

INVESTIGATING THE ASSOCIATION OF SUGARCANE KINASE ANALOGS AND NBS-LRR RESISTANCE GENE ANALOGS WITH SUSCEPTIBILITY AND/OR RESISTANCE TO THE PATHOGENS *USTILAGO SCITAMINEA* (SMUT) AND SUGARCANE MOSAIC VIRUS (SCMV)

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Abstract

Resources have been invested in breeding different sugarcane varieties to cater for the different environmental constraints in South Africa. These varieties show varying levels of resistance and susceptibility to diseases such as mosaic (caused by sugarcane mosaic virus, SCMV), smut (caused by *Ustilago scitaminea*) and pests such as *Eldana saccharina*, which cause yield loss. Identifying possible differences in genetic make-up in varieties leading to disease resistance will make the breeding of new resistant varieties a more efficient process. A number of plant resistance (*R*) genes that have been identified in crops such as maize, barley and rice, and have been observed to contain a nucleotide-binding site (NBS) and a leucine rich repeat (LRR) motif and/or kinase genes. This study gives attention to the identification of possible resistance gene analogs (RGAs) in sugarcane varieties resistant and susceptible to SCMV and smut, by amplifying NBS-LRR and kinase sequences using degenerate primers. Twenty-three different classes of NBS-LRR analogues were identified, and six classes of kinase analogues have so far been observed. Southern blotting was done to identify RGA polymorphisms in the smut and mosaic susceptible and resistant specific varieties. Five polymorphisms were associated with smut resistance. In relation to smut susceptibility, four polymorphisms were identified. With regards to *E. saccharina* resistance, six polymorphisms were observed, and for mosaic resistance, eight polymorphisms were identified. Although some classes included both resistance and susceptibility polymorphisms when measured against *E. saccharina* and mosaic, smut had either resistant or susceptible associations per class. The process of identifying different NBS-LRR and kinase classes is ongoing.

Keywords: SCMV, *Ustilago scitaminea*, resistance genes, NBS-LRR analogues, kinase analogues

Introduction

Sugarcane is produced across very diverse environmental conditions in South Africa. Thus a great deal of resources have been invested in breeding different sugarcane varieties to cater for the different environmental constraints. These varieties show varying levels of resistance and susceptibility to pathogens commonly found in South African sugarcane. Certain pathogens cause diseases that result in severe loss in yield, and which can only sufficiently be contained by breeding disease-resistant varieties. Two diseases, which are well known and cause definite yield loss, are mosaic (caused by sugarcane mosaic virus, SCMV) and smut (caused by *Ustilago scitaminea*) (Anon, 2003). Varieties with high resistance or susceptibility to these diseases have been identified through field trials. Identifying possible varietal

differences in genetic make-up that lead to disease resistance will make the breeding of new resistant varieties a more efficient process. A few plant resistance (*R*) genes against specific pathogens have been identified in various crops, including tobacco, maize, barley, potato, rice, lettuce and citrus. *R* genes can be categorised into four classes on the basis of the conserved amino acid sequences of their protein products. The classes include the NBS-LRR genes, which contain a nucleotide-binding site (NBS) and a leucine rich repeat (LRR) motif, extra-cellular LRR genes, protein kinase genes, and receptor kinase genes. NBS-LRR genes have been found in a number of plant resistance (*R*) genes (Dixon *et al.* 1996; Seah *et al.* 1998; reviewed in Dangl and Jones, 2001). This study gives attention to the identification of possible resistance gene analogs (RGAs) in varieties resistant and susceptible to SCMV and smut, by amplifying NBS-LRR and kinase sequences using degenerate primers designed from conserved NBS-LRR and kinase motifs in rice, barley, maize and wheat. This may lead to the generation of RFLP markers, which would assist in the breeding of resistant varieties, as well as the possible identification and cloning of an *R* gene, which in turn could lead to a transgenic approach to combat mosaic and smut.

Methods and materials

Plant material and DNA preparation

Susceptible and resistant sugarcane varieties were identified according to their performance in field trials, and scored using resistance scales. Plant material was collected from variety plots, field trials and the museum variety plots at the South African Sugarcane Research Institute (SASRI) at Mount Edgecombe. A modified method according to Dellaporta *et al.* (1983) was used to isolate the DNA. Concentrations were determined on a Dyna Quant 200 Fluorometer (Hoefer). Genomic DNA pools were prepared for each group (smut susceptible and resistant, mosaic susceptible and resistant).

PCR conditions

Degenerate oligonucleotide primers were designed according to conserved amino acid motifs among known NBS-LRR resistance genes in rice, barley, maize and wheat. PCR reactions of 25 µl contained 30 ng genomic DNA, using 0.2 mM dNTPs, 0.2 µM of each primer, 2U Roche Taq and 1 x PCR buffer containing 1.5 mM MgCl₂ were subjected to thermal cycling in a Hybaid PCR Sprint machine. The cycling programme included an initial denaturing step at 95°C for 2 min, followed by 35 cycles of denaturing at 95°C for 45 s, annealing at 45°C for 1 min, elongation at 72°C for 1 min 45 s, and a final elongation step at 72°C for 7 min. PCR products were resolved on a 1.5% agarose gel and viewed under UV light.

Cloning and sequence analysis of PCR products

DNA fragments of appropriate sizes were excised from the agarose gel and purified with the Qiaquick gel extraction kit (Qiagen). Cloning was performed using pGEM-T Easy cloning kits (Promega) and colonies were screened for inserts. Plasmids from selected colonies were purified using Qiaprep Spin Miniprep kits (Qiagen). Samples were quantified using the Dyna Quant 200 Fluorometer (Hoefer). DNA samples were sequenced using the ABI 310 Genetic Analyser (Applied Biosystems) and BigDye Terminator v3.1 Cycle Sequencing kits (Applied Biosystems). Sequences were compared with the international database using BLASTX (www.ncbi.nlm.nih.gov/blast) and analyses of sequences were done using Jellyfish (Biowire) and DNAssist.

Southern blot

Genomic DNA for southern blot analysis was digested with Hind III and Dra I restriction enzymes according to the manufacturer's instructions (Roche). The DNA was precipitated by the addition of 1/20 of the volume 5 M NaCl and 2.5 X the volume of absolute ethanol. Restricted DNA was separated on 0.8% agarose gels by electrophoresis at 35 V, in 0.5 X TBE buffer overnight. On completion of electrophoresis, gels were stained with ethidium bromide and viewed. An alkaline transfer method, as described in the Hybond N+ manual (Amersham), was used for southern blotting. For hybridisation of labelled probes, membranes were incubated at 65°C overnight in 30 ml of pre-warmed Denhardt's hybridisation buffer (5x SSC pH7, 5x Denhardt's solution [2% Ficoll, 2% PVP (Polyvinyl pyrrolidone), 2% BSA (Bovine serum albumin)], 0.5% w/v SDS, denatured fragmented, herring sperm DNA 200 µg/ml). Probes were labelled with α -³²P-dCTP using the Megaprime DNA labelling system (Amersham). The denatured probe was added directly to the pre-hybridisation buffer and membranes hybridised overnight at 65°C. Membranes were washed according to the manufacturer's (Amersham) instructions. The membranes were exposed to X-ray film (Hyperfilm MP), for a reading of 2-5 counts per second, and an exposure time of 10 days was sufficient. X-rays were developed and results recorded. Membranes were stripped with a 0.1% SDS solution and re-used.

Results and discussion

Thirty-four NBS-LRR analogues were identified from the smut susceptible pool and 25 from the resistant pool. After obtaining complete sequences, these analogues were classified according to the first three amino acids present immediately following the primer sequence. Eighteen different classes were observed, where nine of the classes were only observed in the smut susceptible group, four classes were only observed in the smut resistant group and the class (VRS) containing the most sequences, included sequences from both the susceptible and resistant pools.

NBS-LRR analogues from SCMV susceptible and resistant pools were amplified with primer set 2 and sequenced. Analogues were again classified according to the first three amino acids present immediately following the primer sequence. Eleven different classes were observed, where five of the classes were only observed in the SCMV susceptible group, two classes were only observed in the SCMV resistant group and two classes included sequences from both the susceptible and resistant pools. Sequencing on the SCMV resistant group and Smut Kinase susceptible and resistant group is still under way. To date, six different classes have been identified in the smut kinase resistant group.

Southern blotting was done to identify RGA polymorphisms in the smut and mosaic susceptible and resistant specific varieties. The expression of RGA polymorphisms and the association with known smut, mosaic and *E. saccharina* resistant ratings were investigated. Five (adjusted values) polymorphisms were associated with smut resistance, of which three were from the same class, namely VSR. In relation to smut susceptibility, four polymorphisms were identified. With regard to *E. saccharina* resistance, six polymorphisms were identified; three belonging to the ARK class, while two out of four polymorphisms associated with susceptibility were from the VSR class. When looking at mosaic resistance, eight polymorphisms were identified, with three polymorphisms in the TLM class and two from the AQH class. Interestingly, some classes included both resistance and susceptibility polymorphisms when measured against *E. saccharina* and mosaic, but smut had either resistant or susceptible associations per class.

Classes of mosaic clones have been identified and probing is currently under way. Kinase analogs are being sequenced and probing will soon follow. The same approach will be applied to identify kinase analogs possibly contributing to susceptibility and/or resistance to smut and mosaic.

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