.321 ($p = 0.005$)	+0.193 (ns)

Table 2. Putative identities of N52/219 (challenged) expressed sequences following subtraction of N52/219 (unchallenged) or Co301 (challenged and unchallenged) sequences.

Signalling	Accession no. of homologue	E-value‡	Comments
Cell-wall bound receptor kinase 403bp	AJ009695	0.001	Pathogen induced¶, S-receptor kinase-like salicylate (SA) induced
Nucleotide Binding Site –LRRs 191bp	AC074283	0.28	Pathogen induced, LRR region
and 635bp	AB019186	$3x10^{-28}$	Carboxy-terminal 107aa plus 3'UTR, probenazole/ SA induced
G-protein coupled peptide receptor 206bp	AF132042	3.1	Pathogen induced
Histidine kinase response regulator 237bp	AB010918	3.2	Recently discovered 2 component system
Protein kinase 407bp	AC006921	4.2	-
Receptor ser/thr protein kinases 312bp	AB012247	6.6	-
and 380bp	AB026649	$5x10^{-19}$	Pathogen induced
Phosphoprotein phosphatases 301bp	AAD21727	$5x10^{-18}$	Pathogen induced, regulator of kinase action
and 252bp	AJ131045	$4x10^{-31}$	Pathogen induced, regulator of kinase action
Uncharacterised transmembrane prot. 498bp	AB026661	$2x10^{-36}$	Pathogen induced
Farnesyl Pyrophosphate synthetase >591bp	P49353	$1x10^{-57}$	Farnesylation of signalling peptides, precursor of isoprenoid phytoalexins
Transcription factors			
MADS-box 1 464bp	AJ249141	$1x10^{-21}$	Pathogen induced
X1 451bp	AF101045	$1x10^{-11}$	Between sh2 and a1 (maize, sorghum), enhancer of c-glycosyl-flavone synthesis
Target of myb1 (TOM) 230bp	T51543	$7x10^{-8}$	Pathogen induced
Zinc finger PR protein 360bp	T11846	$7x10^{-9}$	Pathogen induced, carboxy-terminal 39aa plus 3'UTR
Phenylpropanoid/ flavonoid metabolism			
Isoflavone reductase homologue >569bp	P52581	$7x10^{-48}$	Pathogen induced, lignan phytoalexin synthesis
X1 451bp	AF101045	$1x10^{-11}$	Between sh2 and a1 (maize, sorghum), enhancer of c-glycosyl-flavone synthesis
Peroxidase 326bp	CAA72485	$2x10^{-7}$	Carboxy-terminal 36aa plus 3'UTR
Chalcone reductase 199bp	AJ223291	$5x10^{-7}$	Phytoalexin synthesis
Beta-glucosidase 464bp	T14732	$2x10^{-53}$	Pathogen induced, hydrolysis of glycosyl-phenyl-propanoids/ flavonoids

¶ dbEST search suggests that sequence is pathogen induced.

‡ E-value is an expectation value, the lower the E value, the more significant the homology.

monocots (Rose *et al*., 1998). First strand cDNA was prepared using reverse CODEHOP primer plus SMART™ oligo. After PCR amplification, gel windows of suitable lengths (assuming a 5'UTR of 50 to 250 bases) were cloned and sequenced.

Results and Discussion

The results of cDNA-AFLP analyses are presented elsewhere by Thokoane and Rutherford (2001). SSH experiments subtracted cDNAs of N52/219 (unchallenged), Co301 (unchallenged) and Co301 (challenged) from N52/219 (challenged) in order to detect genes differentially expressed in the resistant variety when challenged. Preliminary results are presented in Table 2.

Three sequences were found to be similar to receptor kinases (Table 2). One of these was similar to a cell-wall bound receptor kinase which might be involved in detecting cell-wall integrity during pathogenesis. According to He *et al*. (1998) wall bound receptor kinase in *Arabidopsis* is induced by salicylic acid and moderates SA activity. This suggests that SA might be involved in signalling sugarcane responses to challenge by smut.

Sequences homologous to genes involved in phenylpropanoid-flavonoid metabolism support the notion of flavonoid involvement in smut resistance. Of immediate interest was the sequence similar to the transcription factor X1 which is located in the 0.1cM interval between sh2 (ADPG-pyrophosphorylase) and a1 (dihydroflavonol-4-reductase) in maize. Guo *et al*. (1999) suggested that X1 interacts with the myb-like transcription factor P1 in maize to enhance C-glycosyl-flavone production and resistance to the corn earworm. P1 alleles in maize are responsible for activating C-glycosyl-flavone, 3-deoxyanthocyanin phytoalexin, and phlobaphene synthesis in pericarp, cob, and silk tissue (Byrne *et al*., 1996).

The X1 fragment was used to probe across genomic DNA (Dra1 and HindIII digested) of 78 clones. Because of the proposed interaction between X1 and P1 in maize, the latter sequence was obtained from the University of Missouri at Columbia. The P1 probe generated two scorable polymorphisms significantly associated with smut rating whilst X1 generated one significant polymorphism, again supporting the notion of flavonoid involvement in resistance. For all three polymorphisms, their

presence is associated with susceptibility. A multiple linear regression (MLR) included two of the polymorphisms, one X1 and one P1, suggesting a possible interaction between X1 and P1 in sugarcane (Table 3).

Of the 71 clones in the MLR model, 40 (56%) were positive for one or other or both polymorphisms. Selection of potential parents based on their known phenotypes and polymorphism scores would allow the concept of MAS to be tested. In a potential cross, for example between N52/219 (rated 1 and scoring 0,0 for the 1st and 2nd polymorphisms) and NCo376 (rated 9 and scoring 1,1), each polymorphism would be present in half of the progeny. Using MAS, i.e. selecting individuals scoring 0,0, should then result in an enrichment of smut resistant individuals among those selected. For example, N23 is a product of this cross; it is rated 2 for smut and scores 0,0.

X1 is expressed in both inoculated and uninoculated breaking buds of the resistant variety (N52/219) and only in inoculated breaking buds of the susceptible variety (Co301) (Figure 1a). This may support the pre-formed glycosyl-flavonoid based resistance proposed by Lloyd and Naidoo (1983). A transcript of approximately 2000 bases is indicated in three separate cDNA preparations suggesting that SMART™ synthesis of full-length cDNAs was successful. The full-length coding sequence for transcription factor X1 is in the process of being isolated from N52/219 SMART cDNA using a 'capture-enrichment' methodology (Ciavatta and Cairney, 2000).

As a test case for specific gene fragment isolation, degenerate primers were synthesised for the small PR5 anti-fungal protein thaumatin, using aligned amino acid sequences of database examples from other monocots (Figure 2). Two full-length coding sequences, including 5'UTRs, were obtained from N52/219 (challenged) RNA. Translations are presented in Figure 2. According to computer predictions both clones seem to have cleavable N-terminal signal sequences and are targeted extracellularly (PSORT: http://psort.ims.u-tokyo.ac.jp/). The mature proteins have estimated isoelectric points of 3.7 and 4.8, i.e. both are acidic.

When one of the thaumatin clones was used to probe a virtual Northern blot, three bands at most could be discerned (Figure 1b). Although expression was apparent in the unchallenged controls, expression in Co301 (challenged) may be considerably less than in N52/219 (challenged). These sequences are now available for further testing.

Conclusions

Table 3. Association of polymorphisms detected by X1 and P1 probes with smut resistance ratings.

enzyme	Dra1	HinDIII	Dra1
probe	X1	P1	P1
% of scored clones (n) scored positive for the polymorphism	(71) 39%	(78) 35%	(78) 53%
Pearson r	+0.386	+0.302	+0.228
p =	0.001	0.006	0.048
cumulative r^2 at each MLR step	0.149	0.262	-

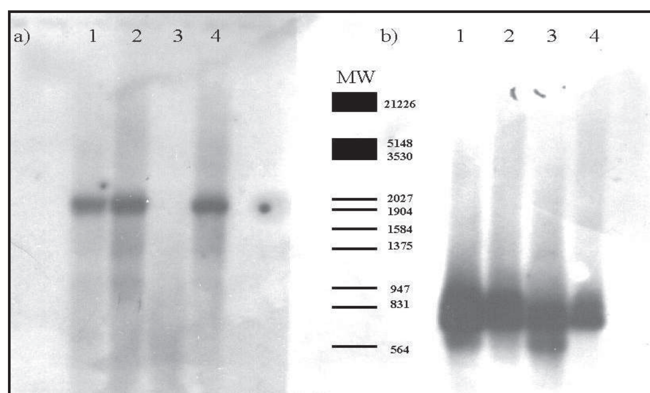

Figure 1. Virtual Northerns; a) probed with sequence homologous to transcription factor X1; b) probed with sequence homologous to thaumatin, Lane 1 - N52/219 (+), Lane 2 - N52/219 (-), Lane 3 - Co301 (-) and Lane 4 - Co301 (+).

The SSH method has been successful in producing a large number of differentially expressed sequences. Preliminary sequence analysis has identified putative receptors involved in the signalling of resistance mechanisms, transcription factors, and enzymes involved in phenylpropanoid-flavonoid metabolism. A large proportion may be pathogen induced (Table 2).

The RFLP results obtained for transcription factors X1 and P1 (Table 3) suggest that differentially expressed sequences can be successfully applied in the discovery of genetic markers in sugarcane. Consequently, as many as is possible of the differentially expressed sequences described in Table 2, and by Thokoane and Rutherford (2001), will be used to probe the set of 78 clones. Ultimately parents might be selected from among these clones which have opposite phenotypes and as many RFLPs significantly associated with their phenotype as possible. The progeny of such a cross might serve both as a genomic mapping population, and in marker validation.

SMART cDNA synthesis generated two full-length cDNAs of the PR5 antifungal protein thaumatin as well as probable full-length X1 cDNAs (Figure 1a). The use of the cloned X1 fragment (451 bp) to capture the full-length (approximately 2000 bp) cDNA can be achieved, without the use of a cDNA library, by employing the method of Ciavatta and Cairney (2000). Once isolated, X1 might be used as a transgene so that its effect on flavonoid composition could be determined.

Thaumatin expression in breaking buds appears to be constitutive (Figure 1b). Vleeshouwers *et al*. (2000) showed that partial resistance to *Phytophthora infestans* in potato can include basal (constitutive) PR-protein gene expression in addition to any inducible component. Coquoz *et al*. (1995) linked basal levels of salicylic acid (SA) in potato to partial resistance. Similarly, Silverman *et al*. (1995) found that increasing basal levels of SA in different rice varieties correlated with increasing partial resistance to rice blast.

Constitutive expression of acidic thaumatin suggests the involvement of SA signalling in sugarcane buds, as does the presence of a putative SA inducible cell-wall bound receptor kinase (Table 2). A common feature of all PR proteins is that the mature protein is targeted extracellularly (acidic forms) or to the vacuole (basic forms). Basic forms tend not be induced by SA,

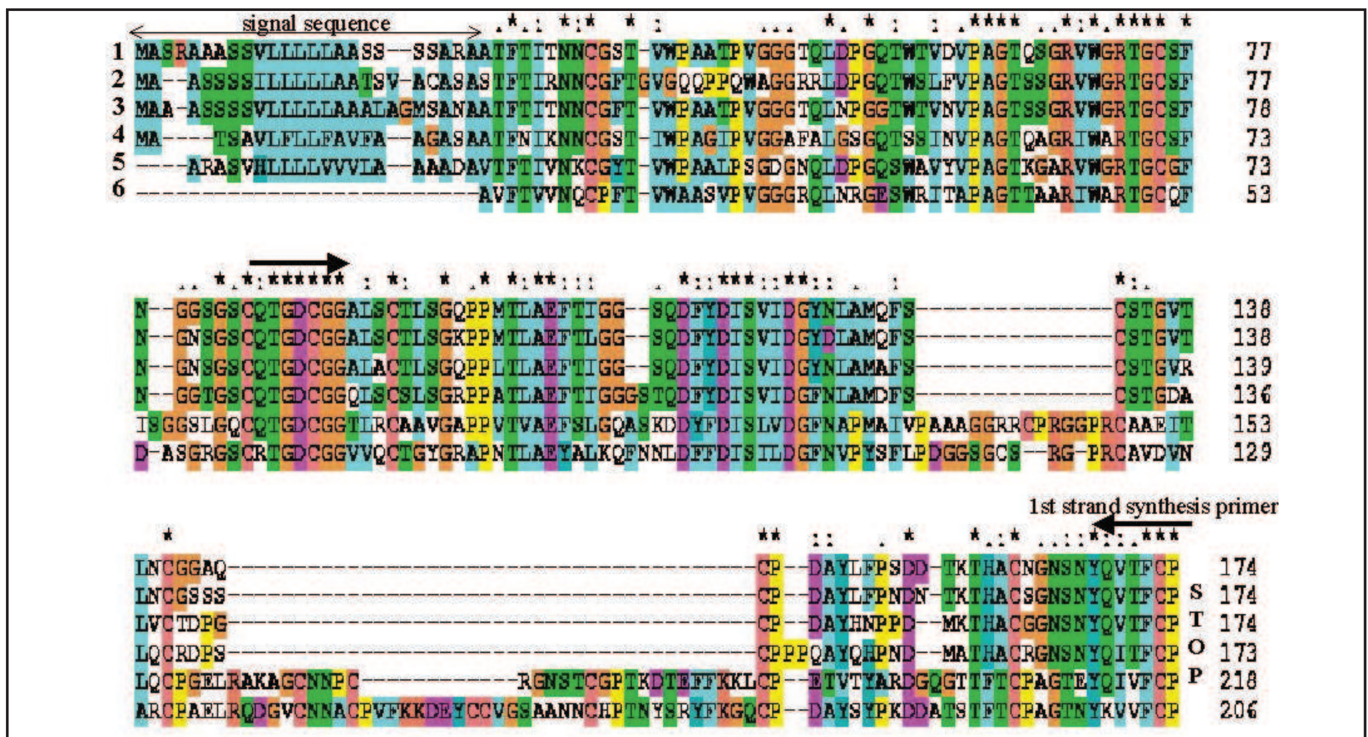

Figure 2. ClustalX multiple amino acid sequence alignment of two cloned sugarcane thaumatin cDNA translations with maize, rice and rye thaumatins / osmotins. 1) Sugarcane thaumatin 28, 2) Sugarcane thaumatin 12, 3) Maize thaumatin 1906392A, 4) Rye thaumatin AAC67259, 5) Rice osmotin AAB67852, 6) Maize osmotin-like P13867.

but by jasmonic acid (JA) and ethylene as a consequence of wounding and herbivory in particular. Acidic forms tend not to be induced by JA, but are induced by SA as a consequence of pathogenesis. Indeed, there appears to be some antagonism between the two signalling pathways. Niki *et al*. (1998) showed that SA-induced expression of acidic PR genes is inhibited by JA in a dose-dependent manner. Conversely, JA-induced expression of basic PR gene expression is inhibited by SA. As a consequence of this it is frequently found that there is an inverse relationship between resistance to certain pathogens and resistance to insect herbivores (Felton *et al*., 1999).

That smut resistance might involve SA signalling is supported by an inverse relationship between smut and *E. saccharina* resistance ratings (Table 1), especially as *E. saccharina* preferentially enters the stalk through the bud (Keeping, pers. comm). The inverse relationship between smut and borer resistance ratings might be explained in terms of higher basal bud salicylate levels and consequent inhibition of a wound response. The results of Dean (1982) are also supportive in that a hypothesised 'physiological' barrier to smut infection was partially overcome by wounding the bud prior to inoculation. In this case JA accumulation following wounding might inhibit a subsequent SA response. In future research, bud SA and JA levels will be determined and related to smut and *E. saccharina* resistance.

The above considerations have implications for both the smut and *E. saccharina* research pertaining to choice of resistant and susceptible sugarcane varieties. The bud as a site of challenge represents an opportunity to investigate bud differential gene expression and signalling pathways induced by herbivory or pathogenesis using the same varieties.

The methodologies and resources outlined in this paper have facilitated the gaining of insight into resistance mechanisms and signalling pathways. Our preliminary results, in the areas of markers and resistance gene isolation, suggest that future work addressing induced responses following challenge by SCMV or *E. saccharina* may have similar potential.

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