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Comparison of transcript profiles between near-isogenic maize lines in association with SCMV resistance based on unigene-microarrays

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Abstract

The molecular mechanisms underlying the development and progression of sugarcane mosaic virus (SCMV) infection in maize are poorly understood. A transcript profiling study based on maize unigene-microarrays was conducted to identify genes associated with SCMV resistance in the near-isogenic line (NIL) pair $F7^+$ (SCMV resistant) and F7 (susceptible). Altogether, 497 differentially expressed genes were identified in 4 comparisons addressing constitutive genetic differences, inducible genetic differences, compatible reaction, and incompatible reaction. Compared to a suppression subtractive hybridization (SSH) approach on the same materials, expression patterns of microarray-ESTs and SSH-ESTs were consistent for the same comparisons despite technical discrepancies. Pathogen-induced transcripts were underrepresented on the unigene-microarray, consequently fewer microarray-ESTs (45.8%) were classified into pathogenesis-related categories than SSH-ESTs (60.5%). Moreover, fewer microarray-ESTs (4) co-segregated with *Scmv* QTL than SSH-ESTs (18). However, our results demonstrate that the microarray experiments complement the SSH-macroarray studies. Good candidate genes (CGs) associated with SCMV resistance can be chosen from three classes: (i) positional CGs co-localized with major *Scmv* QTL, (ii) functional CGs exhibiting the homology to pathogenesis-related genes, or (iii) differentially expressed ESTs showing consistent expression pattern in both approaches.

Keywords: Sugarcane mosaic virus (SCMV); Unigene-microarray; Suppression subtractive hybridization (SSH); Maize; Near isogenic lines (NILs); Expression profiling

1. Introduction

Sugarcane mosaic virus (SCMV) causes mosaic diseases in sugarcane, maize, sorghum and other Poaceous species worldwide [1]. It has resulted in considerable economic losses in sugarcane and failure of commercial clones in several countries. Yield losses of 30–40% and sometimes 60–80% have been recorded in the Western hemisphere [2–4]. SCMV is also responsible for yield losses of 10–30% and 10–50% in China and South Africa, respectively [5,6]. To date, it is one of the most important virus diseases of maize in Europe and causes serious yield losses in susceptible cultivars [7]. Though chemical control of vectors is commonly practiced for the management of viral diseases, control of SCMV is hampered by the non-persistent nature of transmission via aphids. Therefore, cultivation of resistant maize varieties is the most efficient and environmentally sound approach to manage SCMV.

In a study with 122 early maturing European maize inbreds, three lines (FAP1360A, D21, and D32) displayed complete resistance and four lines displayed partial resistance (FAP1396A, D06, D09, and R2306) against SCMV and maize dwarf mosaic virus (MDMV) [8]. In field trials, resistance of all three European lines D21, D32, and FAP1360A seemed to be controlled by one to three genes [9]. Two major QTL regions, *Scmv1* and *Scmv2*, conferring resistance to SCMV were mapped to chromosome arms 6S and 3L [10,11]. Minor QTLs affecting SCMV resistance were identified on chromosomes 1, 5, and 10 [10]. For complete

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resistance to SCMV, presence of both *Scmv1* and *Scmv2* is essential. *Scmv1* suppresses symptom expression throughout all developmental growth stages at a high level, whereas *Scmv2* was mainly expressed at later stages of infection [10,11]. Resistance genes *Scmv1* (*Scmv1a* and *Scmv1b*), and *Scmv2* displayed at least partial dominance in different studies [10–12]. Resistance appears to operate without the hypersensitive response (HR). The underlying defence response to SCMV is poorly understood.

In previous studies, maize resistance gene analogues (RGA) [13] were chosen as starting point for isolation of genes conferring SCMV resistance. Mapping of RGAs in relation to *Scmv1* and *Scmv2* suggested that RGA *pic19* is a candidate for *Scmv1* and *pic13* for *Scmv2* [14]. BAC clones were isolated using *pic19* and *pic13* as probes [15] (unpublished data). Gene expression studies comparing near-isogenic lines (NILs) differing for short chromosome segments offer an alternative to identify candidate genes for QTL located within such segments [16], but also genes from other chromosomal locations involved in subsequent steps leading to resistance or susceptibility after the initial infection by SCMV. Moreover, microarray-based expression profiling methods have become an important tool for characterization of plant pathogenesis-related responses [17]. Baldwin et al. [18] identified 117 differentially expressed out of 1500 genes spotted on a microarray associated with maize reaction to the fungal pathogen *Cochliobolus carbonum*. Nadimpalli et al. [19] identified about 70 differentially expressed genes in an isogenic contrast for the maize lesion mimic mutant *Les9*. In this study, we used publicly available maize unigene-microarrays [20] containing 11,424 distinct ESTs, including 949 mapped sequences. The array actually contains 9841 different unigenes, which represents approximately 20% of the about 50,000 maize genes [21]. Thus, another expression profiling method, combining suppression subtractive hybridization (SSH) [22] and macroarray hybridization, was conducted in a companion study to detect SCMV-related transcripts in maize [23]. Combining both results will provide a more comprehensive understanding of SCMV–maize interaction.

The objectives of our study were to: (1) identify genes associated with SCMV resistance in maize using unigene-microarray hybridization; (2) propose the molecular mechanisms underlying the development and progression of SCMV infection combining the results of SSH-macroarray and unigene-microarray experiments; (3) identify candidate genes underlying major *Scmv* QTL through comparing SSH-macroarray and unigene-microarray results.

2. Materials and methods

2.1. Plant materials

NIL $F7^+$ was developed using phenotypic and marker-assisted selection. The early maturing European maize inbreds, FAP1360A, resistant to SCMV, and F7, highly susceptible to SCMV [8], were crossed to produce an F1 generation, and backcrossed seven times to F7 with two generations per year from 1995 to 1998 [24]. Seed of the homozygous line $F7^+$ was produced by three subsequent selfing steps starting from one SCMV resistant BC7 plant carrying the donor regions from FAP1360A at *Scmv1* and *Scmv2*. No donor segments outside the *Scmv1* or *Scmv2* region were detected using 141 SSR markers (unpublished results). Both donor regions (*Scmv1* and *Scmv2*) together represent about 3% of the total maize genome.

NILs F7 and $F7^+$ [23] were grown and maintained in growth chambers under a 12 h photoperiod at 23 °C and 50% relative humidity. The SCMV isolate Seehausen was maintained in the leaves of susceptible F7 plants [10]. The sap for the inoculation was produced by homogenizing the infected leaves in 0.05 M potassium phosphate buffer, pH 7.2 (1:10, w/v). Two-week-old plants were mechanically inoculated by an air brush technique using a tractor-mounted air compressor at constant pressure of 799 kPa [7]. Non-infected plants and infected plants were kept in separate growth chambers after inoculation. Non-infected and infected leaves were harvested 24 h after inoculation in parallel and immediately frozen in liquid nitrogen. For biological replicates, two independent sets of leaf materials were harvested. To confirm resistance or susceptibility of infected plants used for leaf harvest, plants were grown for additional 2 weeks. After this period, mosaic symptoms were observed on each infected F7 plant, whereas infected $F7^+$ remained without symptoms.

2.2. The introduction of macroarray

The macroarrays containing SSH clones were derived from comparison of NILs $F7^+$ (resistant to SCMV) and F7 (susceptible to SCMV) by Shi et al. [23]. In total, 2688 clones were spotted in duplicate on each macroarray. These clones were randomly picked from five SSH libraries. For two tester/driver cDNA pairs (infected $F7^+$ versus infected F7; non-infected $F7^+$ versus non-infected F7) subtractions were conducted in both directions. For the tester/driver cDNA pair infected $F7^+$ versus non-infected $F7^+$ only forward direction was conducted. Microarray hybridization data were evaluated by the SpotReport™ Alien™ cDNA Array Validation System (Stratagene, La Jolla, CA, USA), including positive, negative, and 10 spiking controls. Of 2688 clones, 672 clones, ranging in length from 96 to 843 bp, were sequenced by MWG (Ebersberg, Munich, Germany) and clustered into 302 genes. Then, their sequences were analyzed in the same way as microarray-ESTs (see Section 2.6).

2.3. Probe preparation and unigene-microarray hybridization

Total RNA from maize leaves was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The same total RNA extracted was also used for probe preparation in SSH-macroarray approach. Poly (A)+ RNA was isolated from Total RNA via Dynabeads® Oligo(dT) 25 (Dynal biotech, Oslo, Norway). According to TIGR Microarray Protocols [25], each mRNA sample was indirectly labeled with Cy3 or Cy5 (Amersham Pharmacia, Piscataway, NJ, USA) and hybridized with maize unigene-microarrays.

Maize unigene-microarrays were generated by the laboratory of Prof. Schnable (Iowa University, USA) and contain

11,827s maize ESTs (http://www.plantgenomics.iastate.edu/maizechip/). Among them, 11,027 ESTs were spotted once, 391 ESTs duplicate, and 6 ESTs triple. Thus, 11,424 unique ESTs, clustered into 9841 unigenes, are on the maize unigene-microarray, and 8.3% (949 of 11,424) of them have been mapped. The EST collection at the maize unigene-microarrray was derived from fifteen EST libraries, including 486 (immature leaf), 605 (endosperm), 606 (ear tissue), 614 (root), 618 (tassel primordia), 660 (mixed stages of anther and pollen), 683 (14-day immature embryo), 687 (mixed stages of embryo development), 707/945 (mixed adult tissues), and ISUM3/4/5/6/7 (seedling and silk), made from plants grown under normal environmental conditions and two stress-induced EST libraries, including 496 (stressed shoot) and 603 (stressed root).

For each comparison, four replications, including two biological replications and dye-swap replications in each biological replication, were conducted. Thus, four maize gene chips were used in each comparison.

2.4. Raw data acquisition

Fluorescence signals were detected using the arrayWoRx® Biochip Reader (Applied Precision, Issaquah, WA, USA). The image data obtained were imported into the software program ArrayVision 7.0 (Imaging Research, St. Catharines, Ont., Canada) for spot detection and quantification of hybridization signals. Local background calculated from the corners between spots, were subtracted using ArrayVision 7.0 to obtain raw signal intensities.

2.5. Unigene-microarray data analysis

Raw data were exported from ArrayVision 7.0 into Excel and converted to TIGR Array Viewer (TAV) format files. Data were normalized using intensity-dependent local regression (Lowess) implemented in MIDAS [26]. All calculated gene expression ratios were log 2-transformed and averaged over dye-swap replicates in each comparison. Differentially expressed genes at the 95% confidence level were determined using intensity-dependent Z-scores (with $Z = 1.96$) as implemented in MIDAS and the union of all genes identified in each comparison was considered significant in this experiment.

2.6. Sequence analysis

Annotation of each gene sequence was taken from the TIGR Maize Gene Index (http://www.tigr.org/tdb/tgi/plant.shtml). Each gene was assigned to a functional class using the Munich

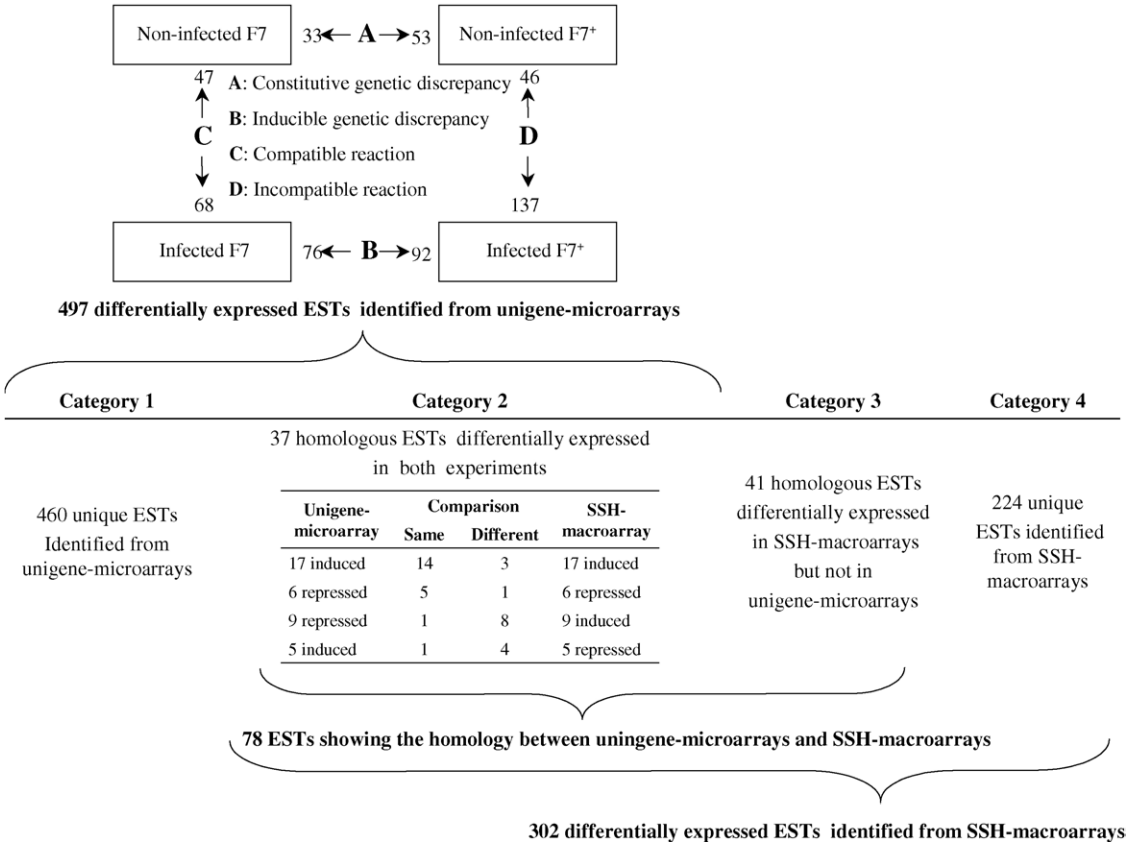

Fig. 1. Comparison of unigene-microarray and SSH-macroarray experiments. In total, 762 differentially expressed ESTs, identified from unigene-microarrays and SSH-macroarrays, were classified into four categories. Categories 1 and 2 include differentially expressed ESTs identified by unigene-microarray experiments in four comparisons, Comparison A (constitutive genetic discrepancy), Comparison B (inducible genetic discrepancy), Comparison C (compatible reaction), and Comparison D (incompatible reaction). The number indicates up-regulated gene numbers identified in each probe (arrow head) from each experiment. Categories 2 and 3 contain ESTs showing homology in both approaches. Differentially expressed ESTs identified by SSH-macroarrays were shown in Categories 2–4.

Information Center for Protein Sequences (MIPS) (http://mips.gsf.de/proj/thal/db/tables/tables_func_frame.html) classification scheme by BLASTX with a threshold *E*-value of 10. The mapped ESTs contain bin information from the Maize GDB (http://www.maizegdb.org) and the IDP mapping project (http://www.plantgenomics.iastate.edu/maizechip/). If an EST sequence investigated in our study was identical to the sequence of a mapped EST or gene cluster, we assigned this EST to the same bin. The distribution of mapped genes was drawn by MapChart [27]. In Fig. 1, the comparison of microarray-ESTs and SSH-ESTs is based on GenBank accession number.

2.7. Data availability

Original microarray expression data presented in this manuscript are available through ArrayExpress (http://www.ebi.ac.uk/arrayexpress) with accession number E-MEXP-253.

3. Results

3.1. Identification of differentially expressed genes by unigene-microarrays

In this study, we compared gene expression in NIL pairs F7 and $F7^+$. NIL $F7^+$ is resistant to SCMV, whereas NIL F7 is susceptible. Four comparisons were made (Fig. 1). Comparisons A and B evaluate the consequences of gene knock-in effect and include different genotypes with the same treatment. Comparison A: F7 non-infected versus $F7^+$ non-infected, here referred to a constitutive genetic difference. Structural and chemical barriers of the plant effectively exclude the majority of organisms. The genotypic difference between NILs might include constitutive resistance or susceptibility factors. Alternatively, differences in gene expression between NILs might be the result of polymorphic genes contained in the donor regions, irrespective of their relevance to the infection process. Comparison B: F7 infected versus $F7^+$ infected, referred to an inducible genetic difference. In addition to constitutive defenses, plants exhibiting resistance can detect foreign pathogens and trigger a rapid response to injury or virus attack. Genetic difference after SCMV inoculation might include induced or repressed resistance factors between NILs. Comparisons C and D reflect pathogenicity design and include different treatments on the same genotype (either F7 or $F7^+$). Comparisons C and D are designed to examine gene expression following infection in susceptible and resistant host combinations, respectively. Comparison C: F7 infected versus F7 non-infected (compatible interaction)—virus replicates and moves systemically in cells of intact susceptible plants. Comparison D: $F7^+$ infected versus $F7^+$ non-infected (incompatible interaction)—virus multiplication is limited to initially infected cells of resistant plants. Since no mock infection was employed, some of the genes identified in Comparisons C and D might be induced by mechanical stress.

The reproducibility of unigene-microarray experiments was high across all comparisons. In dye-swap replications prepared from the same mRNA, over 90% of the ratios calculated from technical replications varied by less than two-fold (Pearson correlation coefficient 0.87 ± 0.03). However, for a particular comparison, when the Cy3 signal of slide 1 and the Cy5 signal of slide 2 were averaged (data set 1), and the Cy5 signal of slide 1 and the Cy3 signal of slide 2 were averaged (data set 2), more than 95% of the ratios varied by less than 1.5-fold (Pearson correlation coefficient 0.94 ± 0.02). After averaging technical replications, more than 88% of all ratios varied by less than 1.5-fold (Pearson correlation coefficient 0.90 ± 0.01 between biological replications).

In total, 497 ESTs were differentially expressed in one or more comparisons, which accounted for 4.1% of 11,827 ESTs deposited on the unigene-microarray. The number of at least two-fold induced ESTs, was 33 for non-infected F7 and 53 for non-infected $F7^+$ in Comparison A, 76 for infected F7 and 92 for infected $F7^+$ in Comparison B, 47 for non-infected F7 and 68 for infected F7 in Comparison C, as well as 46 for non-infected $F7^+$ and 137 for infected $F7^+$ in Comparison D (Fig. 1). In total, 50.4% of these ESTs were induced more than 4-fold up to 25-fold.

The EST collection printed on the maize unigene-microarrray was derived from 17 EST libraries (see Section 2 for details). The discovery rate of differentially expressed genes from EST libraries was 8.0% (111 of 1380), 9.0% (34 of 379), 4.4% (29 of 666), 4.8% (14 of 291), 3.4% (54 of 1598), 3.0% (71 of 2371), 2.6% (15 of 574), 0% (0 of 15), 4.7% (12 of 258), 4.2% (16 of 383), 6.0% (64 of 1060), and 4.6% (132 of 2852) for 486, 496, 603, 605, 606, 614, 618, 683, 687, 707/945, and ISUM3/4/5/6/7, respectively.

In "Digital Northern" analysis [28], 151 (30%) differentially expressed microarray-ESTs were in the rare transcript category with fewer than five sequences in the public EST collection [29]. Forty-three (9%) of differentially expressed ESTs were represented by more than 100 ESTs and can be classified as abundantly transcribed, whereas the remaining 303 (61%) corresponded to genes transcribed at a moderate rate (6–98 ESTs).

3.2. Comparison of SSH-macroarray and unigene-microarray results

We assigned to each differentially expressed SSH-EST to a GenBank accession number (GA) to identify respective microarray-ESTs based on BlastN hits (E-value < 0.0001) against the Maize GDB EST database [23]. In average, PCR fragments printed on microarrays were significantly ($P < 0.0001$) longer (499 bp) than SSH fragments (245 bp).

The comparison of unigene-microarray and SSH-ESTs is summarized in Fig. 1. Four hundred and sixty differentially expressed ESTs were exclusively present on microarrays (Category 1), and 224 on SSH-based macroarrays (Category 4). 78 differentially expressed ESTs were present both on micro- and macroarrays. A Bland–Altman plot [30] revealed no significant difference between both experiments. Among those, 37 homologous ESTs were differentially expressed both in unigene-microarray and SSH-macroarray experiments (Category 2): 17 ESTs were induced in both approaches with 14 in

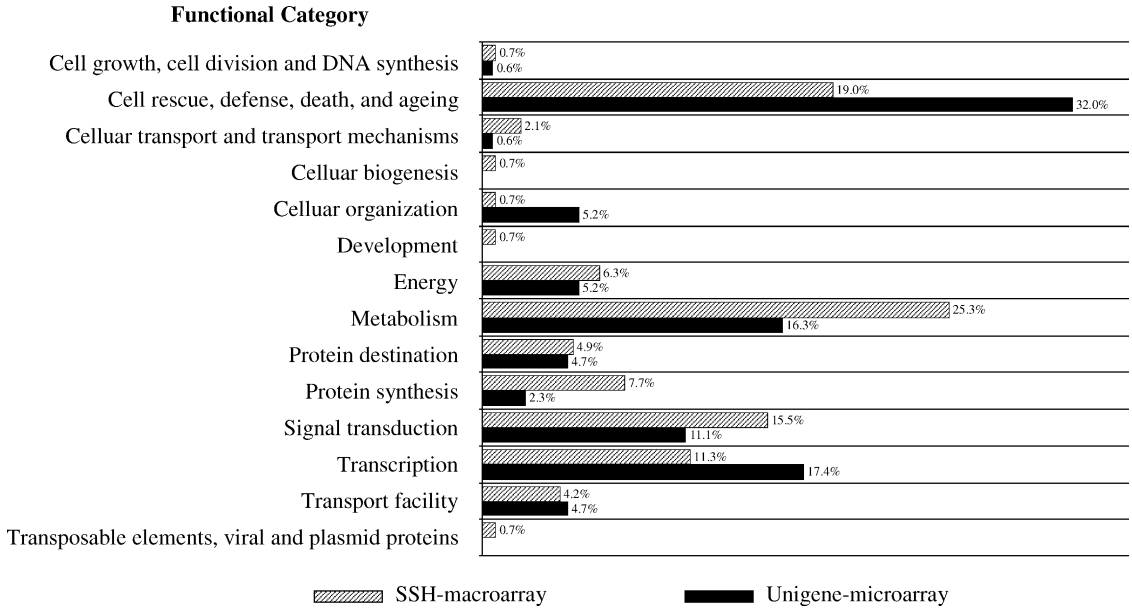

Fig. 2. Comparison of gene distribution in functional classes in unigene-microarray and SSH-macroarray experiments. Each EST was assigned to a functional class using the Munich Information Center for Protein Sequences (MIPS) (http://mips.gsf.de/proj/thal/db/tables/tables_func_frame.html) classification scheme by BLASTX with a threshold *E*-value of 10. Unclassified microarray-ESTs (72%) and SSH-ESTs (42%) were not taken into account.

the same comparison and 3 in different comparisons; 6 ESTs were repressed in both approaches with 5 in the same comparison and 1 in different comparisons; 9 ESTs were repressed in unigene-microarray but induced in SSH-macroarray experiments with 1 in the same and 8 in different comparisons; 5 ESTs were repressed in SSH-macroarray but induced in unigene-microarray experiments with 1 in the same and 4 in different comparisons. Forty-one homologous ESTs differentially expressed in SSH-macroarray were not differentially expressed in unigene-microarray experiments (Category 3). If all 78 ESTs in Categories 2 and 3 are taken into account expression patterns of unigene-microarray and SSH-macroarray experiments from the same comparisons were consistent (Fisher's exact test: $P = 0.0117$).

Although more differentially expressed ESTs were identified based on microarrays (497) than SSH-macroarrays (302), the efficiency of gene discovery, determined as the percent of differentially expressed cDNAs, was much higher in SSH-macroarray (59%) [23] than in unigene-microarray experiments (4.1%). However, due to an approximately five-fold redundancy of SSH clones on the macroarray [23], the actual efficiency of gene discovery by SSH-macroarray experiments was approximately 10%.

3.3. Classification of differentially expressed genes identified by unigene-microarrays and SSH-macroarrays

Each gene was assigned to a functional class using the Munich Information Center for Protein Sequences (http://mips.gsf.de/proj/thal/db/tables/tables_func_frame.html) classification scheme by BLASTX with a threshold *E*-value of 10. In total, more differentially expressed ESTs from the unigene-microarray experiment (72%) were unclassified than in the SSH-macroarray experiment (44%). Among classified ESTs, the largest category was "metabolism" (25.3%) in unigene-microarray experiments (Fig. 2), and "cell rescue, defense, cell death, and ageing" (32.0%) in SSH-macroarray experiments. Further ranking of classification categories was "cell rescue, defense, cell death, and ageing" (19.0%), "signal transduction" (15.5%) and "transcription" (11.3%) for unigene-microarray experiments, and "transcription" (17.4%), "metabolism" (16.3%), and "signal transduction" (11.1%) for SSH-macroarray experiments. In spite of different ranks between both experiments, the top four categories were the same, and three of them ("cell rescue, defense, cell death, and ageing", "signal transduction", and "transcription") are pathogenesis-related. In contrast to 60.5% in SSH-macroarray experiments, 45.8% of differentially expressed ESTs in unigene-microarray experiments were classified into pathogenesis-related categories, such as AI664862 (stress-induced protein OZI1 precursor), AI795699 (peroxidase), AI491543 (hypersensitive-induced response protein), and BM073434 (pathogenesis-related protein-5).

3.4. Comparing map position of differentially expressed ESTs between SSH-macroarray and unigene-microarray

The maize genetic maps are divided into 100 segments (=bins) of approximately 20 cm length. Each bin is determined by two fixed "core markers". The complete list of "core markers" is published in Gardiner et al. [31]. Altogether 20% (100 of 497) of ESTs identified from unigene-microarray experiments were previously assigned to 51 bins distributed over all 10 maize chromosomes (Fig. 3), whereas the same proportion of ESTs (in total 60) identified from SSH-macroarray were assigned to fewer genome regions (29 bins). ESTs were randomly distributed to chromosomes in both unigene-microarray ($P = 0.3979$, $\chi^2 = 9.438$, d.f. = 9) and SSH-macroarray experiments ($P = 0.1806$, $\chi^2 = 12.62$, d.f. = 9).

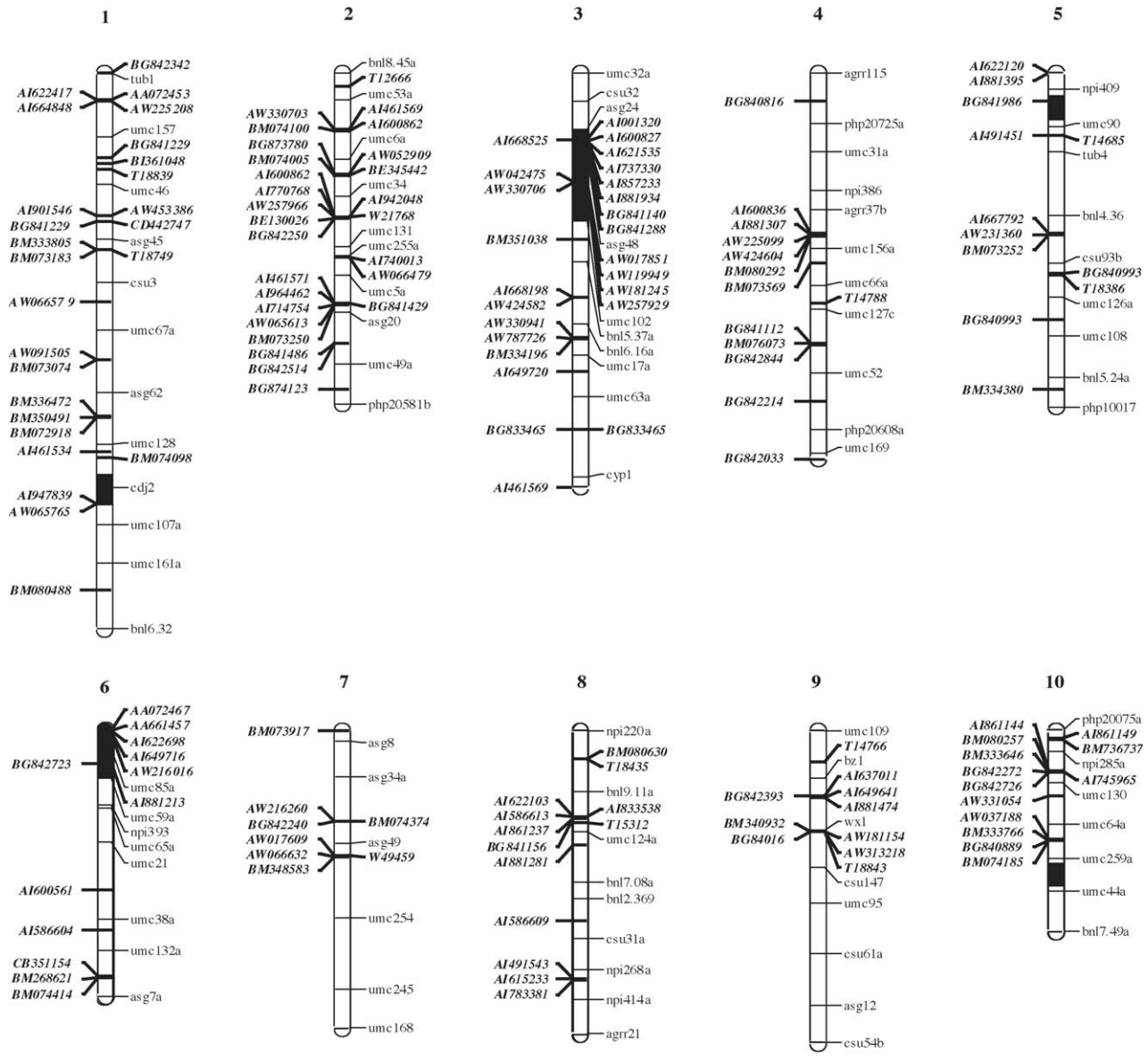

Fig. 3. Distribution of differentially expressed genes on maize chromosomes with respect to SCMV resistance identified in unigene-microarray and SSH-macroarray experiments. Loci in bold and italics placed on the *left* side of each chromosome were in silico mapped ESTs identified from unigene-microarray experiments. Loci placed on the *right* side of each chromosome are a set of core markers that defines a bin boundary [31], while the loci in bold and italics were mapped ESTs identified in SSH-macroarray experiments. EST mapping information was from the Maize GDB (http://www.maizegdb.org) and the IDP mapping project (http://www.plantgenomics.iastate.edu/maizechip/), according to map bins. *Scmv1* is highlighted on chromosome 6, *Scmv2* on chromosome 3, and three minor QSCMs on chromosomes 1, 5, and 10 [10].

In contrast to the 30% (18 of 60) of mapped ESTs located in bin 3.04–3.05 (12) and bin 6.00–6.02 (6) in SSH-macroarray experiments, only 4% (4 of 100) of the mapped ESTs from unigene-microarray experiments were placed in bin 3.04–3.05 (3) and bin 6.00–6.02 (1) (Table 1). The proportion of ESTs mapped in vicinity of *Scmv* QTLs was significantly higher ($P = 0.0013$) in SSH-macroarray than in unigene-microarray experiments. Among the ESTs mapped in bins 3.04–3.05 and 6.00–6.02, no DNA similarity was found between microarray-ESTs and SSH-ESTs. None of these microarray-ESTs, but at least six SSH-ESTs, showed similarity to defense-related genes [23]. Finally, AI947839, AW065765, and BG841986 identified from unigene-microarray experiments were co-localized with QSCM on chromosomes 1, 1, and 5 (Fig. 3).

4. Discussion

4.1. Comparison of SSH-macroarray and unigene-microarray experiments

Microarrays are widely recognized as a significant technological advance providing genome-scale information on gene expression patterns [32]. However, a comprehensive microarray is currently not available for maize. The microarray

Table 1
The list of candidate genes (CGs) associated with SCMV resistance

GA[a]	Annotation[b]	Similarity[b] (%)	Bin[c]	Comparison			
				A	B	C	D
Positional CGs mapping to bins 3.04–3.05 and 6.00–6.02							
AI668525	gb|AAF55168.1||AE003708 CG4913-PA {*Drosophila melanogaster*}	4	3.04			−3.0[d]	
AW330706	Unknown		3.05				2.8
AW042475	dbj|BAB64692.1||AP003683 P0431G06.3 {*Oryza sativa*}	10	3.05			−4.5	
BG842723	dbj|BAB78651.1||AP003022 P0681B11.18 {*Oryza sativa*}	7	6.02	5.8			2.7
Functional CGs showing the homology to pathogenesis-related genes							
AI491543	gb|AAF68389.1 hypersensitive-induced response protein {*Zea mays*}	100	8.07				2.8
AI621822	emb|CAA06925.1 Avr9 elicitor response protein {*Nicotiana tabacum*}	69				6.0	
AI619128	dbj|BAB89081.1 dnaJ-like protein {*Oryza sativa*}	84					−3.3
AI615100	SP|P33890 cold shock induced protein TIR2 precursor—yeast	5		3.0			
AI664862	PIR|S59544 stress-induced protein OZI1 precursor {*Arabidopsis thaliana*}	92			3.5		
AI795699	emb|CAC21392.1 peroxidase {*Zea mays*}	100			5.3		
AI999974	SP|P18123 catalase isozyme 3 {*Zea mays*}	42		6.9	7.2		
BM073434	PIR|T02055 pathogenesis-related protein-5 {*Zea mays*}	98		3.3			
Consistent ESTs in Category 2							
AI461569	PIR|S65781|S54179 acidic ribosomal protein P2 {*Zea mays*}	100					2.5, 3.4[e]
AI600862	dbj|BAB93128.1||AP003196 beta-1,3-glucanase-like protein {*Oryza sativa*}	94	2.04	−9.7, −10.0			
AI621758	dbj|BAB09296.1||AB011476 RNA-binding protein-like {*Arabidopsis thaliana*}	64			4.8, 2.0		
AI649641	dbj|BAB16858.1||AP002537 P0001B06.11 {*Oryza sativa*}	70					3.0, 2.9
AI665633	Unknown					9.0, 5.0	
AI714860	gb|AAM98096.1||AY139778 AT3g13690/MMM17_12 {*Arabidopsis thaliana*}	15		−3.5, −10.0			
AI738263	gb|AAB49338.1 delta-24-sterol methyltransferase {*Triticum aestivum*}	40		8.3, 5.8			
AI855243	Unknown				7.0, 5.3		
AI941971	PIR|S33633|S33633 ubiquitin/ribosomal protein CEP52 {*Oryza sativa*}	100				5.8, 2.0	
AI942048	gb|AAK67147.1 nucleosome/chromatin assembly factor C {*Zea mays*}	25				8.5, 5.3	
AI942105	dbj|BAC55693.1||AP004275 P0453E05.3 {*Oryza sativa*}	14		9.7, 6.1			
AI974914	Unknown				−2.3, −1.9		
AW052909	gb|AAN08216.1||AC090874 ribosomal protein L15 {*Oryza sativa*}	100				6.7, 5.0	
AW330660	Unknown						2.7, 2.1
AW331161	gb|AAO74140.1||AY228468 ORF64c {*Pinus koraiensis*}	100					2.7, 3.1
AW438364	gb|AAL08230.1||AY056374 AT4g22990/F7H19_170 {*Arabidopsis thaliana*}	25			−3.8, −2.3		
BG840993	SP|Q8W425 proteasome non-ATPase regulatory subunit 6 {*Oryza sativa*}	90	5.06				2.3, 6.3
BG841229	SP|P48489 serine/threonine protein phosphatase PP1 {*Oryza sativa*}	98	1.03	−4.3, −10.0			
BG842726	dbj|BAB93128.1||AP003196 beta-1,3-glucanase-like protein {*Oryza sativa*}	84	10.02				24.7, 5.6

[a] GenBank accession number.
[b] Annotation of each gene sequence was taken from the TIGR Maize Gene Index (http://www.tigr.org/tdb/tgi/plant.shtml).
[c] Mapping information is from the Maize GDB (http://www.maizegdb.org) and the IDP mapping project (http://www.plantgenomics.iastate.edu/maizechip/), according to map bins (16).
[d] If the ratio is less than one, the negative reciprocal is listed.
[e] The first ratio is from unigene-microarray experiment, whereas the second one from SSH-macroarray experiment.

used in this study, contained 9841 different unigenes, accounting for approximately 20% of the estimated 50,000 maize genes [21]. In contrast to the unigene-microarray, the macroarray used in a companion study [23] contained 2688 SSH clones specifically developed for studying SCMV resistance. SSH libraries are produced from pairwise comparisons [33]. For the 4 RNA samples (infected F7, infected $F7^+$, not-infected F7 and not-infected $F7^+$) employed [23], only 5 instead of all 12 possible SSH libraries were constructed (6 pairwise comparisons with totally 12 possibilities for subtraction). This might explain non-recovery of 460 differentially expressed ESTs, identified by use of microarrays, by the SSH-macroarray procedure (Fig. 1).

The SSH-macroarray procedure enriches for low-abundant and helps normalize differentially expressed mRNAs [22]. The normalization step is particularly important because abundant pathogenesis-related transcripts (e.g., genes coding for PR proteins) very likely mask important SCMV-specific transcripts expressed at much lower levels. According to Maize EST statistics in GenBank (31/12/2003 assembly), the distribution of high-, medium-, and low-abundant ESTs was 3%, 64%, and 33%, respectively. ''Digital Northern'' analysis revealed a shift towards medium- and low-abundant ESTs within our SSH libraries with 51% and 47%, respectively [23]. This contrasts to a shift towards high-abundant ESTs (9%) on the unigene-microarray. Therefore, the 224 unique SSH-ESTs (Fig. 1) demonstrate the usefulness of the SSH-macroarray procedure to isolate target trait-specific genes. In addition, a greater proportion of ESTs differentially expressed in SSH-macroarray experiments (60.5%) were classified into pathogenesis-related categories than in unigene-microarray experiments (45.8%).

Only 8.8% (1045 of 11827) of the ESTs spotted on the unigene-microarray were derived from stress-induced EST libraries, including library 496 (stressed shoot). The number of differentially expressed genes discovered from library 496 (9%) was substantially higher than from other EST libraries (average: 4%). Regardless of the procedure, the reproducibility was high both in unigene-microarray and SSH-macroarray experiments. Fisher's exact test ($P = 0.0117$) showed consistent expression patterns of microarray-ESTs and SSH-ESTs from the same comparison (Fig. 2). However, some genes such as AI691482 were induced in unigene-microarray experiments but repressed in SSH-macroarray experiments. Other genes showed the opposite response. Discrepancies between both approaches may be explained by: (i) different parts of genes spotted on the two types of arrays, (ii) different labeling procedures, and (iii) different approaches for ratio measurements.

Due to cDNAs of different length, different parts of the genes were deposited on micro- or macroarrays. This might confound changes in transcript levels due to cross-hybridization to gene family members [34]. Furthermore, probes were labeled using different methods. Although same total RNA samples were used, samples were indirectly labeled with the fluorescent dyes Cy3 or Cy5 using random primers in the unigene-microarray experiment, whereas direct labeling with radioactive P^{32} using oligo(dT) was employed in the SSH-macroarray experiment. In another study comparing array-based results with Northern blots, arrays were less sensitive in measuring a subset of the genes [35]. In addition, the methods used to calculate gene expression ratios differed. For the unigene-microarray experiments two cDNA samples were hybridized on the same glass slide in parallel, allowing for direct calculation of gene expression ratios. In contrast, internal controls were included on nylon membranes for the SSH-macroarray experiments. Only one probe was hybridized per membrane and ratios were obtained by indirect comparisons between membranes. Among 21 homologous ESTs from the same comparison in Category 2, the correlation ($r = 0.88$) of the expression levels between both approaches was highly significant ($P < 0.0001$). The finding of 41 ESTs in Category 3 is consistent with a higher risk of smaller fragments cross-hybridizing with other gene family members [36].

4.2. Molecular mechanisms of maize–SCMV interaction

Typical mosaic symptoms were observed in leaves of susceptible F7 after systemic movement and replication of SCMV. Thus, the F7–SCMV interaction is a compatible interaction (Comparison C). In compatible interactions, the distribution of genes among functional classes looked similar to the incompatible reaction, regardless of using the unigene-microarray or the SSH-macroarray. These results are consistent with the hypothesis that viruses induce defense response both in susceptible and resistant plants at early stages [37]. Whitham et al. [38] reported that diverse RNA viruses, including cucumber mosaic cucumovirus, oil seed rape tobamovirus, turnip vein clearing tobamovirus, potato virus X potexvirus, and turnip mosaic potyvirus, elicited the expression of common sets of genes in susceptible *Arabidopsis*. In total, 62% of the differentially expressed genes in Comparison C, 72% (33 of 47) for SSH-macroarray [23] and 58% (67 of 115) for unigene-microarray s, can be assigned to this common set of genes, such as BM501006 (F5M15.13), AI820398 (AIG2 protein homologue F8F16.130), while the remaining genes without annotation could be maize-specific.

Plants of NIL $F7^+$ displayed no SCMV symptoms in all infection trials [23]. Thus, $F7^+$ is completely resistant to SCMV and the $F7^+$–SCMV interaction is an incompatible interaction (Comparison D). The results were generally consistent between unigene-microarray and SSH-macroarray experiments. Gene expression profiles of incompatible reactions, including TMV in tomato and *Chenopodium* [39,40], revealed similarities at the gene level with Comparison D, such as AI491543 (hypersensitive-induced response protein) and BM073434 (pathogenesis-related protein-5) induced after infection. This suggests that common mechanisms for defense against virus attack may operate in resistant plants [37].

Previous evidence suggests that both constitutive and inducible defense mechanisms operate in $F7^+$ plants against SCMV [23]. Our unigene-microarray experiments revealed several putative preformed inhibitors in Comparison A (constitutive genetic difference). AW011679 encodes a putative UMP/CMP kinase, and BM335333 encodes a putative ankyrin-kinase. These results corroborate previous findings that SCMV

can be detected, and thus replicates, in primary infected leaves of resistant genotypes [41].

Two types of inducible defence are defined: hypersensitivity response and extreme response (ER) [37]. HR limits virus infection to a zone of cells around the initially infected cell of the resistant host, usually with the formation of visible necrotic local lesions [37]. ER limits virus multiplication to initially infected cells because of an ineffective virus-coded movement protein, giving rise to latent infection. No HR symptoms have so far been observed on maize leaves infected with SCMV. However, further experiments need to be conducted at the single-cell level, to discriminate between ER and HR for SCMV resistance [37].

ER is most often triggered by dominant or semi-dominant resistance (R) genes and occurring in a strain-specific or ''gene-for-gene'' manner [37]. In potato, two extreme resistance genes (*Rx1* and *Rx2*) to PVX have been cloned, which belong to the nucleotide binding, leucine-rich repeat (NBS-LRR) superfamily of R-genes [42,43]. In addition, ER might be triggered by RNA silencing. In contrast to resistance triggered by the NBS-LRR-type R-genes, resistance through silencing appears not to depend on a gene-for-gene recognition event [44]. In our unigene-microarray experiments, differentially expressed pathogen-related genes, identified from Comparison B (inducible genetic difference), such as AI664862 (stress-induced protein OZI1 precursor) and AI795699 (peroxidase), were found together with R-gene-mediated resistance or RNA silencing. This is in agreement with our previous SSH-macroarray experiments [23]. So far, little is known about genes involved in signal transduction of HR and ER, and it is even possible that they use the same genes for signaling.

4.3. Candidate gene (CG) selection for Scmv QTL

Positional cloning is the major approach used to characterize genes underlying QTL [45]. Maize resistance gene analogues involved in initial pathogen recognition, were chosen as starting point for map-based isolation of genes conferring SCMV resistance [13]. Mapping of RGAs in relation to *Scmv1* and *Scmv2* suggested that RGA *pic19* is a candidate for *Scmv1* and *pic13* for *Scmv2* [14]. *pic19* and *pic13* were used to screen a BAC library of B73 and three paralogues clustering in the *Scmv1* region were isolated from the maize genome [15], currently analyzed in more detail (Xu and Lübberstedt, unpublished results). However, positional cloning of resistance genes, in case of RGA-like sequences, is complicated by tight clustering of similar sequences in the *Scmv1* region [14]. Moreover, recent large-scale comparisons of allelic maize sequences revealed that insertions and deletions are more common than anticipated, with potential negative impact for map-based gene isolation of any type of sequence [46]. Thus, map-based gene isolation using a BAC library from the SCMV susceptible inbred B73 might end in non-detection of the target genes, if they are the result of respective insertion or deletion events. Identification of differentially expressed candidate genes co-segregating with the target genes might help to overcome this problem.

The candidate-gene approach provides an alternative for pinpointing genes underlying SCMV resistance, especially in view of the planned sequencing of major parts of the genome [21]. The CG approach consists of three subsequent steps: the choice, screening, and validation of CGs [47]. This study contributes to the identification of candidate genes for mapped SCMV resistance genes and QTL in three ways: (i) positional CGs mapping to bins 3.04–3.05 and 6.00–6.02, (ii) functional CGs showing homology to pathogenesis-related genes, and (iii) ESTs in Category 2 showing consistent expression patterns in both approaches. Although these three classes contain candidates for genes affecting SCMV resistance, the second two classes of genes might be located outside the mapped SCMV QTL regions.

So far, 18.6% (696 of 3737) of all mapped maize ESTs are located in bins 3.04–3.05 (426) and 6.00–6.02 (270) (http://www.plantgenomics.iastate.edu/maizechip/). In contrast to the 30% (18 of 60) of the mapped ESTs from SSH-macroarray located in bin 3.04–3.05 (12) and bin 6.00–6.02 (6) [23], only 4% (4 of 100) of the mapped microarray-ESTs were located in bins 3.04–3.05 (3) and bin 6.00–6.02 (1) (Table 1). While no sequence similarities was found between SSH-ESTs (18) and microarray-ESTs (4), 50% (9 of 18) mapped SSH-ESTs belong to Category 3 (Fig. 1). One possible explanation is that *Scmv1*- and *Scmv2*-specific ESTs were under represented on the unigene-microarray, whereas respective ESTs were enriched in the SSH libraries after normalization. The larger number of differentially expressed genes mapping to these two regions can be explained by: (i) genes differentially expressed due to the polymorphism between F7 and $F7^+$ in these two regions but without relation to SCMV resistance, or (ii) clustering of genes involved in SCMV resistance in these two regions. Except for positional CGs, several pathogenesis-related CGs were revealed from the 80% non-mapped ESTs, such as AI621822 (Avr9 elicitor response protein), AI999974 (maize catalase isozyme 3).

The 19 consistent ESTs in Category 2 (Table 1) are the most promising candidates for being differentially expressed in the context of SCMV resistance. However, four ESTs (AI665633, AI855243, AW330660, and AI974914) have no tentative annotation in the TIGR Gene Index (http://www.tigr.org/tdb/tgi/plant.shtml), and the annotation of four genes (AI649641, AI714860, AI942105, and AW438364) was based on proteins from the *Arabidopsis* or rice genomes. Gene products of the 11 remaining ESTs were classified into two groups. The first group was related to RNA binding, including AI461569 (acidic ribosomal protein P2), AI621758 (RNA-binding protein-like), AI941971 (ubiquitin/ribosomal protein CEP52), AI942048 (nucleosome/chromatin assembly factor), and AW052909 (ribosomal protein L15). The acidic ribosomal protein P2 has been shown to participate in translation regulation by interacting with eEF-2 elongation factors in the peptide elongation process [48]. Chromatin assembly factor C is involved in the structural organization of DNA into chromatin, which is of key importance to regulate genome function and stability [49]. As the eukaryotic initiation factor 4E (eIF4E) is responsible for recessive resistance against potato virus Y in

pepper [50], these genes related to RNA binding could interact with the SCMV genome. The second group was homologous to catalytic proteins involved in defense response, including AI600862 (beta-1,3-glucanase-like protein), AI738263 (delta-24-sterol methyltransferase), AW331161 (ORF64c), BG842726 (beta-1,3-glucanase-like protein), BG841229 (serine/threonine protein phosphatase PP1), and BG840993 (proteasome non-ATPase regulatory subunit 6). Delta-24-sterol methyltransferase is involved in membrane sterol biosynthesis and was shown to be over-expressed in presence of polyenic antifungal agents [51]. Serine/threonine protein phosphatase PP1 is involved in the regulation of signal transduction and specifically bound to the gluconeogenesis inhibitor FR225659 [52]. The genes AI600862, BG840993, BG841229, and BG842726 were located outside *Scmv* QTL regions in bins 2.04, 5.06, 1.03, and 10.02, respectively. They might be further downstream in the signal transduction pathway and induced by genes located in the *Scmv1* and/or *Scmv2* regions.

Once genes responsible for quantitative variation of SCMV resistance are identified functional markers can be developed [53]. Functional markers are superior to random DNA markers such as RFLPs, SSRs, and AFLPs owing to complete linkage with trait locus alleles. Due to the oligogenic inheritance of SCMV resistance, marker-assisted selection (MAS) with functional markers would substantially increase breeding efficiency.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2005.08.016.

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