Session IV

Biotechnology for sustainable development

Lead lecture

Fauquet, C.	Modern tools for genetic improvement are now available for cassava, the poor farmer's crops

Invited speakers

Tohme, J.	From cassava genomics tools to breeding
Ghislain, Marc	Potato genomics: Resourses and research to contribute to developing country agriculture
Schafleitner, R.	Development of genetic resources for breeding improved sweetpotato
Conner, A.	Intragenic transformation for genetic improvement of root and tuber crops

Oral presentation

Kreuze, Jan	Deep sequencing of plant small RNAs: A generic method for diagnosis, discovery and sequencing of viruses
Si, Huaijun	Control of potato tuber dormancy and sprouting by genetic engineering of inorganic pyrophosphatase Gene
Bohorquez, Adriana	Isolation of genes differentially expressed during the defense response of Cassava (*Manihot esculenta* Crantz) to whitefly *(Aleurotrachelus socialis* Bondar) attack
Chavarriaga, Paul	Combining biotechnology, molecular genetics and breeding to improve the content of carotenes in cassava roots

Posters

Rivera Cristina	Genetic transformation of potato cultivars using the RB gene to increase resistance to late blight disease
Solis, Julio	New SSR markers for sweetpotato from data mining of expressed sequence tags(ESTs)
Ghislain, Marc	Transfer of sweetpotato-like genes expressing Cry proteins into sweetpotato varieties
Zhang, Peng	Gene expression profiling of developing cassava storage and its starch quality improvement by genetic engineering
Agili, Sammy	In Vitro screening of Sweetpotato *(Ipomoea batatas)* genotypes for drought resistance using polyethylene glycol
Lindqvist-Kreuze, Hannele	Molecular assisted assessment of late blight resistance in potato
Chavarriaga, Paul	Structural and functional characterization of an *Ipomoea batatas* Phytoenen Synthase gene

Manrique, S.	Screening for regeneration and transformation efficiencies of African sweetpotato cultivars
Solis, Julio	Insilico prediction and characterization of microRNAs from Sweet potato [*Ipomoea batatas* (L.) Lam.]
Ceballos, Hernan	Identification of regions of cassava genome associated with increased carotenoids content in the roots
Siqueira, Marcos	Protocol for amplification by transferability of micro satellite markers in dioscorea bulbifera
Siqueira, Marcos	Genetic variability in commercial varieties of water yam *(Dioscorea alata)* with microsatellites markers

Development of genetic and genomic resources for breeding improved sweetpotato

Roland Schafleitner, Luz Tincopa, Genoveva Rossel, Jorge Espinoza, Julio Solis, Omar Palomino, Carlos Rivera, Cinthia Quispe, Jack Hou, Ji Young Kim, Wolfgang Gruneberg, Reinhard Simon, Anthony Collins, David Tay and Merideth Bonierbale

International Potato Center – CIP, Avenida La Molina 1895, Apartado 1558, Lima 12, Peru.

*DArT/PL, 1 Wilf Crane Crescent, Yarralumla, ACT 2600, Australia

E-mail: r.schafleitner@cgiar.org

Production of sweetpotato (*Ipomoea batatas* L. (Lam)), an important staple food in Sub-Saharan Africa, is limited by a number of constraints, such as low adaptability of available varieties and landraces, virus diseases, insect pests and drought. Consequently, yields achieved by resource-poor farmers in SSA are low. Improved and well adapted sweetpotato varieties with increased tolerance to biotic and abiotic stresses could significantly contribute to augment productivity and, once available, would have a large positive impact on food and income security in Sub-Saharan Africa. However, breeding efforts are limited by the crop's genetic complexity, lack of information about its genetic resources and access to genomics tools for this crop for modern breeding. To mobilize allelic diversity and to facilitate introgression of desirable alleles into breeding populations, we have established genetic and genomics tools including a well defined Composite Genotype Set and a gene index. Furthermore we have designed and tested more than 200 new microsatellite markers and identified 200 SNP markers in stress response genes. A sweetpotato DArT marker system is under development. For establishing the gene index, we applied Next Generation sequencing technologies to characterize the sweetpotato transcriptome. The index comprises 31.165 contigs and 29.080 singletons and was annotated based on sequence comparisons with known proteins. The Composite Genotype Set and the genomics tools will support trait capture efforts on molecular level, will improve the understanding of the sweetpotato gene pools and finally will enhance access to allelic diversity for breeding improved varieties.

Keywords: Sweetpotato, genetic resources, transcriptome, molecular marker.

Introduction

Sweetpotato, (*Ipomoea batatas* L. (Lam)) the fifth-most important food crop in developing countries, is a rustic crop with generally high levels of stress tolerance. Nevertheless yields are limited by a number of constraints, such as virus diseases, insect pests and drought. Improved and well adapted sweetpotato varieties with increased tolerance to biotic and abiotic stresses could significantly contribute to increasing productivity, which would have a large positive impact on food and income security of resource-poor farmers in Sub-Saharan Africa. However, breeding efforts are limited by the genetic complexity of this hexaploid and highly heterozygous crop and by the lack of genomic resources for this plant.

CIP holds in trust 5,961 sweetpotato accessions including breeding lines, improved varieties, landraces, and wild accessions from 58 countries, which contributes to global conservation efforts. The identification and characterization of a subset of this collection that represents the available diversity for agronomical and resistance traits, nutritional quality and breeding efforts, referred to as a 'composite genotype set' (CGS) will facilitate improving the knowledge of genetic diversity of sweetpotato and contribute to the development of robust molecular tools for exploration of sweetpotato genetic resources. Prior to this work, sequence information for sweetpotato was limited to some 20.000 expressed sequence tags (ESTs) and about 1500 gene sequences deposited in public databases. At PlantGDB an assembly of all genebank-deposited *Ipomoea batatas* sequences is available (//www.plantgdb.org/download/download.php?dir=/Sequence/ESTcontig/Ipomoea_batatas/current_version). This database contains 12.464 sequences, partly contigs and partly singletons. Genomic tools were restricted to a medium density cDNA microarray, medium-density maps and some 100 published SSR markers (Hu et al. 2004, Arizio et al. 2008). Additional genomic

resources such as gene sequences and markers are urgently required to mobilize sweetpotato biodiversity for breeding programs.

Materials and methods

Assembly of the CGS

CIP curators and breeders used CIP databases and evaluation data to establish a sweetpotato CGS. From over 5000 accessions, sweetpotato biodiversity was sampled based on geographical origin and available SSR data. The set was extended with clones with high levels of resistances to abiotic and biotic stresses and high nutritional value. Morphological information and molecular fingerprints (SSR genotypes) are available to facilitate the identification and tracking of the clones of the CGS.

Developing the gene index

For the development of the sweetpotato gene index, shoots from field-grown plants of the sweetpotato *I. batatas* variety "Tanzania" (CIP number 440166) For the development of the sweetpotato gene index, shoots from field-grown plants of the sweetpotato *I. batatas* variety "Tanzania" (CIP accession number 440166) were acclimated to the greenhouse and cultivated in pots for one month. Then drought was imposed by not watering the plants for eight weeks. After that time, leaf and stem tissue was sampled separately and total RNA was produced using the Trizol reagent according to the instructions of the supplier (Invitrogen). Complementary DNA was synthesized from the two RNA batches by Evrogen (www.evrogen.com) and normalized according to Shagin et al., (2002). 7 μg cDNA of each normalized library was submitted to a quarter 454 sequencing run at the School of Biological Sciences, University of Liverpool. The cDNA library of leaves was sequenced with the 454 FLX technology and for sequencing the stem library the 454 FLX TITANIUM system was available.

Sequence cleaning was performed on the CIP High Performance Computer (http://hpc.cip.cgiar.org/). Adaptor primer, oligonucleotide sequences derived from cDNA library construction as well as low complexity regions present in the 454 raw reads were masked using the open source software RepeatMasker 3.2.7 (http://www.repeatmasker.org/RMDownload.html). 20.094 publically available sweetpotato EST sequences were downloaded from http://www.ncbi.nlm.nih.gov/sites/entrez and cleaned from vector sequences using SeqClean (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/download.pl?ftp_dir=software_&file_dir=seqclean/seqclean.tar.gz). The 454 reads were assembled together with the Genbank ESTs with the NGen software (Lasergene) on a 64-bit desktop computer. For optimization purposes, assembly was tried at 85, 80 and 75% minimal match percentage (high, medium and moderate stringency). The final assembly was done using the following parameters: matchsize: 25, gap penalty: 7, mismatch penalty:12, match score: 10, minimal match percentage: 75, match spacing: 40. The quality of the assembly was assessed manually for 400 representative contigs by checking the correct alignment of the fragments.

Assembly quality regarding redundancy and gene representation was assessed by self-blast using a cut-off value of e^{-30} and by blastn comparison of the assemblies with mRNA sequences of *Solanales* (NCBI Genebank). Final annotation of the gene index was done by blastx against the 3.A_thaliana-p protein database (TAIR, www.arabidopsis.org). All blast analyses were run with BLAST 2.2.20 on the CIP High Performance Computer (http://hpc.cip.cgiar.org). Gene ontology attribution was performed using Blast2GO according to Conesa er at. (2005).

Marker development

Microsatellite (SSR) sequences were identified in the sequence assembly with the SSRlocator (http://minerva.ufpel.edu.br/~lmaia.faem/ssr1.html) limiting the hits to motives that consisted of at least 10 dimers, 7 trimers or 5 tetramers. Primers for SSR loci were designed with Primer3 with 100 – 200 bp amplicon size. SNPs were searched in 200 manually selected contigs that represent stress-sensitive genes using Seqman (Lasergene Inc.). Diversity Array Technology (DArT) marker development is in progress at DArT)/PL, as described by Wenzl et al. (2004).

Results and Discussion

A sweetpotato CGS consisting of 480 accessions was established. This set contains a broad range of diversity for agronomical and resistance traits, nutritional quality and breeding including clones with high beta-carotene, starch, iron and zinc contents, nematode, virus and drought resistance. All clones of the CGS are available as pathogen-free in vitro plants ready for international distribution. A complete list of the CGS is available at http://gcpcr.grinfo.net/files/cr_files/gcpcr_file832.xls.

To increase the available gene sequence information for functional genomics approaches on sweetpotato, we have produced two normalized cDNA libraries, one derived from leaves and one from stems of the sweetpotato variety Tanzania (CIP accession no. 440166). To increase the representation of stress-related genes, we have submitted the plants to drought stress before sampling. Both cDNA libraries were submitted to 454 pyrosequencing. Pyrosequencing has become a popular method for high throughput sequencing, as it provides a huge amount of sequence information at relative low error frequency and cost. It is widely used for genome re-sequencing (Bentley 2006), de novo sequencing of small genomes (e.g. Thomson et al., 2008), SNP-detection (Bundock et al. 2009), or transcriptome sequencing (Vera et al., 2008).

In total, we obtained 87.307 raw reads comprising 21.292.096 bases with a 454 FLX quarter run of a normalized cDNA library of leaves and further 436.817 raw reads consisting of 136.844.411 bases from another quarter run of 454 FLX TITANIUM. The average length per read amounted to 213.9 bp for the 454 FLX run and to 313.3 for the 454 FLX TITANIUM run. Short reads (<40 bp) and low quality sequences were eliminated and the remaining 523.914 reads were assembled together with 22.094 ESTs from the Genebank. Assembly was optimized by variation of the assembly parameters, until obtaining an assembly with maximal representativeness and minimal redundancy (Table. 1).

Parameter combination	Very stringent	Stringent	Moderately stringent
Total sequences	77629	65685	60245
Contigs	37593	33079	31165
Singletons	40036	32606	29080
Redundacy between contigs (self-blast matches)	13339	8804	6983
Unique blastn hits in *Solanales* database	3801	4337	4463

Table. 1 Result of hybrid assemblies of ~500.000 454 raw reads with ~22.000 ESTs derived from the genebank at different stringency levels. Lowering the assembly stringency decreased the redundancy and increased the representativeness of the assembly. The assembly parameters at different stringency levels are given in materials and methods.

The final assembly was performed with moderately stringent parameters as suggested by the relatively low redundancy and good gene representation. The assembly and annotation strategy is shown in Fig. 1. From in total 546.013 reads, 424.833 were assembled to 33.165 contigs, while 121.180 sequences remained unassembled. 29 080 unassembled sequences that were larger than 100 bp were considered as singletons. The mean length of the contigs was 787.6 bp and 8011 contigs comprised more than 1000 bp. The average coverage per contig was 14-fold and for the 8011 contigs larger than 1000 bp the mean coverage was 29.6-fold.

The sequence assembly was annotated by blast-x comparison with proteins of the manually reviewed UNIPROT *A. thaliana* protein database. 24.994 contigs and singletons had significant matches to protein sequences of the database, including 14.145 unique hits, while 37.251 remained without significant match. In the next step we have tried to attribute gene ontologies (GO, The Gene Ontology Consortium 2008), and could attribute cellular compartment, biological processes and molecular function to about half of the sequences represented in the assembly. The full information of the assembly is available at http://gcpcr.grinfo.net/index.php?app=datasets&inc=dataset_details&dataset_ id=712.

The relative high number of un-annotated sequences might be due to different causes. First, some of the un-annotated sequences could represent new genes that were not yet identified in other species. Second, these sequences could represent non-protein coding RNAs and therefore we failed to find similar sequences in protein

databases. A part of the un-annotated singletons however could result from contamination of the cDNA with genomic or chloroplast DNA. This issue is currently under investigation. A high number of sequences without significant hit in databases was found also in other 454 transcriptome sequencing projects (e.g. Vera et al., 2008). In spite of the lack of annotation for a significant part of the gene index, the large number of new gene sequences and the high rate of unique genes will strongly facilitate functional genomics approaches in sweetpotato.

The resulting sequence information has been used to design primers for new microsatellite (SSR) markers. Those SSR markers that were successfully amplified and yielded polymorphic bands in a test panel of eight sweetpotato accessions can be found at http://gcpcr.grinfo.net/index.php?app=datasets&inc=dataset_details&dataset_id=712. High heterozygosity of sweetpotato allowed for the mining of the sequence assembly for SNPs, although the majority of the sequences are derived from one sweetpotato clone only. We have identified SNPs in 200 sequences that correspond to stress response genes. These SNPs can be used as gene-based markers to tackle stress gene alleles in germplasm screening and crossing efforts. The SNP data also are available at http://gcpcr.grinfo.net/files/cr_files/gcpcr_file867.xls. DArT markers as well as a diploid reference map for a near relative of sweetpotato, *I. trifida*, are under development and will be available in 2010.

Acknowledgement

The work was supported by the Generation Challenge Program (//www.generationcp.org).

References

Bentley D.R. 2006. Whole-genome re-sequencing. Current Opinion in Genetics & Development 2006, 16:545–552.

Bundock P.C., Eliott F.G., Ablett G., Benson A.D., Casu R.E., Aitken K.S., Henry R.J. 2009. Targeted single nucleotide polymorphism (SNP) discovery in a highly polyploidy plant species using 454 sequencing. Plant Biotechnol J. 7, 347-54.

Conesa A., Götz S., García-Gómez J.M., Terol J., Talón M., Robles M. 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21,3674-3676.

Shagin D.A., Rebrikov D.V., Kozhemyako V.B., Altshuler I.M., Shcheglov A.S., Zhulidov P.A., Bogdanova E.A., Staroverov D.B., Rasskazov V.A., Lukyanov S. 2002. A novel method for SNP detection using a new duplex-specific nuclease from crab hepatopancreas. Genome Res. 12,1935-1942.

The Gene Ontology Consortium. 2008. The Gene Ontology project in 2008. Nuc. acids res. 36, D440–4.

Thomson N.R., Holden M.T.G., Carder C., Lennard N., Lockey S.J., Marsh P., Skipp P., O'Connor C.D., Goodhead I., Norbertzcak H., Harris B., Ormond D., Rance R., Quail M.A., Parkhill J., Stephens R.S., Clarke I.N. *Chlamydia trachomatis*: Genome sequence analysis of lymphogranuloma venereum isolates. Genome Res. 2008. 18: 161-171.

Vera J.C., Wheat C.W., Fescemyer H.W., Frilander M.J., Crawford D.L., Hanski I., Marden J.H. 2008. Rapid transcriptome characterization for a nonmodel organism using 454 pyrosequencing. Mol. Ecol. 17, 1636–1647.

Wenzl, P., Carling J., Kudrna D., Jaccoud D., Huttner D., Kleinhofs A., Kilian A. 2004. Diversity arrays technology (DArT) for whole-genome profiling of barley. PNAS 101, 9915 9920.

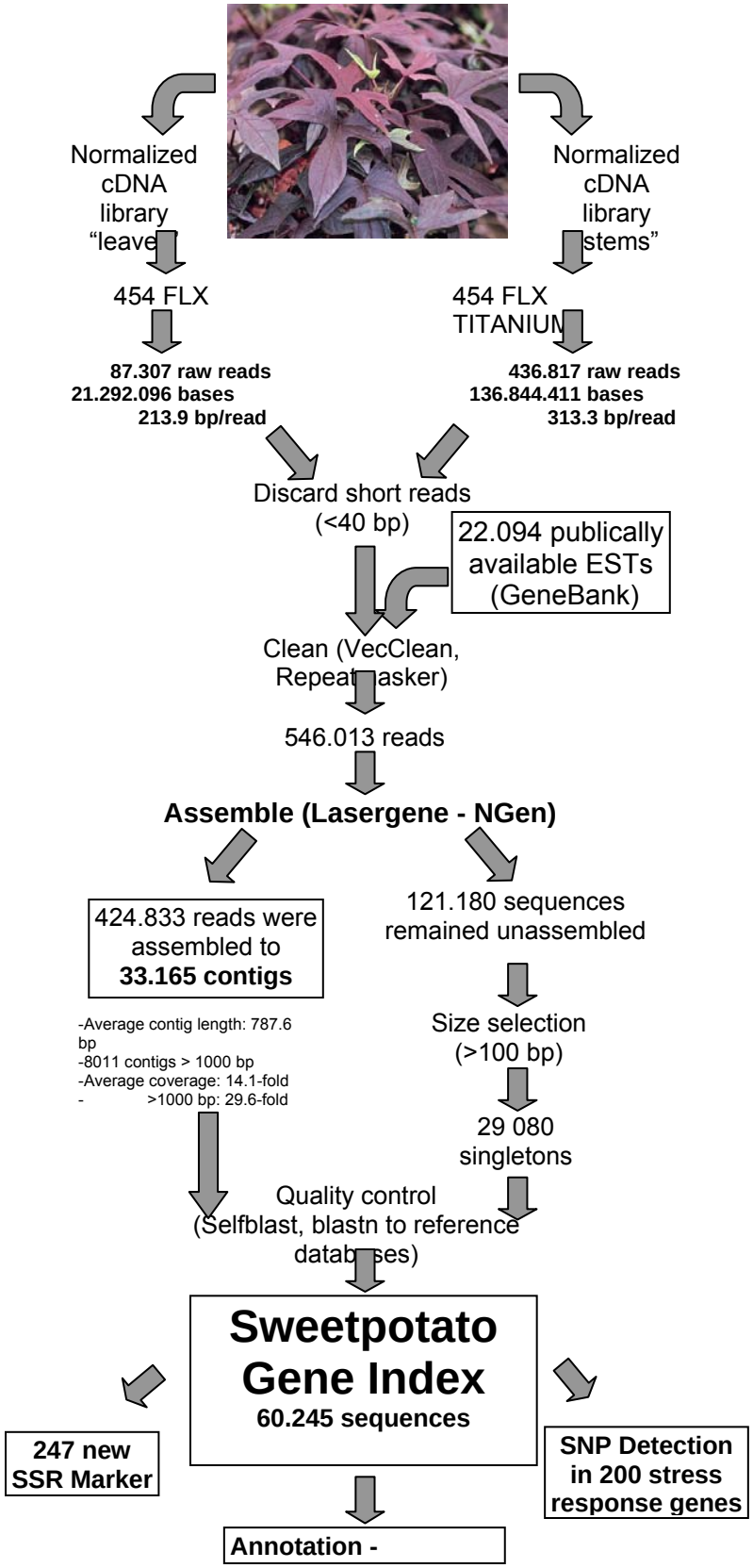

Fig.1: Sweetpotato transcriptome sequencing strategy. Details are given in the text.

Deep sequencing of plant small RNAs: a generic method for diagnosis, discovery and sequencing of viruses

Jan F. Kreuze[1], Ana Perez[2], Milton Untiveros[1], Dora Quispe[1], Segundo Fuentes[2], Giovanna Muller[2], Ratna Kumria[3], Reinhard Simon[1], Ian Barker[2], Claude Fauquet[3] and Wilmer J. Cuellar[2]

[1]Germplasm Enhancement and Crop Improvement Division, and [2]Integrated Crop Management Division, International Potato Center, Apartado 1558, Lima 12, Peru; [3] International Laboratory for Tropical Agricultural Biotechnology, Donald Danforth Plant Science Center, 975 North Warson Road, St. Louis, MO 63132.
Corresponding author: j.kreuze@cgiar.org

Abstract

Vegetative propagated crops are prone to the build up of virus infections and new viral diseases continue to appear. Early detection of the appearance of new viruses followed by rapid and accurate identification of these agents is essential if correct control measures are to be deployed. This is particularly true for entirely new diseases where novel control strategies may have to be developed alongside characterization of novel agents. Plants defend themselves against viruses by RNA silencing which involves the generation and use of small interfering RNA (siRNA): short RNA sequences of 20-25 nt derived from the viral genomic or sub-genomic RNA. We report the first identification of novel viruses from sweetpotato, and sequence of entire viral genomes, by a single step of high-throughput parallel sequencing of short RNAs from diseased, as well as symptomless plants. Contigs were assembled from sequenced total siRNA from plants using small sequence assembly software and could positively identify RNA, ssDNA and dsDNA reverse transcribing viruses, sometimes spanning the entire genome. The results present a novel approach which cannot only identify known viral pathogens, occurring at extremely low titers, but also novel viruses, without the necessity of any prior knowledge

Keywords: plant virus, deep-sequencing, diagnosis, siRNA, virus discovery.

Introduction

Crop losses due to emerging plant diseases, including those of viral origin, are of great current concern particularly in developing countries (Anderson et al., 2004). Strategies to combat plant disease outbreaks often involve early intervention either to stop diseases spreading or to prevent their introduction in the first place. Typically, the identification of a virus requires the application of a number of approaches including physical, biological, serological and molecular methods. Recently technologies such as diagnostic microarrays and mass spectrometry have been proposed as generic tools for identifying viruses (Mumford et al., 2006) although all require some prior knowledge of the agents to be identified. With the advent of next generation high-throughput parallel sequencing ("deep sequencing") platforms, the possibility of random metagenomic sequencing of diseased samples to identify putative pathogens has emerged (Quan et al., 2008). However, elimination of host nucleic acid in these systems is critical to boosting pathogen signals toward the detection threshold. We hypothesized that it would be possible to identify viruses based on the sequences of viral defense related molecules in plants. RNA silencing (RNAi) is a cytoplasmic cell surveillance system to recognize double stranded RNA and specifically destroys single and double stranded RNA molecules homologous to the inducer, using small interfering RNAs (siRNA) as a guide (Fire et al., 1998). Viruses are both inducers and targets of RNAi that constitutes a fundamental antiviral defence mechanism in eukaryotic organisms (Haasnoot et al., 2007). It is particularly important in plants (Pantaleo et al., 2007) that use RNAi to recover from virus disease (Covey et al., 1997). We describe the use of deep sequencing of siRNAs from plants to successfully identify the viruses infecting them, including previously unknown viruses, even in extremely low titre symptomless infections.

Materials and methods

Plant material and virus strains

The following plant materials were used in this study: Sweetpotato (*Ipomoea batatas*) landrace 'Huachano', infected with *Sweet potato feathery mottle* isolate Piu (SPFMV-Piu; Genus: *Potyvirus*, Family: *Potyviridae*), *Sweet*

potato chlorotic stunt isolate M2-47 (SPCSV-M2–47; Genus: *Crinivirus*; Family: *Closteroviridae*) and with both viruses simultaneously; potato (*Solanum tuberosum*) cv. Serrana infected with *Potato virus T* from Peru (PVT-Pe; Family: *Flexiviridae*); cassava (*Manihot esculenta*) infected with *Cassava brown streak virus* isolate Ug (CBSV-Ug; Genus: *Ipomovirus*, Family: *Potyviridae*); *Nicotiana benthamiana* infected with SB29 (a novel suspected viral pathogen of potato); healthy *Physalis floridana.* All these materials were maintained in an insect-proof greenhouse at CIP, Lima, Peru, except CBSV-Ug infected cassava, which was maintained in a growth chamber at the Donald Danforth Plant Science Center, Missouri, USA.

Nucleic acid extraction and sequencing

Total RNA was isolated from 3 g of fresh leaf material using Trizol (Invitrogen, CA, USA) following the manufacturer's instructions. Lyophilized RNA was sent to Fasteris Life Sciences SA (Plan-les-Ouates, Switzerland) for processing and sequencing on the Illumina Genome Analyzer. DNA was extracted using the CTAB method. PCR amplification of virus specific fragments was performed using Taq DNA polymerase (Promega) according to the manufacturer's recommendations together with virus specific primers. Sequencing of PCR amplified fragments using the Sanger method was performed by Macrogen (Seoul, Korea).

Sequence analysis

For siRNA sequence assembly three different short read assemblers were tested: SSAKE v3.2 (Warren et al., 2007), VCAKE v2.0 (Jeck et al., 2007) and Velvet v0.6.04 (Zerbino and Birney, 2008). Different, overlapping, contigs were produced depending on the program used and the parameters set, and they could be further assembled into greater contigs using the program ContigExpress included in the Vector NTI package (Invitrogen, Carlsbad, CA). Assembled contigs were used to search the GenBank/EMBL/DDBJ database using BLASTn (nucleotide blast) or BLASTx (translated nucleotide blast). Primers (data not shown) were designed for amplification and Sanger sequencing based on the identified viral contigs using Vector NTI (Invitrogen). Guide strand mediated assembly was performed using the program MAQ (http://maq.sourceforge.net). Coverage and distribution of virus specific contigs by siRNAs were also determined using MAQ under default parameters, and results were exported to Microsoft Excel for further analysis.

Results and discusion

Sweetpotato viruses

In a first experimental setup we isolated and sequenced sRNAs from single (SPCSV or SPFMV) and double (SPFMV and SPCSV) infected sweetpotato plants using the Illumina deep sequencing platform (Kreuze et al., 2009). Between 1 and 1.2 million sRNA (1–28 nt) reads were obtained from each different sample. The far majority of sequences (>95%) were between 21 and 24 nt in size.

The obtained sRNA sequences could be assembled into contigs of up to more than 1000 nts using any of the three tested short sequence assembly programs. Among the programs tested Velvet was the fastest and generally more accurate than SSAKE or VCAKE. Velvet worked best with the hash length set between 13 and 17, whereas the ideal coverage cut-off parameter varied considerably depending on the hash length used. Searches of nucleotide and protein databases using Blast with the assembled contigs and their corresponding translated peptides successfully identified the expected viruses in each plant, and, surprisingly, also identified several contigs with similarity to badnaviruses (family *Caulimoviridae*; dsDNA reverse transcribing viruses) and mastreviruses (family *Geminiviridae*; ssDNA viruses)(Table 1).

Table 1. Number of contigs assembled by Velvet using 21–24 nt sRNA, with virus specific hits as identified using Translated Nucleotide Blast (Blastx) and % coverage and average depth of viral genome sequenced

Plant infected with	siRNAs sequenced (21-24nt)	Contigs identified	Contigs with Blastx hits All sequences k=15, cov=3	Coverage of complete genome and average sequencing depth
Sweetpotato SPFMV	1'072'019	Total contigs	1633	
		SPFMV	71	93%/93x
		SPCSV	0	-
		Badnavirus	62	A:92% /79x B: 99%/107x
		Mastrevirus	6	95%/73x
Sweetpotato SPCSV	1'169'787	Total contigs	1675	
		SPFMV	0	-
		SPCSV	64	92%/16x
		Badnavirus	63	A: 92%/90x B: 99%/135x
		Mastrevirus	10	95%/113x
Sweetpotato SPFMV + SPCSV	984'490	Total contigs	1363	
		SPFMV	43	100% / 470x
		SPCSV	41	86% /13x
		Badnavirus	63	A[1]: 92%/130x B: 99%/165x
		Mastrevirus	8	91% /123x
Cassava CBSV	879'337	Total contigs	760	
		CBSV	60	97% / 153x
		Begomovirus	55	SLCMV:93%/20x ClCuRaV: 91%/57x
		Beta satellite	4	89%/18x
		Alpha satellite	1	ND[2]
Potato PVT	1'591'500	Total contigs	2276	
		PVT	56	98.59% / 32x
		Cavemovirus	16	ND
Nicotiana benthamiana SB-29	784'718	Total contigs	276	
		Torradovirus	2	ND
Physalis floridiana Healthy	757'310	Total contigs	635	
		Cavemovirus	4	ND

[1] Two different viruses identified; [2] ND: not done

Moreover, contigs of SPFMV generated in dually infected plants were found to span the entire genome and could be further assembled to generate the complete genomic sequence at a sequencing depth of 470x (Table 1). The accuracy was confirmed by Sanger sequencing and found to concur to 99.8%. Although the contigs produced for SPCSV were too few and short to be able to assemble it's entire genome de-novo, guide strand aided assembly using the published SPCSV-Ug sequence and the MAQ software was able to assemble up to 92% of the SPCSV genome at an average depth of 16x (Table 1). Further investigation of the badna- and mastrevirus specific contigs revealed that they corresponded to at least two distinct badnaviruses and one mastrevirus and covered more than 50% of their respective genomes. Guide strand aided assembly using MAQ was not able to further assemble the genomes of these viruses because they were too different from any known

virus sequences. Therefore primers were designed based on the available contigs, which successfully amplified fragments of the expected sizes filling the gaps between the contigs found by siRNA assembly. Thus, the complete genomes of both Badnaviruses and the mastrevirus were determined. Subsequent analysis using MAQ showed that >90 % of the genomes of each of the new viruses was covered by siRNAs (Table 1). The significance of these new, apparently symptomless, viruses is currently under investigation. It is noteworthy however that the amount of siRNAs corresponding to these viruses increase in SPCSV infected plants and even more so in SPVD affected plants (Table 1), suggesting that they may have a role in the aetiology of both diseases.

Viruses of other plants

To validate our method in other plant species, deep sequencing of sRNA samples from PVT infected potato, *N. benthamiana* infected with the previously un-characterized potato virus SB29*,* CBSV infected cassava and healthy *Physalis floridiana* (as a negative control) was performed. As expected PVT and CBSV could be positively identified in infected potato and cassava plants respectively. As with SPCSV and SPFMV in sweetpotato, almost the entire genomes of both PVT and CBSV could be assembled using a combination of MAQ and Velvet (Table 1). The assembled PVT sequence showed 98% nucleotide identity with the one reported in database (NC011062). The CBSV isolate sequenced in this study however showed only about 85% nt identity with the Tanzanian highland strain MBL3 (NC012698), which was confirmed by Sanger sequencing.

Only two virus specific contigs could be identified from SB29 infected *N.benthamiana* samples and they matched to two regions separated by ~1.7 Kbp in the RNA2 of *Tomato torrado virus* (ToTV; EU563947). PCR primers designed from these two contigs amplified a product of the expected size and Sanger sequencing of this PCR product revealed a region corresponding to Vp35 (one out of 3 CP in ToTV) which had a 33-35% amino acid identity with the corresponding region in other torradoviruses reported in database. This result suggests SB29 represents a new virus that is most closely related to, but still significantly different from torradoviruses. Low sequence similarity to any known viruses is probably the reason only two torradovirus specific contigs were identified in SB29 infected *N. benthamiana* and illustrates both the potential and the limitations of this technology.

Interestingly, as in the case of sweetpotato, in all samples additional contigs with similarity to plant viruses were also identified. CBSV infected cassava samples yielded siRNA contigs with similarity to Begomoviruses and beta satellite virus (Table 1). On closer inspection these appeared to be homologous to *Sri-Lankan cassava mosaic virus* (SLCMV DNA-A and –B; Genus: *Begomovirus*, Family *Geminiviridae*), *Cotton leaf curl Rajastan virus* (ClCuRaV DNA-A; Genus: *Begomovirus*, Family *Geminiviridae*) and Cotton leaf curl beta satellite, and could be assembled to ~90% of the genome using MAQ (Table 1). siRNAs corresponding to these viruses were however very few as compared to CBSV and their presence has yet to be confirmed by independent methods.

Contigs with similarity to cavemovirus (Family: *Caulimoviridae*) sequences were recovered from both PVT infected potatoes and "healthy" *P. floridiana* samples. PCR primers designed from the contigs in *P. floridiana* to amplify the whole genome gave PCR products of expected size. Additional PCR products were also obtained, and Sanger sequencing showed that all amplified products corresponded to cavemovirus-like sequences. No follow up was made with the cavemovirus like sequences identified in potato yet. Because cavemoviruses are known to integrate into their host genomes, it will be an interesting question to determine whether these sequences correspond to ancient, non-active integrated viruses, or to infectious symptomless viruses in these plants, and how these two alternatives could be distinguished using this technology.

Distribution of siRNAs over viral genomes

The quantity of virus specific RNAs among the different size classes of sequenced RNA was determined and are shown in Fig 1. The major proportion of virus specific siRNAs were found in the 21 or 22nt class, regardless of the host plant or type of virus (DNA or RNA). A difference could however be observed between plant species, in that sweetpotato had the majority of virus specific siRNAs of the 22nt size class, and potato in the 21nt size class, whereas the situation was ambiguous for cassava. More data would be required to determine if this is due to differences in the host plants or the viruses that are targeted. Nevertheless because virus specific siRNAs represent a higher proportion of the 21 and 22nt siRNAs, sequencing only this size class would reduce the number of sequences required to identify a new virus and thus increase sensitivity of the method. Indeed analysis using the sweetpotato siRNA sequences indicated that similar results could be obtained if using only the 22nt siRNAs (Kreuze et al., 2009). Furthermore simulations using random subsets of sequences indicated that as

few as few as 30,000 total siRNA sequences were enough to assemble at least one contig recognizable as SPCSV, the virus for which the fewest siRNA sequences were found in sweetpotato.

Analysis of the distribution of siRNAs over the viral genomes revealed that they are not homogenously distributed, but concentrated in certain regions (Fig 2). This effect was highly reproducible between samples and is most likely due to a bias produced in the sample preparation technique (Linsen et al., 2009). Therefore the use of improved sample preparation methods that are currently available, and are less biased may improve the coverage and thus the ability to produce longer contigs with fewer sequences.

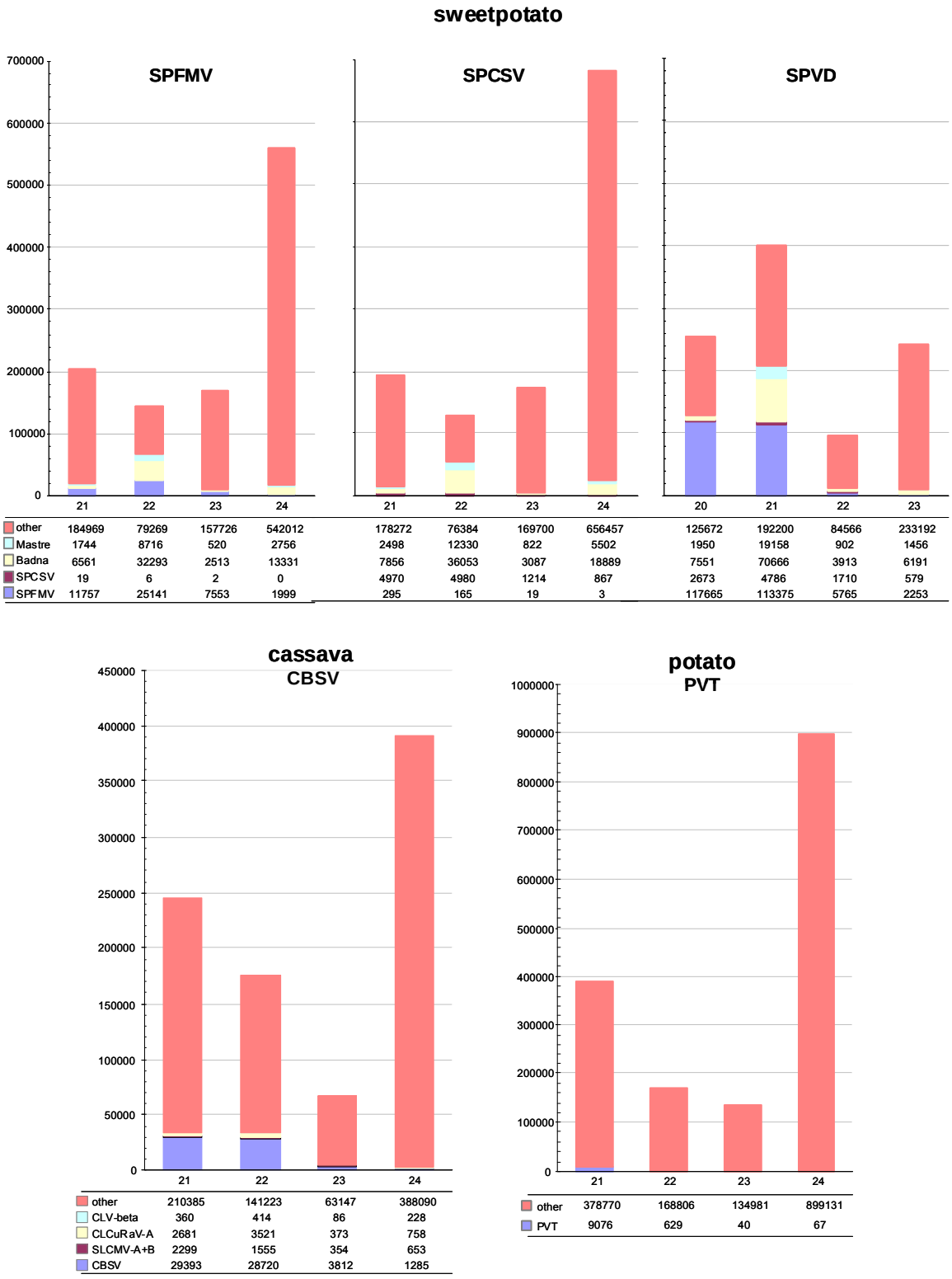

Figure 1 Bare graph showing the frequency of 21-24nt siRNAs corresponding to different viruses as compared to the total number of siRNAs sequenced in virus infected sweetpotato, potato and cassava.

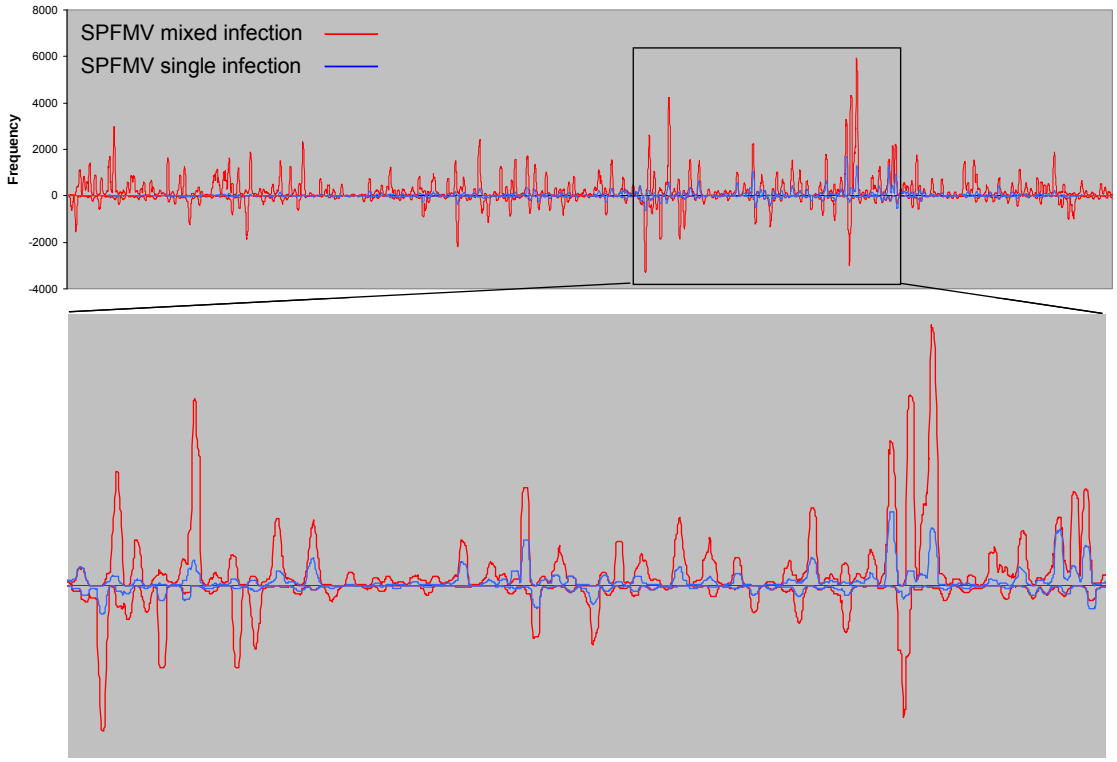

Figure 2. Graphic showing the sequence coverage of the SPFMV genome by siRNAs in single and mixed infected plants. Strong and consistent preference for certain regions over others can be observed.

Conclusions

This methodology, as applied in plants, was thus able to detect both RNA and DNA viruses, from widely different families and with different tissue trophisms and intracellular replication sites, even in extremely low titre and seemingly symptomless infections. It offers an entirely generic, specific and apparently sensitive approach to identify plant viruses, as compared to other techniques, which are all in some way limited to a subset of viruses that can be identified or require additional confirmatory steps for virus identification. The fact that similar results could be obtained with viruses from different families and in diverse plants suggests it may be universally applicable. The apparent sensitivity combined with increased throughput obtained by massive parallel sequencers may eventually lead to the technique becoming widely applicable. On the other hand the frequent identification of unexpected viral sequences even in seemingly healthy plants suggests that apparently symptomless viral infections are more common than previously thought, and poses the problem of what significance should be assigned to them.

References

Anderson, P.K.; Cunningham, A.A.; Patel, N.G.; Morales, F.J.; Epstein, P.R.; Daszak, P. 2004. Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. Trends Ecol. Evol. 10, 535–544.

Covey, S.N.; Al-Kaff, N.S.; Langara, A.; Turner, D.S. 1997. Plants combat infection by gene silencing. Nature 285, 781-782.

Fire, A.; Xu, S.Q.; Montgomery, M.K.; Kostas, S.A.; Driver, S.E.; Mello, C.C. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806-811.

Haasnoot, J.; Westerhout, E.M.; Berkhout, B. 2007. RNA interference against viruses: strike and counterstrike. Nature Biotech. 12, 1435-1443.

Jeck, W.R.; Reinhardt, J.A.; Baltrus, D.A.; Hickenbotham, M.T.; Magrini, V.; Mardis, E.R.; Dangl, J.L.; Jones, C.D. 2007. Extending assembly of short DNA sequences to handle error. Bioinformatics 23, 2942–2944.

Kreuze, J.F.; Perez, A.; Untiveros, M.; Quispe, D.; Fuentes, S.; Barker, I.; Simon, R. 2009 Complete viral genome sequence and discovery of novel viruses by deep sequencing of small RNAs: a generic method for diagnosis, discovery and sequencing of viruses. Virology 388, 1-7.

Linsen, S.E.V.; de Wit, E.; Janssens, G.; Heater, S.; Chapman, L.; Parkin, R.K.; Fritz, B.; Wyman, S.K.; de Bruijn, E.; Voest, E.E.; Kuersten, S.; Tewari, M.; Cuppen, E. 2009. Limitations and possibilities of small RNA digital gene expression profiling. Nature Meth. 6, 475-476.

Mumford, R.; Boonham, N.; Tomlinson, J.; Barker, I. 2006. Advances in molecular phytodiagnostics - new solutions for old problems. European J. Plant Pathol. 116, 1–19.

Quan, P.; Briese, T.; Palacios, G.; Lipkin, W.I. 2008. Rapid sequence-based diagnosis of viral infection. Antiviral Res. 79, 1–5.

Pantaleo, V.; Szittya, G.; Burgyan, J. 2007. Molecular bases of viral RNA targeting by viral small interfering RNA-programmed RISC. J. Virol. 81, 3797-3806.

Warren, R.L.; Sutton, G.G.; Jones, S.J.M.; Holt, R.A. 2007. Assembling millions of short DNA sequences using SSAKE. Bioinformatics 4, 500–501.

Zerbino, D.R.; Birney, E. 2008. Velvet: Algorithms for de novo short read assembly using de Bruijn graphs. Genome Res. 18, 821-829

Genetic transformation of potato cultivars using *R* genes to increase resistance to late blight of potato caused by *Phytophthora infestans*

Rivera C., Tovar J.C., Roman, M., Orbegozo J., Serna-Chumbes F., Manrique-Trujillo S., Núñez J., Trujillo-Luján G, Medrano-Gallardo G, Perez W, Kreuze J., Forbes G, Ghislain M.

Applied Biotechnology Laboratory, International Potato Center, P.O. Box 1558, Lima 12, Peru; m.ghislain@cgiar.org

Abstract

Several resistance (*R*) genes have recently been isolated and used in a transgenic approach. The *RB* gene of *Solanum bulbocastanum* confers partial resistance to many isolates of *Phytophthora infestans*, the causal agent of late blight (LB) disease in potato. We have selected three recipient varieties: (1) Desiree, which is easy to transform and has wide adaptability; (2) Granola which is the principal fresh market variety in Indonesia; and (3) Victoria (Asante) which is a well adopted variety in Uganda (and Kenya). Genetic transformation of Desiree was done with 2 batches of events. The first one resulted in 14 putatively transformed plants regenerated from wounded leaves and internodes. Out of these, 10 transgenic events have been selected as being independent events and 7 of them devoid of non-TDNA backbone vector sequences. These will be field trialed to determine their level of resistance to LB and other agronomical characteristics. The second batch resulted in 41 putative transformed plants. These plants are currently undergoing molecular characterization. With the variety Granola, 15 putative transformed events with the *RB* gene construct were produced. This variety displays much lower regeneration efficiency in addition to rapid oxidation of explants. Finally, the variety Victoria is being tested for regeneration efficiency and susceptibility to kanamycin and carbenicillin to select for transformed events while eliminating *Agrobacterium* cells. Two additional *R* genes, *Rpi-blb2* and *Rpi-vnt1.1* from *S. bulbocastanum* and *S. venturii* respectively, are now used in combination with *RB* gene in order to increase durability of resistance to late blight.

Keywords: Potato, late blight resistance, genetic engineering.

Introduction

Late blight caused by the oomycete *Phytophthora infestans* is the most devastating diseases of potato worldwide, causing an average production loss of 15% in developing countries which means approximately $2.75 billion loss as estimated by the International Potato Center (Anonymous, 1997). Since the middle of the 19th century when this pathogen turned out to be responsible of a major food crisis in Europe (the "Irish potato famine"), active efforts by plant breeders to control this disease have been carried out.

In the early days of breeding for LB resistance, eleven single resistance (*R*) genes from *Solanum demissum*, a wild potato species indigenous to México, were introgressed into modern potato varieties (Malcolmson and Black, 1966, Gebhardt and Valkonen, 2001). Even though *S. demissum* is highly resistant to late blight, their *R* genes only conferred race-specific resistance when transferred into potato cultivars. This resistance was rapidly overcome by new isolates (Wastie, 1991).

Other *R* genes were recently identified and isolated from *Solanum* species with resistance to a broad spectrum of isolates of *P. infestans*. Taking advantage of genetic engineering, a new strategy under development consists of transferring these *R* genes either as single events or (preferably) stacked into potato varieties and deploying these in such a way that the pathogen will not be able to overcome this multiple *R* gene mediated resistance. We report here our progress made in transferring the *RB, Rpi-blb2* (*Solanum bulbocastanum*) and *Rpi-vnt1.1* (*S. venturii*) in three potato varieties.

Materials and methods

Gene constructs

The plasmid pCLD04541 containing the 8,569 bp fragment corresponding to the *RB* gene was sent to the Applied Biotechnology Lab of CIP (ABL-CIP) from Dr. Jiang's lab at University of Wisconsin (Song et al., 2003). The *Rpi-vnt1.1* gene was obtained from the Sainsbury Lab (UK) but due to delays it was ultimately obtained from chemical synthesis, as well as the *Rpi-blb2* gene.

Plant material and genetic transformation

Solanum tuberosum varieties Desiree and Granola (CIP800048 and CIP800959, respectively) were obtained from CIP's genebank and propagated in vitro as published previously (Cuellar *et al.*, 2006). We used the strains EHA105 of *Agrobacterium tumefaciens* to transfer these R genes into the potato varieties using petioles and internodes as explants. Standard regeneration and transformation protocol were used (Cuellar *et al.*, 2006).

Analysis of transgenic plants

PCR analysis was done with genomic DNA of putative transformed plants using specific-primers to the *RB* and *npt*II genes for a rapid detection of transformants. The presence of the *RB* and the *nptII* genes were confirmed by PCR using specific primers. The extent of non-T-DNA sequence insertion was monitored by PCR using primer from three regions extending from the left border (LB) to the right border (RB) of the T-DNA. Southern blot hybridizations were performed using as probe a 597 bp DNA fragment of the *nptII* gene and genomic DNA digested with *Eco*RI.

Phytophthora infestans assays

Detached leaf and whole plants assays were performed with a complex isolate POX 067 which is virulent on the following *S. demissum* R genes: R1, R2, R3, R4, R5, R6, R7, R10, and R11). The isolate was inoculated on the transgenic events and six potato genotypes with known level of resistance using standard conditions developed at the CIP pathology lab.

Results and discusion

Development of R gene constructs

The *RB* gene from *S. bulbocastanum* is available in a single gene construct (pCIP68 and pCIP56 with and without the *nptII* kanamycin selectable marker gene, respectively). The *Rpi-vnt1.1 and Rpi-blb2* obtained through chemical synthesis were moved into a single R gene constructs (pCIP93 with *Rpi-vnt1.1* + *nptII*; pCIP95 with *Rpi-blb2* + *hptII* hygromycin selectable marker gene). We have also developed one double R gene construct (pCIP96 with *Rpi-blb2* + *Rpi-vnt1.1* + *nptII*). We plan to develop additional gene constructs (1) *Rpi-vnt1.1* + *Rpi-blb2* + *hptII* to stack them in *RB* events (kmR), using selection on hygromycin; a triple gene construct (*RB* + *Rpi-vnt1.1* + *Rpi-blb2* + *nptII*). A marker-free triple R gene construct will also be developed to adopt a cisgenic strategy when a good marker-free transformation protocol will be available at CIP.

Potato genetic transformation

Genetic transformation with the gene construct pCIP68 was carried out using 120 explants of the potato cultivar Desiree which produced 14 regenerants. Out of these, 11 plants have been characterized by PCR using primers for the TDNA (*RB* and *nptII* genes), and non-TDNA (backbone vector sequences, three pairs of primers) (Table 1). Two plants (50 and 62) were shown to have non-TDNA sequence and two others (47 and 83) were shown to be negative for all PCR primers (regenerants that are resistant to kanamycin but not transformed = "escapes"). Hence, 7.5% transformation efficiency was achieved with the gene construct pCIP68 in Desiree. Ten of the 11 putative transformed plants were characterised by Southern blotting (SB) using the *nptII* gene as probe (*RB* is homologous to many *R* genes present in the potato). One plant (67) has not yet been assessed by SB due to delays in plant propagation (in progress). The SB revealed that the 8 transgenic plants are independent events and that 4 events (9.1, 43, 53, 66) have one copy of the TDNA, 3 events (27, 50, 62) have two copies, and 1 event (28) has three copies.

Table 1. Molecular characterization of 11 independent events from the potato variety Desiree transformed with the RB gene

Event #	T-DNA	non-T-DNA	Copy number
Desiree [RB] 9.1	+	-	**1**
Desiree [RB] 27	+	-	**2**
Desiree [RB] 28	+	-	**3**
Desiree [RB] 43	+	-	**1**
Desiree [RB] 47	-	-	**none**
Desiree [RB] 50	+	+	**2**
Desiree [RB] 53	+	-	**1**
Desiree [RB] 62	+	+	**2**
Desiree [RB] 66	+	-	**1**
Desiree [RB] 67	+	-	**n.d.**
Desiree [RB] 83	-	-	**none**

Additional transformed events have been obtained. A second genetic transformation event with the pCIP68 gene construct was carried out in the variety Desiree. Three hundred and sixty explants (180 leaves with petioles and 180 internodes) were transformed which produced 41 regenerants from selection on kanamycin. For the variety Granola, we obtained 15 regenerants resistant to kanamycin from 630 explants from two events of transformation (210 leaves, 210 leaves with petioles and 210 internodes). More recently, the variety Victoria (Asante) has been tested for its ability to be genetically transformed through agroinfection. The plant propagation and regeneration *in vitro* appears to be very similar to Desiree. Preliminary result indicated that 50mg/l kanamycin and 250 mg/l carbenicillin are more appropriate to use in the first genetic transformation event.

Late blight resistance assay

A bio-assay using detached leaves to assess activity against *Phytophthora infestans* revealed a high level of resistance (hypersensitive reaction) for 2 of the 9 plants tested (7 transgenic events and 2 regenerants). The results were confirmed in whole plants assays (Table 2). Curiously, these 2 events (50 and 62) have non-TDNA sequence which makes them unsuitable for future product development. The other plants, including the untreated control Desiree, presented disease symptoms at 7 days post infection. The 9 transgenic events from Desiree will be characterized for transcriptional activity by real-time PCR under no infection and 5 days after inoculation and with the pathogen.

Table 2. Bio-assay using detached leaves of 9 transgenic events and 6 genotypes with known resistance levels against the isolate POX 067 of *Phytophthora infestans*

Event/Genotype	Severity (%)		
	Detached leaf		Whole plant
	Rep. 1	Rep.2	Rep. 1
Desiree[RB] 9.1	100	100	100
Desiree[RB] 27	100	100	100
Desiree[RB] 28	100	100	100
Desiree[RB] 43	100	100	100
Desiree[RB] 50	HR*	HR	HR
Desiree[RB] 53	100	100	100
Desiree[RB] 62	HR	HR	HR
Desiree[RB] 66	100	100	100
Desiree[RB] 67	100	100	100
Atzimba	100	100	100
Desiree	100	100	100
LBr 40	0	0	0
Monserrate	100	100	100
Pimpernell	100	100	100
Tomasa condemayta	100	100	100

*Hypersensitive reaction (rapid cell death

Acknowledgement

The authors are grateful to continuous financial support from USAID.

References

Anonymous (1997). "CIP in 1996. The International Potato Center Annual Report.", International Potato Center, Lima, Peru. 59pp.

Cuellar W, Gaudin A, Solórzano D, Casas A, Ñopo L, Chudalayandi P, Medrano G, Kreuze J, Ghislain M (2006). Self-excision of the antibiotic resistance gene *nptII* using a heat inducible *Cre-loxP* system from transgenic potato. Plant Molecular Biology 62: 71-82

Gebhardt C, Valkonen JPT (2001) Organization of genes controlling disease resistance in the potato genome. Annu. Rev. Phytopathol. 39, 79-102.

Malcolmson JF, Black W (1966) New R genes in *Solanum demissum* Lindl. and their complementary races of Phytophthora infestans (Mont.) de Bary. Euphytica 15: 199–203.

Song J, Bradeen JM, Naess SK, Raasch JA, Wielgus SW, Haberlach GT, Liu J, Kuang H, Austin-Phillips S, Buell CR, Helgeson JP, Jiang J (2003) Gene RB cloned from *Solanum bulbocastanum* confers broad spectrum resistance to potato late blight. Proc Natl Acad Sci USA 100:9128–9133.

Wastie RL (1991) in *Phytophthora infestans*: The Cause of Late Blight of Potato, Advances in Plant Pathology, eds. Ingram, D. S. & Williams, P. H. (Academic, London), Vol. 7, pp. 193–223.

Transfer of sweetpotato-like genes expressing Cry proteins into sweetpotato varieties

J.C. Tovar[1*], K. Prentice[1], M. Ormachea[1], N.L. Wamalwa[1,4], R.O.M. Mwanga[1,3], W.J. Moar[2], J. Machuka[4], **M. Ghislain[1]**

[1] Applied Biotechnology Laboratory, International Potato Center, P.O. Box 1558, Lima 12, Peru; [2] College of Agriculture, Auburn University, USA; [3] National Crop Resources Research Institute, Kampala, Uganda, [4] Kenyatta University, Nairobi, Kenya; *j.tovar@cgiar.org

Abstract

Seven proteins from *Bacillus thuringiensis* (*Bt*) were previously tested for activity against the two African weevil species, *Cylas puncticollis* and *C. brunneus*. Three of them (Cry7Aa1, CryET33/CryET34, Cry3Ca1) were effective against both African weevils with an LC_{50} below 1 ppm. Therefore, *cry7Aa1*, *cry3Ca1*, and *cryET33/cryET34* gene constructs were developed taking into account sweetpotato optimized codon usage, promoters with storage root expression and wound response, and two selectable marker genes. Combinations of two weevil resistant (WR) genes were also included as an insect-resistance management component. A total of 10 WR gene constructs have been developed and 5 of them are currently used for direct gene transfer into relevant African sweetpotato varieties. Regeneration and genetic transformation protocols were applied to two African cultivars ("Tanzania" and "Wagabolige") selected as the most prominent cultivars in Uganda and neighboring countries. After almost two years of testing different regeneration and transformation protocols for the African varieties, scientists at CIP have obtained only one transformed shoot (from "Wagabolige") with one of the single-WR gene constructs. Non-African varieties "Jewel" and "Huachano" were genetically transformed with three single-WR and two double-WR gene constructs producing 18 and 12 putative transformed regenerants respectively. These plants, when confirmed to be genetically transformed with the WR gene constructs, will be used to produce leaves and storage roots in the biosafety greenhouse to be forwarded to the US and African partners for testing activity against weevils.

Keywords: Sweetpotato, weevil, *Bt*, genetic transformation.

Introduction

Sweetpotato (*Ipomoea batatas*) is an important crop in sub-Saharan Africa (SSA), especially during seasons when other staples are scarce. The African weevils *Cylas puncticollis* and *C. brunneus* are the main biological constraints, accounting to losses between 50 and 100%. Sweetpotato-weevil larvae attack the tuberous root, causing the most economic damage, even during storage, which is important for marketability and in dry seasons. Piecemeal harvesting is commonly practiced in SSA, which in turn favors weevil infestation, due to the exposure of the plants in the field for long periods (Stathers *et al.*, 2005).

Resistance to sweetpotato African weevils has not been achieved through conventional breeding due to the lack of the necessary genes in the sweetpotato gene pool. A potential source of resistance that could be used in conventional breeding is found in the latex (Stevenson *et al.*, 2009), however, varieties with this resistance have not been adopted widely in SSA. A biotechnological approach to attain this resistance could be used.

Bacillus thuringiensis (*Bt*) toxin-encoding genes have been used extensively in modern agriculture, through modification of the genome (James, 2008). *Bt* toxins active against weevils have been reported (Bravo *et al.*, 1998; Donovan *et al.*, 1992; Génissel *et al.*, 2003; Guzov *et al.*, 2007; Höfte *et al.*, 1987; Lambert *et al.*, 1992; Mettus & Baum, 2006; Peferoen *et al.*, 1998; Sekar *et al.*, 1987), some of which are being used commercially (Agbios, 2009). Based on the evidence of successful application of *Bt* technology to control insect pests, we have devised a strategy to introduce *Bt* genes into sweetpotato.

Previous experiments developed essentially at NARO in Uganda under the direction of Prof. Moar revealed that seven *Bt* toxins (CryET70, CryET33/CryET34, Cry3Aa3, Cry3Ba2, Cry3Bb3, Cry3Ca1 and Cry7Aa1) displayed activity against the two African sweetpotato weevil species. Three proteins had a LC_{50} below 1 ppm: CryET33/CryET34,

Cry3Ca1, Cry7Aa1 which made these good candidates for engineering resistance to weevils. We report here the progress in transferring the corresponding genes into sweetpotato.

Materials and methods

Transformation plasmids

All three *Bt*-gene coding sequences were optimized for sweetpotato codon usage and synthesized. The sporamin promoter and 3' regions were attached to both *cryET33/cryET34* and *cry3Ca1* coding sequences. The gene *cry7Aa1* was attached to the ß-amylase promoter and 3' region. All genes were inserted into pCAMBIA1305.1 (CAMBIA, Canberra, Australia) or into pCIP100, a modification of pCAMBIA1305.1 conferring kanamycin resistance to plants. Six binary vectors bearing single Bt genes were developed, three on pCAMBIA1305.1 and three on pCIP100. Another four binary vectors were constructed bearing either the *cry7Aa1* and the *cryET33/cryET34* genes or *cry7Aa1* and *cry3Ca1*, two of these vectors on pCAMBIA1305.1 and two on pCIP100.

Plant transformation

Sweetpotato varieties Tanzania, Jewel, Wagabolige and Huachano were obtained from CIP's germplasm bank. The genetic transformation method used was that described in Medina-Bolivar *et al*. (2003). Regeneration was performed using a two-step protocol described in Ormachea (2008) for Wagabolige and Tanzania, Cruzado (2009) for Huachano and Luo *et al*. (2006) for Jewel.

Molecular analysis

Shoots were screened by callus testing, as described in Cruzado (2009), PCR amplification of the transgene, Southern blotting for transgene copy number and NCM-ELISA detection of the expressed protein. Polyclonal antibodies for NCM-ELISA detection were produced in rabbits by inoculating the purified *Bt* protein and collecting sera after 2 weeks. Transgene expression was quantified through real-time PCR based on SYBER Green I methodology. The results obtained were carried out using the software REST©. The housekeeping *cox* gene was used as reference of analysis.

Tuberous root and leaf production

Shoots confirmed to be positive were grown in the Biosafety greenhouse for tuberous root production. Plantlets were transferred from *in vitro* conditions directly into soil pellets. After one week they were planted on plastic pots and remained there until tuberous root formation.

Results and discusion

Transformed plants

Genetic transformation of the African variety Tanzania yielded no shoots, even after transforming more than 10,000 explants. Using approximately the same amount fo explants, we obtained one regenerating shoot for Wagabolige. This shoot has been confirmed to bear the transgenes and seems to have three copies of the *cry7Aa1* gene and two copies of the Kanamycin resistance *nptII* gene. This could be due to incomplete transfer of the T-DNA in one of the insertions.

Five Huachano shoots were obtained bearing the double gene construct *cry7Aa1* and *cryET33/cryET34*. These shoots were positive by callus and PCR screening.

Jewel transformation yielded one shoot bearing the *cryET33/cryET34* gene, 8 shoots with the *cry7Aa1* gene and 9 shoots with the *cry3Ca1* gene, 2 shoots bearing the double gene construct *cry7Aa1* and *cryET33/cryET34*, and 6 shoots with the double gene construct *cry7Aa1* and *cry3Ca1*. All shoots were positive by PCR and callus

testing. NCM-ELISA was assayed for 6 different shoots bearing the *cry7Aa1* gene and 4 bearing the *cry3Ca1* gene. All shoots were positive by NCM-ELISA.

Transgene expression

Five transformed events bearing the *cry7Aa1* gene were analyzed for transgene expression in the leaf. The event with the lowest expression (LE) is used as reference. Two events were found to have statistically significantly higher expression than the LE reference with expression levels of 10.01 and 1.74 times higher (Figure 1), whereas the other two events did not have significantly higher expression levels.

The transgene expression ratios in four plants were not higher in comparison with the housekeeping *cox* gene. Because the promoters used to guide gene expression were taken from the genes of the two major proteins in the tuberous root (Hattori & Nakamura, 1988; Nakamura *et al.*, 1991), it is expected that gene expression in the tuberous roots will be higher. This will allow us to localize the expression, a strategy used for insect resistance management (Ferré *et al.*, 2005).

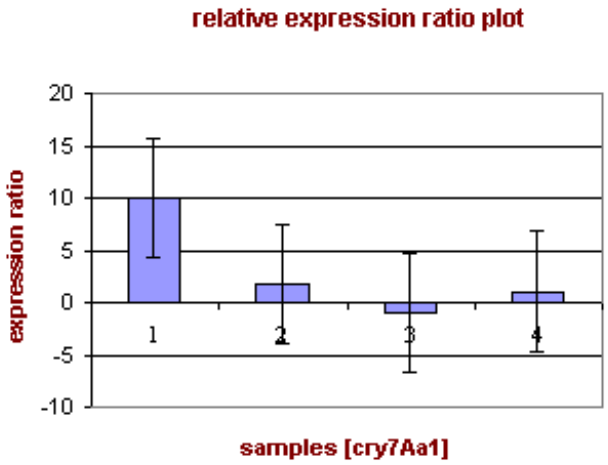

Figure 1. Quantitative determination of gene expression in transformed events of sweetpotato var. jewel with *cry7Aa1* inserted

Tuberous root and leaf assays

Tuberous roots and leaves produced in the Biosafety greenhouse will be used for toxin quantification by DAS-ELISA and gene expression by Real-time PCR.

Acknowledgement

The authors are grateful to continuous financial support from The Rockefeller Foundation, the Belgian Directorate General for Development Cooperation, and the USAID.

References

Agbios. 2009. GM Database (on line). http://www.agbios.com/dbase.php

Bravo, A.; Sarabia, S.; Lopez, L.; Ontiveros, H.; Abarca, C.; Ortiz, A.; Ortiz, M.; Lina, L.; Villalobos, F.J.; Peña, G.; Nuñez-Valdez, M.E.; Soberón, M.; Quintero, R. 1998. Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. Applied and Environmental Microbiology 64(12):4965-4972.

Cruzado, R.G. (2009). Identificación de las proteínas responsables del sinergismo del complejo viral de camote (SPVD). Mg. Sc. Thesis. Universidad Nacional agraria La Molina, Lima, Peru.

Donovan, W.P.; Rupar, M.J.; Slaney, S.C.; Malvar, T.; Gawron-Burke, M.C.; Johnson, T.B. 1992. Characterization of two genes encoding *Bacillus thuringiensis* insecticidal crystal proteins toxic to *Coleoptera* species. Applied and Environmental Microbiology 58(12):3921-3927.

Ferré, J.; Escriche, B.; González-Cabrera, J.; Herrero, S. 2005. Estrategias de manejo de la resistencia para preservar la utilidad de las plantas Bt. beSEG 20:13-17.

Génissel, A.; Leplé, J.C.; Millet, N.; Augustin, S.; Jouanin, L.; Pilate, G. 2003. High tolerance against *Crysomela tremulae* of transgenic poplar plants expressing synthetic *cry3Aa* gene from *Bacillus thuringiensis* ssp *tenebrionis*. Molecular Breeding 11:103-110.

Guzov, V.M.; Malvar, T.M.; Roberts, J.K.; Sivasupramaniam, S. 2007. Insect inhibitory *Bacillus thuringiensis* proteins, fusions, and methods of use therefore. U.S. Patent 7,214,788.

Hattori, T.; Nakamura, K. 1988. Genes coding for the major tuberous root protein of sweet potato: identification of putative regulatory sequence in the 5′ upstream region. Plant Molecular Biology 11:417-426.

Höfte, H.; Seurinck, J.; Van Houtven, A.; Vaeck, M. 1897. Nucleotide sequence of a gene encoding an insecticidal protein of *Bacillus thuringiensis var. tenebrionis* toxic against Coleoptera. Nucleic Acids Research 15(17):7183.

James, C. 2008. Global status of commercialized biotech/GM crops: 2008. ISAAA Brief No. 39. ISAAA, New York, United States of America.

Lambert, B.; Höfte, H.; Annys, K.; Jansens, S.; Soetaert, P.; Peferoen, M. 1992. Novel *Bacillus thuringiensis* insecticidal crystal protein with a silent activity against coleopteran larvae. Applied and Environmental Microbiology 58(8):2536-2542.

Luo, H.R.; Santa María, M.; Benavides, J.; Zhang, D.P.; Zhang, Y.Z.; M. Ghislain. 2006. Rapid genetic transformation of sweetpotato (Ipomoea batatas (L.) Lam) via organogenesis. African Journal of Biotechnology 5: 1851-1857.

Medina-Bolivar, F.; Wright, R.; Funk, V.; Sentz, D.; Barroso, L.; Wilkins,T.; Petri Jr., W.; Cramer, C. 2003. A non-toxic lectin for antigen delivery of plant-based mucosal vaccines. Vaccine 21, 997–1005.

Mettus, A.M.L.; Baum, J.A. 2006. Polynucleotides encoding δ-endotoxins toxic to Lepidoptera and Coleoptera, and method of use. U.S. Patent 7,022,897B2.

Nakamura, K.; Ohto, M.; Yoshida, N.; Nakamura, K. 1991. Sucrose-induced accumulation of ß-amylase occurs concomitant with the accumulation for starch and sporamin in leaf-petiole cuttings of sweet potato. Plant Physiology 96:902-909.

Ormachea, M. 2008. Estandarización de un protocolo de cultivo *in vitro* para la regeneración vía organogénesis de camote (*Ipomoea batatas* (L.) Lamarck, 1793) variedades Tanzania y Wagabolige. BSc. Thesis. Universidad Ricardo Palma, Lima, Peru.

Peferoen, M.; Lambert, B.; Van Audenhove, K. *Bacillus thuringiensis* strains and their genes encoding insecticidal toxins. U.S. Patent 5,723,756.

Stathers, T.; Namanda, S.; Mwanga, R.O.M.; Khisa, G.; Kapinga, R. 2005. Manual for Sweetpotato Integrated Production and Pest Management Farmer Field Schools in Sub-Saharan Africa. International Potato Center. Kampala, Uganda. 168 pp.

Sekar, V.; Thompson, D.V.; Maroney, M.J.; Bookland, R.G.; Adang, M.J. 1987. Molecular cloning and characterization of the insecticidal crystal protein gene of *Bacillus thuringiensis* var. *tenebrionis*. Proc. Natl. Acad. Sci. 84:7036-7040.

Stevenson, P.C.; Muyinza, H.; Hall, D.R.; Porter, E.A.; Farman, D.I.; Talwana, H.; Mwanga, R.O.M. 2009. Chemical basis for resistance in sweetpotato *Impomea batatas* to the sweetpotato weevil *Cylas puncticollis*. Pure Appl. Chem. 81(1):141-151.

In vitro screening for drought tolerance of orange-fleshed Sweetpotato genotypes

1.Sammy Agili, 2.Bernard Nyende, 2.Kamau Ngamau, 3.Peter Masinde, 1. Regina Kapinga

1. International potato center, Nairobi, Kenya
2 Jomo Kenyatta University of Agriculture and Technology.
3 International potato centers, Kampala, Uganda

Abstract

Orange-fleshed sweetpotato is rich in beta carotene that the body easily converts into vitamin A. This reduces maternal and child mortality. However, drought susceptibility of OFSP is a major draw back in the promotion in the sub Saharan Africa. Simulation of drought stress under invitro condition using tissue culture regeneration process constitutes a conventional way to study the effects of water stress.

In this study, drought induced alterations in early shoot and root development of 59 sweetpotato genotypes from Lima, Peru and two Kenyan checks cultivars Marooko (drought tolerant) and K566632 susceptible) were assessed with different concentrations of polyethylene glycol (PEG 6000MW) at three levels 0, 10 and 15 g litre with three replications in factorial completely randomized design. . Data on shoot and root growth was recorded during tissue culture regeneration. Analysis of variance was performed and significant differences among treatment means calculated by the least significant differences (LSD) test at a probability level of 0.01.

Analysis of variance indicated evidence that all effects: genotype, Salt levels and Salt Level X genotype interaction, were highly significant ($p<0.01$) with respect to all the responses.. At 15 g/l concentration of PEG, genotypes 189135.9, 194515.5, 440024, 441724 and 440001 exhibited long roots that were above that of Marooko. This level of stress severely affected the production of biomass for most of the genotypes .Genotypes 189135.9, 192033.5, 194515.5, 194539.3, 401055, 441724, 440429, 441097, 441538,441768 were observed with outstanding ability to continue root and shoot growth under *in vitro* stress conditions at all salt levels indicating their ability to fight with sever water stress situation. Susceptible genotypes observed were 189151.38, 420027, 440034, 440166, 440132, 441755, 421111 and 440104.

In-vitro technique was shown to be useful in identifying relatively salt-tolerant genotypes at early stages of development and this can be a very useful tool for screening large number of breeding lines of genotypes within a short time

Keywords: drought tolerance, genotype, in-vitro screening; polyethylene glycol salt.

Introduction

Sweetpotato (Ipomea batatas) is one of the most important staple crops in densely populated parts of Eastern Africa and is quickly becoming an important supplementary staple in the southern part of the continent (Silver et al, 2004). Sweet potato is vital to small scale farmers with limited land, labor and capital. They are easy to grow and the average consumer can afford them. One of the greatest values is its ability to be harvested piecemeal for home consumption or income generation. Presently, the predominant Sweetpotato cultivars for Eastern and Southern Africa are white-fleshed varieties that contain negligible amounts of beta-carotene, a micronutrient that the body uses to produce vitamin A (Silver et al 2004).

Orange-fleshed Sweetpotato is rich in beta-carotenes that the body converts easily into vitamin A. Adding 100 g of the Sweetpotato to the daily diet can prevent vitamin A deficiency in children, dramatically reduce maternal mortality and lower the risk of mother-to-child transmission of HIV/AIDS. The drought susceptibility of OFSP is a major drawback when promoting OFSP in SSA. Lower yields and increased susceptibility to pests on water stressed plants decrease the farmer acceptability of this otherwise very valuable crop type

Drought is one of the most common environmental stresses affecting plant growth and productivity (Boyer, 1982). Under field conditions, drought severity, timing and duration vary from year to year and a cultivar, which is successful in one year, might fail to in another year hence the need to do in-vitro screening. The unpredictable and variable forms in which drought stress manifest, complicates the selection of superior plant materials as well as breeding programs .Plant cell and tissue culture has been a useful tool to study stress tolerance mechanism under in vitro conditions (Baijji et al 2000). In vitro culture techniques minimize environmental variations due to defined nutrient media, controlled conditions and homogeneity of stress application (Sakthivelu et al 2008). In addition, the simplicity of such manipulations enables studying large plant population and stress treatments in a limited space and short period of time. Polythylene glycols (PEG) of high molecular weights have been long used to stimulate drought stress in plants as non-penetrating osmotic agents lowering the water potential in away similar to soil drying (Larher et al, 1993). Selection for drought tolerance at early stage of seedlings is most frequently carried out by including chemical drought induced molecules like poly ethylene glycol (PEG6000) in the medium. This can be used to modify the osmotic potential of nutrient solution culture and thus induce plant water deficit in a relatively controlled manner, appropriate to experimental protocols (Zhu et al., 1997.

Simulation of drought stress under in vitro conditions during the regeneration process constitutes a convenient way to study the effects of drought on morphogenic responses

In vitro selection for drought tolerant genotypes or breeding lines has been conducted for various crops like for wheat genotypes Asif et al; 2006; Tomatoes Shtereva et al 2007; Rice Biswas et al 2002, Lestari,(2005, 2006)**;** Soya bean cultivars Sakthivelu et al 2008, Husni et al; 2006 green grams mungbean (Vigna radiate L.) Gulati and Jaiwal, 1993) and hence can also be used for sweetpotato

The possibility of using in vitro screening for orange-fleshed Sweetpotato genotypes for drought tolerance was investigated, with the aim of identifying at early stages of development those genotypes that are drought tolerant and drought susceptible.

Materials and methods

Trial site

The experiment was conducted in the tissue lab at Kenya Plant Health Inspectorate Quarantine station, Muguga, Kenya

Planting material and preparation of growth media

This consisted of 59 mega sweetpotato genotypes having CIP accession no were received as in-vitro plantlets from International potato centre (Lima, Peru) with contrasting drought resistance, beta carotene and mineral content levels. These were transferred on in vitro for regeneration of apical cuttings with 2-3 nodes.

Treatment and experimental design

Murashige and Skoog (1962) basal media with various concentration of polyethylene glycol salt (PEG6000) at 0, 10 and 15g/lit was prepared, poured into Kilmer jars and autoclaved at 121 ^{o}C and 15lb/sq inch for 15 minutes. Five cuttings per genotype with 2 -3 nodes each were placed onto the media in Kilmer jars. All the planted jars were maintained under optimum culture conditions at 10 photoperiod 70 µmol M^{2}/s and 28^{o}C temperature. The experiment was laid out in factorial complete randomized design with three replications. Plantlet growth study was recorded on regeneration. Data analysis was done using the SAS package (SAS version 8 of SAS Institute, Inc, 1999

Data collected

Root length (cm); this was determined by measuring the length of the longest root from each sample plant using a meter scale

Root weight (g); Root samples from plants from each jar were heated to a constant weight in an oven for 48 h at > 65 ^{0}C and re-weighed to determine the dry weight

Leaf area (cm2); the linear dimensions of Length (L) and width (W) at the broadest part of the lamina of each 3rd leaf from the bottom of the plant were measured with a ruler. The leaf area was then calculated as A= LXW

Shoot length (cm); this was determined at harvest by measuring the plants in each treatment from the surface of the media in the jar to the tip of the tallest leaf

Shoot fresh and dry weight (g); the shoot samples from plants from each jar were collected and weighed and then heated to a constant weight in an oven for 48 h at 65 ^{0}C. These were re-weighed to determine the dry weight.

Results

Fisher's F-test indicates existence of adequate evidence that all effects i.e. Salt levels, Clones and Salt Level X Clone interaction, are highly significant ($p<0.01$) with respect to all the responses (table 1) . Virtually all major processes contributing to crop yield including, leaf expansion, shoot and root growth were inhibited as stressed increased. These growth-supporting processes showed no further net growth (i.e. increase in biomass) at 15g/lit of PEG (Figures 1 and 2).

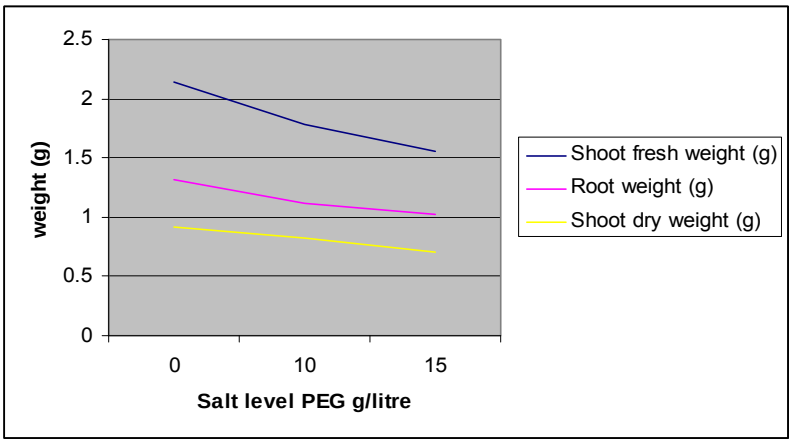

Figure 1. Effect of different salt levels on shoot fresh weigh, Root weight and shoot dry weight for the screened 59 Sweetpotato genotypes

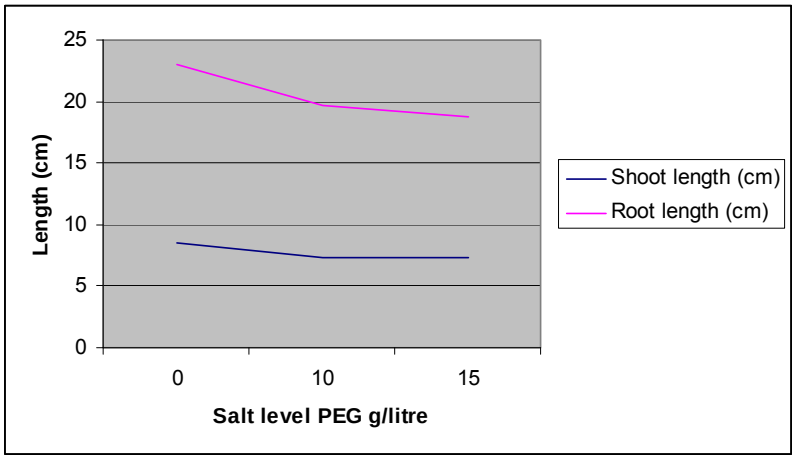

Figure 2. Effect of different salt levels on shoot and Root length for the screened 59 Sweetpotato genotypes

Leaf area

Leaf expansion and growth is one of the major processes that contribute to crop yield Significant decrease in leaf area for genotypes 420027, 440034, 440104, 194549.6 and 440643 was observed with increasing **PEG** concentration. This decrease ranged from 0.17cm2 to 0.57 cm2 At the same higher concentration of 15g/litre genotypes 189135.9, 194515.5, 441097 and 441768 recorded higher leaf expansion that ranged from 5.70 to 6.63 cm2 although not significantly different from that of the check (5.67 cm2) (table 2)

Root length

Under controlled treatment genotypes 189135.9, 421066, 440396, 440429, and 441097 formed the longest roots that ranged from 32cm to 38cm this was above the tolerant (table 2) check length (25.97cm) although not significantly different. At 15 g/l concentration of PEG, genotypes 189135.9, 194515.5, 440024, 441724 and 440001 exhibited long roots that ranged from 29.67 cm to 40.17 cm (Table 2). Poor root growth at the same level was observed for genotypes 440031 (4.71cm) and 440286 (5.10), 440025 (3.53cm), 440132 (1.87) and 420027 (2.63cm). The performance of genotypes 440024, 194515.5, 441077 and 189135.9 are worth noting. These genotypes registered high mean root length across the salt levels

Root weight

Under control treatment genotypes 189135.9, 441538 and 441768 registered higher root weight that ranged from 5.23 to 6.0 g. These were significantly different from that of the check (1.27cm) table 2.There was significant root weight reduction as stressed increased. Genotypes that exhibited higher root weight at 15 g/l concentration of PEG were 189135.9 (5g), 194569 .1 (5.03g), 440429(4.37g) and 441768(5.37g). These were significantly higher than of the check (2.20g). Higher mean root weight across the salt levels was recorded for genotypes 194515.5, 441538, 440378 (figure 3), 441097 (Figure 4) and 441768 and this ranged from 3.05 g to 5.62 g

Figure 3. Tolerant genotype 440378- (0,5,15 g/lit of PEG)

Figure 4. Tolerant genotype 441097 (0,5,15 g/lit of PEG)

Shoot dry weight

Shoot dry weight reflects a fundamental trade off in plant functioning between a rapid production of biomass and an efficient conservation of nutrients. In the control treatment genotypes 189135.9,440328, 440170, 440378 and 441538 produced significantly high root dry matter content that ranged from 2.13g to 2.79g compared to the check (0.70g), where as genotypes 440429, 194539.36, 441538, 401055, 194515.5 and 189135.9 recorded higher shoot dry weight at 15g/litre of PEG concentration .The same genotypes recorded higher mean shoot dry weight across the salt levels that were significantly higher than that of the check (0.70g). Lowest mean shoot weight were recorded for genotypes 194541.45, 4200014, 420027, 440024, 440050, 440167, 440240 and 440286, and this ranged from 0.11 g to 0.29 g (Table 3)

Shoot fresh weight (g)

Under controlled treatment high shoot fresh weight above that of the tolerant check(1.63g) were recorded for genotypes 189135.9 (6.48g), 440170 (4.90g), 440328 (5.30g), 441538 (5.48g). A sharp and significant decrease in

shoot fresh weight was recorded for genotypes 194541.45, 420027, K566632, 440167 and 440027 at high 15g/lit PEG concentration. At the same level of stress genotypes 194515.5, 194573.9, 401055, 440429, 441097, 441538 and 441768 recorded high fresh root weight (Table 3).

Shoot length

Increased stress at 15g/litre induced longer shoot length for genotypes 187016.2, 187017.1, 194539.36, 420064, 440378, and 441097 which ranged from 13.83 to 18.23 way above that of the tolerant check (8.50cm). The same genotypes registered high mean shoot values across the salt level. Significant reduction in growth was observed for genotypes 189148.65, 194541.45, 440286 (Table 3).

Discussion

The present study revealed different response of genotypes to various levels o PEG concentrations. Higher concentration of PEG at 15g/litre reduced significantly growth parameters in susceptible genotypes like 420027, 440034, 440104, 440643, 189148.65, 194541.45, 420014 and 440131. Such negative effects have been observed for susceptible genotypes in wheat Razi (2003**),** Soya bean *(*Sakthivelu et al 2008).At the same level of stress genotypes 189135.9, 194515.5, 194539.3 401055, 440429,441097,441538 and 441768 were observed with outstanding ability to continue root and shoot growth indicating their ability to tolerate stress. Leaf expansion is among the most sensitive of the processes that are affected by water deficit. High concentration of PEG severely reduced leaf expansion in the susceptible genotypes like 440034,440104, 420027,189140 and 421111 unlike in tolerant genotypes 189135.9, 194515.5, 440131, 441097 and 441768 that showed high leaf expansion. This reduced expansion results to drastic reduction in transpiration surface (Alfredo et al 2004, Barta et al 2002)) resulting to low biomass production. This reduction may be due to inhibition of cell division which results to fewer cells per leaf (Tardeo et al 2000; Alfredo et al 2004). Early Detection of such genotypes with low leaf expansion under moisture stress condition can save resources in the breeding process. . Two major dimensions describe the root: root depth and root-length density. Early and rapid elongation of roots is important indication of drought tolerance; this facilitates deep soil moisture extraction under limited water conditions. Ability of continued elongation of the root under situation of water stress was remarkable character of some of the genotypes screened. Genotypes 189135.9, 194515.5, 441097, 187017.1, 440034,441768 and 441538 observed with high root length and weight have the ability to survive under high moisture stress conditions. Drought stress significantly reduced dry matter production in susceptible genotypes 194541.45, 420014, 420027, 440167 and 440394, their means were not significantly different from the of the susceptible variety K566632 Genotypes 189135.9, 194515.5, 194539.36, 440027, 440429, 441538, 401055 were observed to be relatively tolerant with her dry matter production at high PEG concentration of 15g/lit. Similar observation has been made in crops like Alfalfa (Berta et al 2002). Stress affects rate of photosynthesis thus reducing the supply of assimilate to various parts of the plant (Hall and Twidwell, 2002).

Conclusion

The results showed significant variations among the genotypes for salt tolerance based on plant growth characters. Higher concentration of the salt at 15g/litre severely affected the production of biomass for most of the genotypes .Genotypes 189135.9, 192033.5, 194515.5, 194539.3, 401055, 441724, 440429, 441097, 441538,441768 were observed with outstanding ability to continue root and shoot growth under *in vitro* stress Conditions at all salt levels indicating there ability to fight with sever water stress situation. Most susceptible genotypes observed were 189151.38, 420027, 440034, 440166, 440132, 441755, 421111 and 440104. Greater leaf area expansion under high moisture stress condition was observed for genotypes 189135.9, 194515.5, 441097 and 441768. Poor leaf expansion area was recorded for genotypes 194549.6, 420027 and 440034. All major processes contributing to crop yield including, leaf expansion, shoot and root growth were inhibited as stressed increased. In-vitro techniques were shown to be useful in identifying relatively salt-tolerant genotypes and can be a very useful tool for screening large number of breeding lines

References

Alfredo. Alves A. C; Tim setter, L. Response of Cassava Leaf Area Expansion to Water Deficit: Cell Proliferation, Cell Expansion and Delayed Development Annals of Botany 2008, doi:10.1093/aob/mch179

Barta, A. L.; Sulc, R. M; Ogle, M. J; Hammond, R. B. 2002. Interaction between flooding or drought stress and potato leafhopper injury in alfalfa. Online. Plant Health Progress doi:10.1094/PHP-2002-0502-01-RS.

Bajji, M; Lutts, S; Kinet, J. M. 2000. Physiological changes after exposure to and recovery from polyethylene glycol-induced water deficit in callus culture issued from durum wheat (Triticum durum) cultivars differing in drought resistance. J. Plant Physiol., 156, 75–83.

Boyer, J.S., 1982. Plant productivity and environment. Science 218, 443–448.

Din, Razi ud (2003) in situ and in vitro studies in wheat (triticum aestivum l.) genotypes for drought tolerancE. PhD thesis, NWFP Agriculture University, Peshawar.

Larher, F., L. Leport, M. Petrivalsky, M. Chappart, 1993. Effectors for the osmoinduced praline response in higher plants. Plant Physiol. Biochem., 31(6), 911–922

Sakthivelu, G; Akitha Devi M. K; Giridhar, P; Rajasekaran, T; Ravishankar, G. A; Nedev,T ; Kosturkova, G; *Gen. Appl. plAnt physiology, 2008, speciAl issue, 34 (1-2), 103-112*

Murashige, T; Skoog, F 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant., 15, 473–497.

Robert. Hal C; Edward. Twidwell K, Effects of Drought Stress on Corn Production.ExEx 8033Updated June 2002 F&F 1.4-1.3

Sakthivelu *1*, G. ; Akitha Devi *1*, M. K.; Giridhar *1*, P; Rajasekaran *1*, T; Ravishankar *1,* G. A; Nedev*2* T; Kosturkova*,* G. Drought-induced alterations in growth, osmotic potential and in vitro regeneration of soybean cultivars *Gen. Appl. plAnt physioloGy, 2008, speciAl issue, 34 (1-2), 103-112*

Tumwegamire, S., Kapinga R., Zhang D., Crissman, C. , and Agili S.. 2004. Opportunities for promoting orange-fleshed sweetpotato among food based approach to combat Vitamin-A deficiency in sub-Saharan Africa. African Crop Science Journal 12 (3): 241-253

Table 1. Summarized analysis of variance table showing mean square values for various variables measured during the in vitro drought screening of sweet potato genotypes

Source of variation	Root length (cm)	Root dry weight (g)	Shoot length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Leaf area (cm2)
Genotype	907.9**	20.88**	135.22**	14.19**	3.52**	2.24**
Salt level	1889.3**	10.33**	195.03**	45.53**	7.07**	
Clone* salt	907.9**	1.77**	18.52**	9.50**	8.02**	90.59**
						7.96**

****=significant at P < 0.001; * Significant at P< 0.005.**

Table 2. Effect of three salt levels on Leaf Area, Root Length and root dry weight of 59 orange-fleshed sweetpotato genotypes screened for drought tolerant

Clone	Leaf Area (cm2)					Root length (cm)					Root dry weight (g)				
	Salt level					Salt level					Salt level				
	0	10	15	Mean	STR	0	10	15	Mean	STR	0	10	15	Mean	STR
Marooko*	5.50	6.47	5.67	5.9	**T**	25.97	22.00	21.33	23.10	**T**	1.27	1.57	2.20	1.7	T
187016.2	2.33	1.67	1.50	1.8	**S**	17.67	14.67	13.33	15.22	**S**	0.33	0.30	0.06	0.2	S
187017.1	3.17	2.67	2.33	2.7	**S**	27.00	26.33	27.00	26.78	**T**	0.33	0.99	0.39	0.6	S
189123.68	4.00	0.60	1.67	2.1	**S**	18.00	16.67	15.33	16.67	**S**	0.22	0.38	1.33	0.6	S
189135.9	7.00	7.33	5.20	6.5	**T**	33.00	33.53	35.33	33.95	T	6.00	7.87	5.00	6.3	S
189140	1.90	1.63	1.20	1.6	**S**	29.13	28.83	25.77	27.91	T	0.07	0.01	0.04	0.03	S
189148.21	3.83	2.50	3.23	3.2	**S**	17.33	14.80	17.50	16.54	S	0.03	0.04	0.02	0.03	S
189148.65	5.10	1.70	2.50	3.1	**S**	10.33	5.67	8.00	8.0	S	0.43	0.02	0.02	0.2	S
189150.1	5.33	4.33	3.43	4.4	**S**	15.33	7.17	5.33	9.28	S	0.57	0.23	0.13	0.30	S
189151.38	2.63	5.67	5.50	4.6	**S**	11.00	17.67	13.33	14	S	0.70	2.00	1.07	1.30	S
192033.5	4.17	6.33	6.42	5.6	**S**	12.67	17.67	15.67	15.34	S	0.73	1.00	0.88	0.9	S
194515.5	5.10	9.50	5.77	6.8	**T**	31.33	33.33	30.67	31.78	T	4.77	0.63	3.77	3.1	T
194521.2	5.33	2.10	2.00	3.1	**S**	26.33	21.67	12.17	20.06	S	1.34	0.64	0.42	0.8	S
194539.36	4.33	6.00	5.30	5.2	**S**	23.83	23.67	23.67	23.72	T	1.50	2.27	3.17	2.3	T
194541.45	4.17	3.00	4.50	3.9	**S**	10.53	21.50	24.93	18.91	S	1.03	0.78	0.65	0.8	S
194549.6	0.57	0.60	1.50	0.9	**S**	17.33	9.00	24.33	16.89	S	0.13	0.21	1.03	0.5	S
194555.7	2.20	3.77	1.40	2.5	**S**	7.33	29.00	16.10	17.48	S	0.49	0.11	0.05	0.3	S
194569.1	3.53	2.50	4.83	3.6	**S**	21.00	4.00	9.17	11.39	S	4.80	0.01	5.03	3.3	T
194573.9	2.00	0.82	3.83	2.2	**S**	8.50	4.67	9.73	7.63	S	0.39	0.60	0.29	0.37	S
400011	5.33	4.33	4.33	4.7	**S**	16.33	25.00	20.33	20.55	T	0.51	0.23	0.60	0.40	S
401055	5.13	7.17	5.63	6.0	**S**	9.83	6.33	10.90	8.99	S	0.46	0.81	0.39	0.60	S
420001	7.00	1.17	1.83	3.3	**S**	28.33	23.67	14.50	9.02	S	1.81	0.80	0.62	1.1	S
420014	4.60	6.13	3.37	4.7	**S**	19.30	16.27	13.50	22.14	S	1.73	1.73	0.54	1.3	S
420027	1.07	1.53	0.57	1.1	**S**	17.83	3.60	2.63	16.36	S	0.06	0.01	0.02	0.03	S
420064	2.20	1.83	6.60	3.5	**S**	20.00	23.00	17.83	8.02	S	3.15	0.82	0.70	1.6	S
421066	2.47	2.33	1.50	2.1	**S**	38.00	16.67	21.67	20.28	S	0.97	0.33	0.30	0.50	S
421111	3.80	2.83	1.23	2.6	**S**	2.06	27.27	5.00	11.44	S	0.06	0.08	0.17	0.10	S
422656	8.00	2.43	4.40	4.9	**S**	29.67	25.17	16.67	23.84	T	2.21	1.21	1.13	1.50	T
440001	2.20	3.00	2.17	2.5	**S**	24.67	15.00	31.67	23.78	S	1.67	0.30	0.20	0.56	S
440017	1.50	2.33	3.50	2.4	**S**	24.33	33.67	5.43	21.14	S	2.7	1.40	0.01	0.90.	S
440023	4.00	4.33	2.83	3.7	**S**	21.00	10.50	13.17	14.89	S	0.87	0.44	0.37	1.40	S
440024	1.10	8.73	4.63	4.8	**S**	28.77	69.67	40.17	27.87	T	0.03	0.87	0.48	0.60	S

Clone	Leaf Area (cm2)					Root length (cm)					Root dry weight (g)				
	Salt level					Salt level					Salt level				
	0	10	15	Mean	STR	0	10	15	Mean	STR	0	10	15	Mean	STR
440025	3.50	5.00	3.33	3.9	**S**	17.83	25.80	3.53	15.72	S	1.17	0.19	0.03	0.50	S
440027	2.23	5.50	1.50	3.1	**S**	30.33	28.33	16.67	25.11	T	0.68	2.33	0.04	1.00	S
440031	5.93	5.67	1.80	4.5	**S**	5.33	4.33	3.83	4.50	S	0.03	0.02	0.01	0.02	S
440034	2.80	0.87	0.83	1.5	**S**	29.80	27.20	28.07	28.26	T	0.07	0.02	0.05	0.04	S
440050	2.00	4.20	4.00	3.4	**S**	13.30	10.20	9.83	11.11	S	0.01	0.04	0.42	0.20	S
440104	7.33	3.87	0.17	3.8	**S**	21.97	18.17	22.00	20.71	S	0.21	0.00	0.02	0.10	S
440131	5.70	10.20	1.47	5.8	**T**	13.63	10.83	9.83	11.43	S	4.33	0.06	0.06	1.50	T
440132	4.80	6.17	0.00	3.7	**S**	17.87	16.77	1.87	12.17	S	0.04	0.04	0.00	0.30	S
440166	2.33	1.50	1.67	1.8	**S**	18.67	17.33	12.00	16.0	S	0.30	0.63	0.04	0.10	S
440167	1.20	2.10	2.37	1.9	**S**	30.67	7.53	6.50	11.57	S	0.06	0.05	0.04	1.10	S
440170	2.50	2.83	2.93	2.8	**S**	20.67	20.17	24.00	21.61	S	2.00	1.73	1.90	1.90	T
440240	4.17	0.38	2.17	2.2	**S**	21.33	13.80	12.63	15.93	S	0.04	0.16	0.14	0.10	S
440286	5.20	0.00	0.00	1.7	**S**	11.87	2.93	1.43	5.41	S	0.34	0.00	0.00	0.10	S
440287	6.10	5.77	0.00	4.0	**S**	27.30	29.87	3.50	20.22	S	0.31	0.40	0.07	0.30	S
440328	8.25	3.33	4.20	5.3	**S**	28.33	26.00	17.67	24.0 0	T	2.22	1.39	1.73	1.80	S
440378	2.00	2.33	2.50	2.3	**S**	22.00	48.33	12.67	21.00	T	1.93	0.93	0.83	1.20	S
440394	1.50	1.17	2.50	1.7	**S**	19.67	10.33	5.13	11.71	S	2.00	1.50	0.05	1.20	S
440396	5.00	8.33	4.00	5.8	**S**	36.00	14.00	8.73	19.58	S	2.13	1.93	1.23	1.80	T
440429	3.67	3.50	4.50	3.9	**S**	32.33	25.67	21.17	23.06	T	2.30	1.70	4.37	2.80	T
440643	1.17	5.57	0.57	2.4	**S**	16.90	9.33	3.80	10.01	S	1.10	0.24	0.01	0.50	S
441097	5.67	10.00	6.50	7.4	**T**	32.50	34.17	25.33	30.17	T	1.30	1.27	3.53	2.00	T
441538	5.67	7.30	5.83	6.3	**S**	31.33	21.47	13.67	22.16	S	5.23	6.13	3.07	4.80	T
441724	3.50	4.33	2.00	3.3	**S**	29.33	25.00	29.67	28	T	0.77	2.73	0.90	1.5	S
441725	4.13	5.60	6.33	5.4	**S**	31.33	25.50	19.33	25.39	T	0.50	0.04	0.07	0.2	S
441755	0.30	5.03	3.43	2.9	**S**	8.70	9.67	10.83	9.78	S	1.93	0.75	0.68	1.10	S
441768	7.03	11.33	3.80	7.5	**T**	32.67	25.80	22.17	26.88	T	5.87	0.27	5.37	3.90	T
K566632**	11.23	2.00	1.70	5.0	**S**	13.67	8.00	4.53	8.73	S	0.05	0.02	0.04	0.04	S
Mean	**3.82**	**4.09**	**3.06**	**3.37**		**22.95**	**19.73**	**15.47**	**18.81**		**1.32**	**1.12**	**0.95**	**1.03**	
LSD(0.01)	**0.35**					**3.49**					**0.23**				
	LSD(0.01)- - for salt level= 0.06; clone= 0.25; means with the same level= 0.49; means with different salt level = 0.49					**LSD(0.01)**- means- for salt level= 3.49; clone= 8.88; means with the same level= 17.84; means with different salt level = 17.77					**LSD(0.01)**- means- for salt level= 0.23; clone= 0.50; means with the same level= 1.01; means with different salt level = 1.00				

Table 3. Effect of three salt levels on shoot dry weight, shoot fresh weight and shoot length of 59 orange-fleshed sweetpotato genotypes screened for drought tolerant

Clone	Shoot dry weight (g)					Shoot fresh weight (g)					Shoot length (cm)				
	Salt level					Salt level					Salt level				
	0	10	15	Mean	INF	0	10	15	Mean	INF	0	10	15	Mean	INF
Marooko*	0.70	0.68	0.99	0.80	T	1.63	1.63	2.20	1.80	T	7.23	5.17	8.50	7.00	T
187016.2	0.31	0.36	0.13	0.25	S	1.10	0.97	0.33	0.80	S	10.33	12.17	14.00	12.20	T
187017.1	1.05	1.01	0.26	0.80	T	2.30	1.94	0.75	1.7	S	10.33	13.83	14.90	13.00	T
189123.68	1.23	0.70	1.43	1.10	T	1.23	0.70	1.43	1.10	S	8.00	4.33	6.67	6.30	S
189135.9	2.79	2.35	1.22	2.10	T	6.48	5.57	2.83	5.00	T	15.33	12.83	13.17	13.80	T
189140	0.29	0.05	0.74	0.40	S	0.63	0.14	1.90	0.90	S	5.50	5.33	4.83	5.20	S
189148.21	0.29	0.44	0.44	0.40	S	0.67	1.00	0.97	0.90	S	5.50	5.00	8.63	6.40	S
189148.65	0.23	0.22	0.10	0.18	S	0.55	0.52	0.23	0.40	S	6.87	3.00	1.83	3.90	S
189150.1	0.87	0.87	0.90	0.90	T	1.93	2.00	2.13	2.20	T	4.83	4.33	5.00	4.70	S
189151.38	0.67	1.77	0.98	1.10	T	1.58	4.50	2.30	2.80	T	7.00	11.00	9.67	9.20	T
192033.5	0.86	1.97	0.74	1.19	T	1.46	4.77	2.19	2.80	T	10.33	14.67	12.17	12.40	T
194515.15	1.99	1.90	1.21	1.70	T	4.80	4.33	2.73	4.00	T	12.57	10.00	11.00	11.20	T
194521.2	1.26	0.38	0.69	0.80	T	2.95	0.88	1.60	1.80	S	14.00	8.40	8.30	10.2	T
194539.36	1.83	2.08	2.58	2.2	0T	4.07	4.53	5.73	4.80	T	9.33	10.00	16.67	12.00	T
194541.45	0.69	0.30	0.03	0.30	S	1.64	0.69	0.06	0.80	S	6.67	4.50	1.33	4.20	S
194549.6	0.38	0.32	0.65	0.50	S	0.73	0.70	1.43	0.70	S	1.83	2.83	7.67	4.10	S
194555.7	0.44	0.26	0.09	0.30	S	1.31	0.63	0.17	0.70	S	6.67	4.83	6.50	6.00	S
194569.1	0.68	0.10	0.76	0.50	S	1.60	0.24	1.67	1.70	S	8.00	1.50	7.33	5.60	S
194573.9	0.45	0.44	0.42	0.40	S	1.06	1.08	2.50	1.50	S	7.00	7.67	9.67	8.10	T
400011	0.80	0.69	0.69	0.70	S	2.03	1.27	1.93	1.70	S	5.67	4.83	5.57	5.40	T
401055	0.53	0.55	1.19	0.80	T	1.16	2.00	2.65	1.90	T	8.00	8.37	10.17	8.80	T
420001	1.03	0.39	0.44	0.60	S	4.34	1.03	1.69	2.40	T	16.33	8.17	8.33	10.90	T
420014	0.58	0.32	0.10	0.30	S	1.39	0.85	0.24	0.80	S	6.70	5.87	5.77	6.10	S
420027	0.14	0.10	0.05	0.09	S	0.31	0.21	0.04	0.19	S	6.70	1.93	2.80	3.80	S
420064	0.68	0.33	0.45	0.46	S	1.55	1.16	0.97	1.20	S	10.33	11.47	18.23	13.30	T
421066	0.77	0.47	0.33	0.41	S	2.20	1.03	0.73	1.30	S	11.00	6.00	6.33	7.80	S
421111	0.31	0.41	0.44	1.00	T	0.73	0.81	1.01	0.90	S	8.93	10.07	4.77	7.90	T
422656	1.63	0.67	0.70	0.60	S	4.40	1.58	1.65	2.50	T	16.33	11.20	9.73	12.40	T
440001	0.91	0.54	0.32	1.00	T	2.50	1.33	0.83	1.60	S	9.33	5.33	8.33	7.70	T
440017	1.78	1.09	0.13	0.60	S	4.43	2.30	0.29	2.30	T	9.00	10.33	1.27	6.90	T
440023	1.04	0.38	0.45	0.30	S	1.97	0.87	1.00	1.30	S	5.67	2.00	2.67	3.40	S
440024	0.05	0.54	0.19	0.40	S	0.14	1.36	0.49	0.70	S	2.80	7.13	6.83	5.60	S
440025	0.48	0.51	0.23	1.9	T	1.07	0.93	0.50	0.80	S	2.00	8.17	2.77	4.30	S
440027	1.43	4.33	0.07	0.20	S	1.43	4.33	0.07	1.90	T	7.00	10.33	8.00	8.40	T
440031	0.25	0.14	0.08	0.90	T	0.57	0.42	0.21	0.40	S	7.33	4.23	7.67	6.40	T
440034	1.06	0.91	0.64	0.20	S	3.13	2.00	1.47	2.20	T	0.23	5.50	4.57	3.40	S

Clone	Shoot dry weight (g)					Shoot fresh weight (g)					Shoot length (cm)				
	Salt level					Salt level					Salt level				
	0	10	15	Mean	INF	0	10	15	Mean	INF	0	10	15	Mean	INF
440050	0.08	0.03	0.35	0.30	S	0.21	0.28	0.83	0.40	S	4.10	5.57	9.00	6.20	S
440104	0.66	0.04	0.10	0.60	S	0.99	0.10	0.24	0.40	S	14.73	1.50	7.57	7.90	T
440131	1.43	0.02	0.40	0.20	S	3.50	0.05	0.81	1.50	S	7.93	2.30	11.60	7.30	T
440132	0.35	0.31	0.00	0.40	S	0.80	0.68	0.06	0.50	S	5.57	5.80	1.27	4.20	S
440166	0.44	0.47	0.17	0.10	S	1.17	1.13	0.40	0.90	S	9.00	15.17	13.17	12.40	T
440167	0.14	0.12	0.03	1.60	T	0.34	0.26	0.07	0.20	S	5.67	1.80	6.33	4.60	S
440170	2.43	1.27	0.97	0.10	S	4.90	2.83	1.70	3.10	T	7.67	12.33	12.00	10.70	T
440240	0.16	0.04	0.15	0.11	S	0.34	0.16	0.34	0.30	S	6.77	1.73	5.40	4.60	S
440286	0.44	0.00	0.00	0.10	S	1.02	0.06	0.03	0.40	S	5.83	1.03	1.17	2.70	S
440287	0.42	0.69	0.00	0.39	S	0.97	1.67	1.93	1.50	S	5.50	5.23	3.50	4.70	S
440328	2.09	0.69	0.86	1.20	T	5.30	1.67	2.08	3.00	T	17.00	9.17	10.97	12.40	T
440378	2.13	1.22	0.50	1.30	T	5.20	2.87	1.40	3.20	T	10.33	12.00	13.83	12.10	T
440394	1.65	1.06	0.13	0.89	T	3.93	2.47	0.29	2.20	T	7.00	6.50	2.00	5.20	S
440396	1.74	1.67	0.47	1.30	T	3.77	3.53	1.03	2.80	T	8.00	7.33	4.67	6.70	T
440429	1.57	1.40	1.74	1.60	T	3.77	3.07	3.87	3.60	T	15.33	9.33	12.33	12.30	T
440643	0.50	0.88	0.13	0.46	S	2.27	0.88	0.13	1.1	S	6.90	8.23	1.53	5.60	S
441097	0.67	2.22	4.35	1.20	T	1.90	3.13	4.30	3.10	T	12.67	15.67	13.67	14.00	T
441538	2.48	2.22	4.35	3.00	T	5.48	4.83	2.24	3.20	T	14.67	9.50	11.83	12.50	T
441724	0.77	2.56	0.64	1.30	T	1.80	6.20	1.47	3.20	T	9.00	10.67	8.00	9.20	T
441725	0.56	0.77	0.62	0.70	S	1.29	1.47	1.83	1.50	S	9.13	4367	7.33	7.08	T
441755	0.18	0.20	0.20	0.30	S	0.22	1.43	0.61	0.80	S	1.33	1.50	1.47	1.40	S
441768	2.54	1.28	1.28	1.70	T	6.08	5.70	3.10	5.00	T	16.17	16.00	12.67	14.90	T
K566632**	0.12	0.03	0.03	0.10	S	0.22	0.30	0.07	0.20	S	4.50	3.50	1.93	3.30	S
Mean	**0.92**	**0.82**	**0.61**	**0.70**		**2.14**	**1.78**	**1.32**	**1.56**		**8.50**	**7.27**	**7.66**	**7.35**	
	LSD(0.01)- for salt level= 0.06; clone= 0.25; means with the same level= 0.49; means with different salt level = 0.49					**LSD(0.01)** means- for salt level= 0.16; clone= 0.44; means with the same level= 0.88; means with different salt level = 0.87					**LSD(0.01)** means- for salt level= 0.76; clone= 1.67; means with the same level= 3.37; means with different salt level = 3.34				

Molecular assisted assessment of late blight resistance in potato

Hannele Lindqvist-Kreuze, Matilde Orrillo, Willmer Perez, Leticia Portal, Manuel Gastelo, Juan Landeo, Merideth Bonierbale

International Potato Center (CIP), Apartado 1558, Lima 12, Peru
Email: h.kreuze@cgiar.org

Abstract

Late blight is one of the most important diseases of potato causing an estimated yearly economic loss of $3.25 billion in potato-growing areas worldwide. We have identified molecular markers associated with late blight resistance in wild relatives of potato and advanced breeding materials available at CIP. The research focuses on wild species *S. paucissectum* that displays R-gene dependent and/or quantitative resistance, as well as on the advanced tetraploid breeding population B3, which displays high levels of quantitative resistance under high epidemic pressure in a wide range of tropical and subtropical agro ecologies. To understand the genetic components of resistance in these materials we have developed and characterized diploid experimental populations PCC1 (resistant *S. paucissectum*/ susceptible *S. chomatophilum*), and B3C1HP (resistant haploidized B3 clone/ susceptible *S. phureja*). Both of these populations express high levels of field resistance under high endemic late blight pressure, and quantitative trait loci (QTL) for late blight resistance have been located in chrXI in PCC1, and in chrIX in B3C1HP. We have identified molecular markers by 10K potato cDNA microarray and tested these and other markers for association with the resistance phenotype in our mapping populations. Our objective is to develop knowledge of the genetic base of the resistance and tools to facilitate its utilization or transfer across populations.

Keywords: Potato, late blight, molecular markers, resistance breeding.

Introduction

Late blight is one of the most devastating diseases of cultivated potato world wide. Despite a long history of breeding, durable resistance has not been achieved and the disease is mainly controlled by pesticide applications. In searches for durable resistance focus has been turned to the close wild relatives of potato and indeed, many wild Solanum species have proven to contain promising levels of late blight resistance (Perez et al., 2000). New R-genes (for example: Ewing et al., 2000; Foster et al., 2009; Song et al., 2003), as well as quantitative trait loci (for example: Villamon et al 2005; Bisognin et al., 2005) for late blight resistance have been discovered in several of these species. QTL for late blight resistance have been identified on almost every potato chromosome and in many cases the same region of the genome also contains clusters of R-genes (Gebhardt and Valkonen, 2001), thus R-genes are candidates for the QTL effect.

Screening plant material for late blight resistance is normally done by inoculating plants with different isolates or planting the tested material in field locations with endemic disease. However, this type of screening is laborious and its success dependent on environmental factors. Tracking the resistance phenotype by molecular assisted methods could cut back the costs considerably and allow for marker assisted selection of resistant plant material for breeding. Furthermore markers would be helpful in resolving the architecture of late blight resistance in and among available genetic resources.

In this study we are reporting the identification of genetic resistance components in an advanced tetraploid potato clone from the B3C1 population and in a wild species *S. paucissectum* with the help of molecular markers. We also demonstrate that the PCC1 progeny contains at least two new R-genes that locate in the major QTL in the chrXI.

Material and methods

Genotype 391011.17 from the tetraploid population B3C1, highly resistant to late blight, as challenged with *Phytophthora infestans*, and leaves were collected for RNA extraction. The cDNA synthesized was hybridized to

TIGR microarray consisting of approximately 10 000 probes originating from various potato cDNA libraries. Differentially regulated cDNA clones were selected by significant analysis of microarray (SAM) (Tusher et al., 2001) and placed in functional categories by MapMan (Thimm et al., 2004).

Marker CT182 is linked to the quantitative trait locus (QTL) in chr XI, in population PCC1 (*S. paucissectum* / *S. chomatophilum)* (Villamon et al., 2005). PCR based positional candidate markers were selected based on their location in proximity to this marker in published genetic maps of potato and tomato and tested for association with resistance phenotype in PCC1 population. We also generated AFLP (Amplified Fragment Length Polymorphism) and RGA (Resistance Gene Analog) markers for the same purpose. The significant (P≤0.05) effects of single marker alleles on AUDPC values were tested by Students t-test. A detached leaf assay was performed using the PCC1 progeny and *Phytophthora infestans* isolates PE84006, POX-067, PCO-093 and PCO-002 to test for presence of R-genes.

Results

Molecular markers in B3. Of the approximately 10000 probes present on the TIGR potato microarray 274 showed differential responses in B3 genotype due to late blight inoculation as revealed by SAM. The functional classification of these probes by MapMan indicated that 82 had no assigned function. The remaining probes belonged into various functional categories, of which the most interesting, stress related, is shown in Figure 1. According to the classification R-genes involved in recognition, R-gene dependent signaling genes as well as genes involved in hormone signaling, respiratory burst, proteolysis and several pathogenesis related (PR) genes were affected in the B3 genotype during late blight attack. Also secondary metabolism- and abiotic stress related genes and WRKY type transcription factors were affected. With the help of cDNA microarray we have identified a number of candidate genes responsible for the late blight resistance phenotype in B3 population. The location of eight of these candidate genes was determined in the B3C1HP (resistant haploidized B3 clone/ susceptible *S. phureja*) genetic map, and were found to tag chromosomes II, III, IV, VI, IX and XII.

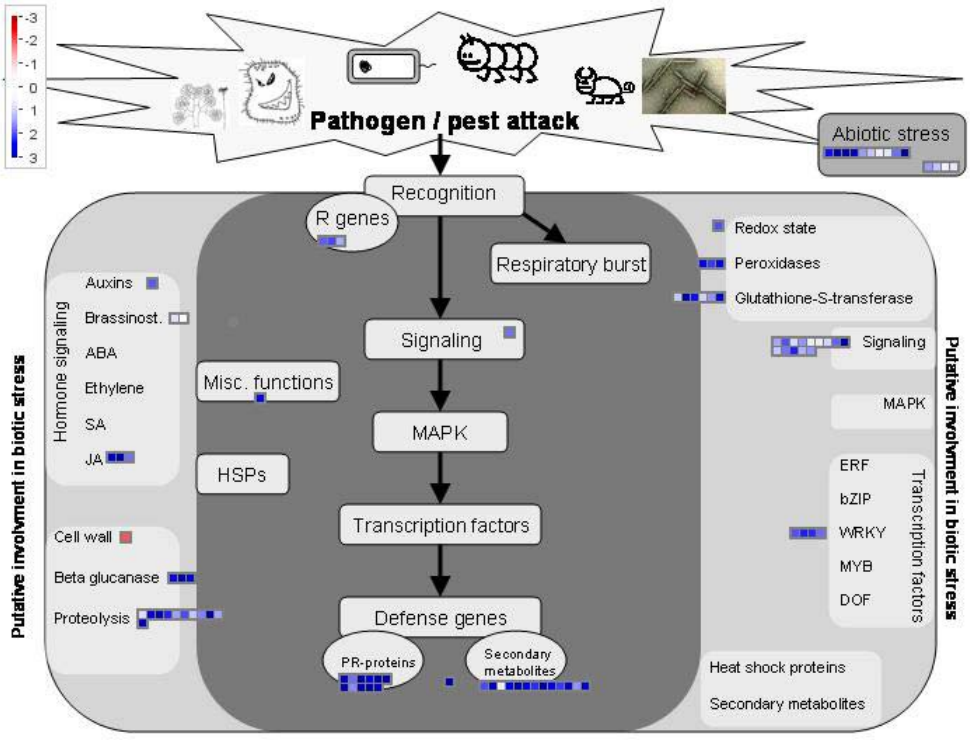

Figure 1. MapMan classified stress related genes differentially regulated in B3 during late blight attack. Each colored square represents a gene and the color of the square indicates it's level of expression as compared to un-inoculated sample.

Molecular markers in PCC1. We used a range of techniques to identify molecular markers associated with the field resistance phenotype in PCC1 population. In total five informative positional candidate marker alleles were found. Four of these mapped into chromosome XI and were associated with the field resistance phenotype in both locations, while one located on chromosome I and was not significantly associated with field resistance. AFLP, NBS and SSR analysis yielded 44, 49 and 15 informative marker alleles, respectively. These marker alleles tagged all 12 chromosomes. In total six AFLP markers located in chromosomes I, II, V and XI, were associated with field resistance at least in one of the test locations (Table 1). Four NBS markers mapping into chromosomes IV and XI were associated with resistance. None of the SSR markers were associated with resistance.

Table 1. Marker type, number of loci scored and number of alleles associated with field resistance in PCC1 progeny in Comas 2002 and Oxapampa 2006

Marker type	loci	Associated with resistance	
		Comas	Oxapampa
AFLP	44	4	5
NBS	49	3	4
SSR	15	0	0
Cand gene	5	0	0
Position candidate	5	4	4
Resistance specificity	4	4	3
Total	122	15	16

R-genes in PCC1 progeny. There was a high frequency of incompatible interactions with the *P. infestans* isolates tested within the group of individuals that had low AUDPC values in the field experiment. The characteristic compatibility pattern in this group was incompatibility with all isolates (Table 2, pattern 3). The most susceptible groups of individuals had no incompatible interactions with any of the complex isolates (PCO93, POX67 and PCO002), but instead had a high number of incompatible reactions with isolate PE84006 (Table 2, pattern 2). The inability of the race 0 -isolate to infect a plant is considered indicative of presence of at least one of the known R-genes originating from S. demissum (Vleeshouwers et al 2000) or it may simply indicate that the isolates' avr factors match additional, previously unidentified R-genes. Although incompatibility to PE84006 was the most common reaction in the resistant progeny, there were a few individuals that displayed compatibility to PE84006 but were incompatible with the complex races suggesting the presence of a new R-gene (Table 2, pattern 1). This R-gene was absent from the susceptible progeny since all individuals with high AUDPC values showed incompatibility to PE84006 and compatibility with the complex races (Table 2, pattern 2). Therefore, it seems that the susceptible progeny has an R-gene with similar specificity as *S. demissum* R-genes other than R9, R5 or R8 originating from the *S. chomatophilum* parent, whereas the progeny with low AUDPC values contain at least two new R-genes originating from the *S. paucissectum* parent (Table 2, patterns 1 and 3). The association of resistance spcificities against each P. infestans isolate with the field resistance phenotype were tested by T-test. All four resistance specificities were associated with the field resistance phenotype in test location Comas, and three in test location Oxapampa (Table 1).

Table 2. Compatibility patterns with four P. infestans isolates detected in DLAs of progenitors of PCC1 and 60 individuals of PCC1 progeny and the average AUDPC of the individuals having the indicated compatibility pattern

Pattern	P. infestans isolates				AUDPC Comas				Progenitors of PCC1	
	race 0 PE84006	avr 5, 8, 9 PCO93	avr 8, 9 POX67	avr 5, 9 PCO002	60-517	1100-1760	2370-3074	Total	chm PI 310991-1	pcs PI 473489-1
1	C	I	I	I	6	1	0	7		
2	I	C	C	C	2	4	19	25	1	
3	I	I	I	I	15	4	0	19		1
4	C	C	C	C	1	7	1	9		
				Total	**24**	**16**	**20**	**60**		

Discussion

The TIGR 10K potato array was used to monitor global gene expression in an advanced tetraploid clone B3C1, under late blight attack. The inoculated samples were compared to healthy non-inoculated plants to filter out the genes expressed due to other reasons than the pathogen attack. cDNAs annotated as putative disease resistance proteins were found differentially expressed in B3, indicating the potential usefulness of identification of functional R-genes by transcriptome profiling. So far R-genes are likely candidates responsible for QTL effect only because of their co-localization on genetic maps. To date, we have preliminary indication of an expression of a major QTL in chr9 in a progeny of a cross where the resistance originates from one of the genotypes of the advanced B3 population, thus we may begin testing hypothesis of co-localization of some candidate R-genes in this genomic region. We have identified several new candidate genes for late blight resistance. We are interested in testing the effect of these genes in entire the B3 population to understand the genetic base of resistance in this population which shows good late blight resistance across a variety of tested tropical and subtropical environments.

Attempts to identify molecular markers associated with the field resistance phenotype by NBS profiling, AFLP and by utilizing positional candidates yielded markers linked with resistance but the best approach was by far the positional candidate method. As described previously by Villamon et al., (2005) a major QTL in the long arm of chr XI (designated QTLpcs11) explained the largest amount of the variation in Comas at 2002, and in a green house experiment. This map segment is a resistance hot spot containing several R-genes against viruses, wart disease and root knot nematode as well as QTL for resistance to other diseases including late blight (Leonards-Schippers et al. 1994, Ewing et al 2000). We identified several molecular markers in this QTL and associated with the field resistance phenotype. Molecular markers based on gene sequences previously mapped in this particular map segment either in potato or tomato were linked with field resistance phenotype. The utility of these markers for marker assisted selection (MAS) remains to be evaluated in *S. paucissectum* accessions.

The phenotypic resistance markers (incompatibilities to isolates POX-067, PCO-093, and PCO-002) indicative of presence of major genes show significant association with field resistance phenotype in both environments tested suggesting possible involvement in quantitative resistance. However, more research is needed to confirm this hypothesis.

References

Bisognin, D.A.; Douches, D.S.; Buszka, L.; Bryan, G.; Wang, D. 2005. Mapping Late Blight Resistance in *Solanum microdontum* Bitter. Crop Sci. 45:340–345.

Ewing, E., Simko, I., Smart, C.D., Bonierbale, M.W., Mizubuti, E.S.G., May, G.D. and Fry, W.E. (2000) Genetic mapping from field tests of qualitative and quantitative resistance to Phytophthora infestans in a population derived from *Solanum tuberosum* and *Solanum berthaultii*. Mol. Breed. 6:25-36.

Foster, S.J., Park, T.-H., Pel, M.A., Brigneti, G., Śliwka, J., Jagger, L., Van der Vossen, E.A.G. and Jones, J.D.G. (2009) Rpi-vnt1.1, a Tm-22 homolog from *Solanum venturii* confers resistance to potato late blight. Mol. Plant-Microbe Interact. 22:589-600.

Gebhardt, C., and Valkonen, J.P.T. (2001) Organization of genes controlling disease resistance in the potato genome. Annu. Rev. Phytopathol. 39: 79-102.

Leonards-Schippers, C.; Gieffers, W.; Schäfer-Pregl, R.; Ritter, E.; Knapp, S.J.; Salamini, F.; Gebhardt, C. 1994. Quantitative resistance to *Phytophthora infestans* in potato: a case study for QTL mapping in an allogamous plant species. Genetics 137, 67–77.

Pérez, W.; Salas, A.; Raymundo, R.; Huaman, Z.; Nelson, R.; Bonierbale, M. 2000. Evaluation of wild potato species for resistance to late blight. CIP Program Report 1999-2000, 49-62.

Song, J.; Bradeen, J.M.; Naess, S.K.; Raasch, J.A.; Wielgus, S.M.; Haberlach, G.T.; Liu, J.; Kuang, H.; Austin-Phillips, S.; Buell, C.R.; Hegelson, J.P.; Jiang, J. 2003. Gene RB cloned from *Solanum bulbocastanum* confers broad spectrum resistance to potato late blight. PNAS 100, 9128-9133.

Thimm; O.; Blaesing, O.; Gibon, Y.; Nagel, A.; Meyer, S.; Krüge, P.; Selbig, J.; Müller, L.A.; Rhee, S.Y.; Stitt, M. 2004. MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J. 37, 914-39.

Tusher, V.G.; Tibshirani, R.; Chu, G. 2001. Significance analysis of microarrays applied to the ionizing radiation response. PNAS 98, 5116–5121.

Villamon, F.; Spooner, D.; Orrillo, M.; Mihovilovich, E.; Perez, W.; Bonierbale, M. 2005. Late blight resistance linkages in a novel cross of the wild potato species *Solanum paucissectum* (series Piurana). Theor. Appl. Genet. 111,1201-1214.

Vleeshouwers, V.; van Dooijeweert, W.; Govers, F.; Kamoun, S.; Colon, L. 2000. The hypersensitive response is associated with host and nonhost resistance to *Phytophthora infestans.* Planta 210, 853-864.

Screening for regeneration and transformation efficiencies of African sweetpotato cultivars

Manrique-Trujillo S.[1†], Wamalwa N. L.[1,2†], Díaz D.[1], Tovar J.C.[1], Kreuze J.[1], Machuka J.[2] and Ghislain M.[1]

[1] Applied Biotechnology Laboratory, International Potato Center, P.O. Box 1558, Lima 12, Peru
[2] Department of Biochemistry and Biotechnology, Kenyatta University, P.O. Box 43844 Nairobi, Kenya
s.manrique@cgiar.org
†Both authors have contributed equally to the reported research

Abstract

Sweetpotato has been shown to be generally recalcitrant to genetic transformation which is a major constraint to develop biotech varieties with key traits not available in the crop's natural gene pool. Regeneration protocols have been reported for few African cultivars but their efficiency remains largely genotype-dependent. In this study, 31 African sweetpotato cultivars from CIP genebank were screened for regeneration and transformation efficiencies by organogenesis and embryogenesis, including "Jewel" and "Jonathan" varieties as organogenic and embryogenic controls respectively. First three leaves with petioles, or the three first lateral meristems were used as explants in organogenic and embryogenic experiments respectively. Regeneration by organogenesis was conducted using a two-step protocol including 2,4-D then thidiazuron, zeatin or kinetin while regeneration by embryogenesis was performed using a three-step protocol, each one using a different hormone (2,4,5-T, ABA and AG_3). More than 40% regeneration efficiencies were obtained for 6 cultivars (Imby, Kawogo, Luapula, Mafutha, Mugande and Zambezi) with organogenesis and 8 cultivars (Bwanjule, Imby, K51/3251, Luby, Malawiala, Mugande, New Kawogo and SPK004) with embryogenesis protocol after the second-step (culture media with ABA). Transformation efficiencies of these cultivars are currently estimated using GUS transient expression assay. Preliminary results show efficiencies between 30-90%. Our results suggest that our pre-screening for high regeneration and transformation efficiency has identified a dozen African cultivars amenable to genetic transformation.

Keywords: Sweetpotato, embryogenesis, organogenesis, genetic transformation.

Introduction

Sweetpotato production in Sub-Saharan Africa is mainly produced by small-scale farmers both for consumption and as a source of income (Dapaah, 1994) contributing to food security in the region. Its production in Eastern Africa yields as low as 4.17 tons as compared to its potential of 50 tons per hectare, reported in FAOstat (2006).

Constraints to production are numerous ranging from socio-economical, agronomical to biological factors. Biological constraints such as pests and diseases have been reported to cause losses between 50-100%, with weevils and virus diseases ranking the highest (Stathers *et al.*, 2005). Efforts have been made towards addressing the weevil problem with little success (Abidan *et al.*, 2005; Braun and Fliert, 1999; Dhir *et al.*, 1998; Yaku, 1992). Biotechnology offers the possibility of expanding and optimizing the use and importance of sweetpotatoes through genetic engineering, by developing a sweetpotato expressing a protein active against weevils. In order to obtain such resistant variety, a robust regeneration and transformation protocol must be established first.

Regeneration and transformation protocols have been developed for sweetpotato (Chen *et al.*, 2006; Liu *et al.*, 2001; Luo *et al.*, 2006; Sihachakr *et al.*, 1997). This crop is known to be highly recalcitrant and all protocols published so far are genotype-dependent. Genetic transformation can be achieved but for very few cultivars and with low efficiencies (Opabode, 2006). Hence, improved protocols for regeneration and transformation of African sweetpotato cultivars are in great needs.

Material and methods

Plant materials and propagation

Thirty one African sweetpotato cultivars from CIP genebank were screened for regeneration capacity using Jewel and Jonathan cultivars as controls. These cultivars are Rusenya RWA, Rusenya BDI, Mohc, Luby, Imby, Chifukama, Chihongo, Chiuva, Namagizi, Kemb10, Kemb37, Gikanda, Muibai, KSP11, SPK004, SPK013, Mafutha, Kamchiputu, Mugande, K51/3251, Malawiala, Budagala, Mwanamonde, Sinia, SPN/O, Kawogo, Bwanjule, New Kawogo, Chingowva, Luapula and Zambezi.

Organogenesis assay

The three apical leaves with petioles were used as explants, which were collected after 3 to 4 weeks from propagation. Regeneration was assayed using a two-step protocol reported by Blasco (2007).

Embryogenesis assay

Regeneration through somatic embryogenesis was assayed using the first three lateral meristems as explants. A three-step protocol having each step using a different hormone was applied (Liu *et al.*, 2001; Al-Mazrooei *et al.*, 1997; Dhir *et al.*, 1998). The initial step had MS with 2,4,5-T (in the dark), followed by MS with ABA and finally with GA_3.

Genetic transformation

Plants were infected with the hypervirulent strain EHA105 of *Agrobacterium tumefaciens* carrying the plasmid pCIP100, which differs from pCAMBIA1305.1 by conferring kanamycin resistance. This step was done according to protocols described in Medina-Bolivar *et al.* (2003), Dhir *et al.* (1998), Luo *et al.* (2006) and Xing *et al.* (2008). Genetic transformation efficiencies were assessed using a GUS expression assay following the CAMBIA protocol (www.cambia.org).

Results and discussion

Organogenesis

Regeneration commenced after three weeks for some of the cultivars while others remained recalcitrant during the two months in culture. Regeneration efficiencies expressed in percentage were calculated from the number of regenerating shoots divided by the explants. These were highly genotype-dependent ranging between 0–86% depending on the phyto-hormones used in the regeneration media. Six cultivars showed regeneration efficiencies above 40% (Table 1).

Table 1. Organogenic regeneration efficiencies for the best sweetpotato African cultivars from the CIP collection

Cultivar	CIP number	Percentage (%)
Zambezi	CIP 441772	86
Luapula	CIP 441763	60
Kawogo	CIP 440165	48
Mugande	CIP 440163	60
Mafutha	CIP 441862	46
Imby	CIP 440037	83

NB: Results are likely to change once all the cultivars have all samples included

All cultivars had swollen petioles after the auxin treatment followed by calli formation within a week; contrary to a recent protocol developed by Santa-Maria *et al.* (2009) who differed with the use of 2, 4-D on some sweetpotato varieties from the USA. Our results coincide with previous studies with African sweetpotato cultivars (Blasco, 2007; Oggema *et al.*, 2007) in that none of our hormone treatment avoided the genotype-dependence.

Embryogenesis

After two or three weeks of the explants in culture, three different type of callus were observed: pro-embryogenic callus (yellowish, compact, and slow growing), non-embriogenic callus (white or cream, friable and fast growing) and some with pro-embryogenic and non-embryogenic parts on the same callus, as reported previously by Otany & Shimada (1996).

Table 2. Efficiencies of embryogenic calli formation of the best African sweetpotato cultivars from the CIP collection

Accession name	CIP number	% embryogenic calli
K51/3251	CIP 440164	62.9
Luby	CIP 440036	46.7
Mugande	CIP 440163	43.6
New Kawogo	CIP 441745	43.5
Imby	CIP 440037	42.9
Malawiala	CIP 440172	41.5
Bwanjule	CIP 440168	40.0
SPK004	CIP 441768	40.0

NB: Results are likely to change once all the cultivars have all samples included

Eight cultivars had embryogenic calli above 40% (Table 2) in the second step of regeneration while other cultivars evaluated did not show embryogenic tissues. Inability to form embryogenic calli even using different phyto-hormones to induce embryogenesis has been reported in sweetpotatoes (Al-Mazrooei *et al.*, 1997; Triqui *et al.*, 2008).

Preliminary results of the GUS assays showed efficiencies between 30-90%. Final results of regeneration and transformation efficiencies are still pending but this step is known to be less limiting and genotype dependent than the regeneration.

In conclusion, we report here the identification of twelve sweetpotato African cultivars with workable regeneration efficiencies. This represent about one third of the genotypes tested. Preliminary results with GUS assays for these cultivars show high transformation efficiencies. We hope that using these improved protocols, researchers will be able to genetically engineer this important crop to withstand devastating production constraints, such as weevils and virus diseases.

Acknowledgement

The authors are grateful to continuous financial support from The Rockefeller Foundation, the Belgian Directorate General for Development Cooperation, and the USAID.

References

Abidin, P. E.; van Eeuwijk, F. A.; Stam, P.; Struik, P. C.; Malosetti, M.; Mwanga, R. O. M.; Odongo, B.; Hermann, M.; Carey, E. E. 2005. Adaptation and stability analysis of sweetpotato varieties for low-input systems in Uganda Plant Breeding 124: 491–497

Al-Mazrooei, S.; Bhatti, M.H.; Henshaw, G.G.; Tayor, N.J.; Blakesley, D. 1997. Optimization of somatic embryogenesis in fourteen cultivars of sweet potato [*Ipomoea batatas* (L.) Lam.]. Plant Cell Rep. 16: 710–714

Blasco, C. 2007. Regeneracion 'in-vitro' via organogenesis de cinco cultivares de camote (*Ipomoea batatas* Lam.): Tanzania, SPK004, Wagabolige, 199004.2 y nanshu. Tesis para optar el titulo de bióloga; Facultad de Ciencias, Universidad Nacional Agraria la Molina

Braun, A.R.; Van De Fliert, E. 1999. Evaluation of the impact of Sweetpotato weevil (Cylas formicarius) and of the effectiveness of Cylas sex pheromone traps at the farm level in Indonesia. International Journal of Pest Management 45: 101-110

Chen, L.; Bhasagari, A.; Carter, J. 2006. Effects of medium composition and culture duration on in vitro morphogenesis of sweetpotatoes. Biologia plantarum 50: 114-117

Dapaah, S.K. 1994. Contributions of Root and Tuber Crops to Socioeconomic Changes in the Developing World: The Case of Africa, with Special Emphasis on Ghana. Acta Hort. (ISHS) 380:18-25

Dhir, S.K.; Oglesby, J.; Bhagsari, A.S. 1998. Plant regeneration *via* somatic embryogenesis, and transient gene expression in sweet potato protoplasts. Plant Cell Rep. 17: 665–669

Liu, Q.C.; Zai, H..; Wang, Y.; Zhang, D.P. 2001. Efficient plant regeneration from embryogenic suspension cultures of sweetpotato. In Vitro Cellular and Developmental Biology-Plant 37: 564-567

Luo, H.R.; Santa Maria, M.; Benavides, J.; Zhang, D.P.; Zhang, Y.Z.; Ghislain, M. 2006. Rapid genetic transformation of sweetpotato *(Ipomoea batatas* (L.) Lam) via organogenesis. African Journal of Biotechnology 5: 1851-1857

Medina-Bolivar, F., Wright, R., Funk, V., Sentz, D., Barroso, L., Wilkins,T., Petri Jr., W., Cramer, C., 2003. A non-toxic lectin for antigen delivery of plant-based mucosal vaccines. Vaccine 21, 997–1005.

Oggema, J.N.; Kinyua, M.G.; Ouma, J.P. 2007. Optimum 2, 4-D concentration suitable for embryogenic callus induction in local Kenyan sweetpotato cultivars. Asian Journal of Plant Science 6: 484-489

Opabode, J.T. 2006. *Agrobacterium*-mediated Transformation of Plants: Emerging Factors that Influence Efficiency. Biotechnology and Molecular Biology Review 1: 12-20

Otany, M.; Shimada, T. 1996. Efficient embryogenic callus formation in sweet potato (*Ipomea batatas* (L.) Lam.). Breeding Science 46: 257-260

Sihachakr, D.; Haicour, R.; Cavalcantes, A.J.M. ; Umboh, I.; Nzoghe, D.; Servas, A.; Ducreux, G. 1997. Plant regeneration in sweetpotato (*Ipomoea batata* L., Convolvulaceae) Euphytica 96: 143-152

Santa-Maria, M.; Pecota, K.V.; Yencho, C.G.; Allen, G. and Sosinski, B. 2009. Rapid shoot regeneration in industrial high starch sweetpotato (Ipomoea batatas L.) genotypes. Plant cell Tiss Organ Cult 97: 109-117

Stathers, T.; Namanda, S.; Mwanga, R.O.M.; Khisa, G.; Kapinga, R. 2005. Manual for Sweetpotato Integrated Production and Pest Management Farmer Field Schools in Sub-Saharan Africa. International Potato Center. Kampala, Uganda. 168 pp.

Triqui, Z.E.A.; Guedira, A.; Chlyah, B.; Chlyah, H.; Souvannavong, V. ; Haicour, R.; Sihachakr, D. 2008 . Effect of genotype, gelling agent, and auxin on the induction of somatic embryogenesis in sweet potato (*Ipomoea batatas* Lam.) C R Biol. 331:198-205

Yaku, A. 1992. Effects of intercropping sweetpotato on the population density of sweetpotato weevil (Cylas formicarius (F.) Coleoptera: Curculionidae). MSc. thesis, University of Montreal, Department of Entomology, Quebec Canada

Xing, Y.J.; Ji, Q.; Yang, Q.; Luo, Y-M.; Li, Q.; Wang, X. 2008. Studies on *Agrobacterium*-mediated genetic transformation of embryogenic suspension cultures of sweet potato. African Journal of Biotechnology 7: 534-540

Genetic variability in commercial varieties of water yam with microsatellites markers

Marcos V.B.M. Siqueira[1*], Gabriel Dequigiovanni[2], José Carlos Feltran[3], Elizabeth A. Veasey[1]

[1]Genetic Department, "Luiz of Queiroz" College of Agriculture, Piracicaba, Brazil; [2]Rio Grande do Sul Federal University, Bento Gonçalves, Brazil; [3]Agronomic Institute, Campinas, Brazil; *E-mail: mvbms@esalq.usp.br.

Abstract

This study aimed at evaluating the genetic diversity of commercial water yam varieties with microsatellite markers. Yam is the fourth more important crop from the tuber and root crops in the world. We assessed 27 varieties of *Dioscorea alata*, collected in markets of several cities in Brazil, and from the germplasm collection of the Agronomic Institute (IAC), in Campinas, SP. CTAB 3% method was used for DNA extraction. We used 12 microsatellite primers for the amplification reactions. The amplified material was separated on 6% polyacrylamide gels and stained with silver nitrate. We performed clustering analysis and principal coordinates, using the NTSYS-pc software. High polymorphism was found among loci verified by the high PIC values (0.46 - 0.87). The accessions were classified in two main groups, showing great genetic variability, verified by the magnitude of the Jaccard's similarity coefficient (0.29 to 1.0). One of the main groups ranked most of the IAC's collection, with accessions originating from Africa, the Belgian Congo, being similar to varieties marketed today. In the second group, there was a high similarity of an accession originated from Puerto Rico, which led to the Florida variety, the most planted now-a-days, with several commercial varieties. The results, therefore, suggest the hypothesis of separate ancestry of the varieties currently under cultivation in Brazil. Four duplicates were found with accessions acquired in different cities, revealing the marketing of the same variety in different locations. The analysis of principal coordinates, with the first two coordinates explaining 49.2% of total variation, confirmed the groupings formed.

Keywords: *Dioscorea alata*, SRR, genetic diversity, germplasm bank.

Introduction

Yam is one of the most important crops in the tropics and subtropics, being mainly cultivated in the West and Central regions of Africa, where it is also widely consumed (Mignouna et al., 2005). It belongs to the Dioscoreaceae family and *Dioscorea* genus, which contains approximately 600 species, where only 10 are economically viable for consumption (Pedralli, 2002).

The world-wide yam production, estimated in 2005, was approximately 44.3 million tons, with Nigeria being the largest world-wide producer. The remaining is produced mainly in Latin America, the Melanesia and Japan (FAO, 2005). The Brazilian yam production is concentrated in the Northeast and Southeast regions, in the states of Paraíba, Rio de Janeiro, Minas Gerais, Pernambuco, Espírito Santo and São Paulo, responsible for approximately 90% of total national yield (Mesquita, 2001). In the Southeast region, 83.766 tons of *Dioscorea alata* L. were produced in the year 2000, and this number was not higher due to gaps in public and private sector which do not allow the markets supply and a quality standard of these tubers to be reached (Mascarenhas and Resende, 2002).

Studies with molecular markers, including microsatellites (SSR), have become an important tool for the identification of cultivars, and mainly for population genetics studies (Caixeta et al., 2006). Besides, since this marker is, in general, more abundant in the majority of the genomes, possessing a high informative content, it becomes interesting to use it as tool for the assessment of distinct yam (*D. alata*) varieties. Microsatellite primers have already been developed for a few *Dioscorea* species (Hochu et al., 2006; Bousalem et al., 2006), including *D. alata* (Tostain et al., 2006). Fundamentally, SSR markers have been used in studies of segregation patterns and genetic characterization of accessions of *Dioscorea* species (Terauchi and Konuma, 1994; Mignouna et al., 2003a; Mignouna et al., 2003b; Scarcelli et al., 2005).

This study aimed to access the genetic diversity of 27 yam commercial varieties obtained in fairs, markets and germplasm banks using SSR markers, in order to verify the level of genetic vulnerability. The work aimed to contribute for *in situ* and *ex situ* conservation strategies, emphasizing the role of local breeders for the propagation and maintenance of this crop.

Materials and methods

This study used 27 accessions belonging to the germplasm collections from "Luiz of Queiroz" College of Agriculture/São Paulo University (ESALQ/USP), in Piracicaba, SP, and the Agronomic Institute (IAC), in Campinas, SP. The accessions were obtained from different municipalities in Brazil, and also from Puerto Rico, Singapore and Belgian Congo (Table 1).

For DNA extraction, the Siqueira et al. (2009) methodology, based in CTAB extraction buffer, with modifications, was used. Thirteen microsatellite loci (primers), based on Tostein et al. (2006) and Mignouna et al. (2003) were used. The polymerase chain reactions (PCR) were conducted in a final volume of 10.2 µL, in accordance with Siqueira et al. (2009). The reactions were accomplished in the thermocycler BioRad®, MyCycler® model, in the following amplification conditions: initial denaturalization at 94ºC for 5 min, followed by 35 cycles of denaturalization at 94ºC for 30 s, 1 min at the defined annealing temperature for each primer (Table 1), and 1 min at 72ºC, with a final extension at 72ºC for 8 min (Tostein et al., 2006). The amplification products were submitted to electrophoresis in a polyacrylamide gel (6%) using a silver staining procedure (Bassam et al., 1991).

Table 1. List of the 27 *Dioscorea alata* accessions used in this study, including their origin and common names

Germplasm Number[1]	Origin (municipalities/state/country)	Introduction year	Origin	Variety name
SRT 3.0	Campinas – SP - Brazil	1936	IAC	Mimoso
SRT 24.0	Sorocaba – SP – Brazil	1947	IAC	Sorocaba
SRT 29.0	Puerto Rico	1947	IAC	Flórida
DGC 36.0	Iguape – SP – Brazil	2002	Local market	---
DGC 38.0	Araras – SP – Brazil	2002	Local market	---
DGC 40.0	Piracicaba – SP – Brazil	2002	Local market	---
DGC 43.0	Matão – SP – Brazil	2002	Local market	---
DGC 45.0	Campinas – SP – Brazil	2002	IAC	---
DGC 46.0	Piracicaba – SP – Brazil	2002	Local market	---
SRT 66.0	Congo Belga – Jamgambi	1949	IAC	Angola II
SRT 71.0	Congo Belga – Jamgambi	1949	IAC	Bira
SRT 75.0	Congo Belga – Jamgambi	1949	IAC	Leno Dandino
SRT 78.0	Singapore	1949	IAC	Singapura roxo
SRT 80.0	Minas Gerais – Brazil	1949	IAC	Branco Viçosa
SRT 84.0	Campinas – SP – Brazil	1951	IAC	Cova Campinas
SRT 89.0	Araraquara – SP – Brazil	1959	IAC	Araraquara I
DGC 97.0	Cuiabá – Mato Grosso – Brazil	2006	Municipal market	---
DGC 107.0	Botucatu – SP – Brazil	2006	Local market	---
SRT 112.0	Mato Grosso do Sul – Brazil	2000	IAC	Cará do Mato
DGC 115.0	Fortaleza – CE – Brazil	2006	Municipal market	---
DGC 116.0	Cuiabá – Mato Grosso – Brazil	2006	Municipal market	---
DGC 123.0	Mogi-Guaçu – Brazil	2006	Small farmer	---
DGC 124.0	Campo Grande – Mato Grosso – Brazil	2007	Municipal market	---
DGC 127.0	Santa Mercedes – SP – Brazil	2007	Small farmer	---
DGC 128.0	Belo Horizonte – Minas Gerais – Brazil	2007	Municipal market	---
DGC 129.0	Espírito Santo – Brazil	2007	Municipal market	---

DGC 132.0	Fernandópolis – SP - Brazil	2007	Local market	---

[1]SRT – germplasm from the Agronomic Institute (IAC); DGC – germplasm from ESALQ/USP.

For the statistical analysis a similarity matrix was obtained for the 27 yam accessions using binary data and the Jaccard similarity coefficient method. With this coefficient and the UPGMA (Unweighted Pair Group Method with an Arithmetic Mean) (Sneath and Sokal, 1973) method, cluster analyzes were accomplished, using the NTSYSpc software (Rohlf, 1992). The precision of the generated groupings was estimated from sampling simulations, considering 10.000 bootstraps, using BOOD, version 2.0 software (Coelho, 2001). An analysis of principal coordinates was also accomplished with the NTSYSps software (Rohlf, 1992). The number of alleles per loci as well as the Polymorphism Information Content (PIC) were also determined.

Results and discussion

In this study, all 13 loci (primers) used showed polymorphism between the analyzed accessions, producing well definite and reproducible fragments. A total of 83 alleles (fragments) were amplified with an average of 7 alleles per loci (Table 2). The highest number of alleles (10) was verified for loci Dab2E07, and the lower number (4) was found for primers Da1D08 and YM-28. The polymorphism information content (PIC) values varied from 0.87 to 0.46, with 0.71 on average. The highest value was obtained for primer Dab2E07 and the lower value for primer Da1D08, demonstrating that the SSRs used in the present study presented, on average, a high level of information.

Table 2. Polymorphism detected based on SSR primers, including allele number, annealing temperature, fragment size and polymorphism information content (PIC) when assessing 27 *Dioscorea alata* accessions

Primers	Allele number	Annealing temperature (°C)	Fragments size (pb)	PIC
Dpr3D06	8	56	140 – 180	0.7206
Dpr3F04	8	51	100 – 145	0.7372
Da1A01	7	50	210 – 270	0.7562
Da1C12	5	55	165 – 190	0.6932
Dab2C05	9	50	145 – 205	0.8073
Dab2D06	8	50	260 – 325	0.8041
Da1D08	4	50	310 – 325	0.4629
Dab2E07	10	50	150 – 220	0.8696
Da1F08	9	53	190 – 250	0.6905
YM-19	5	50	230 – 255	0.6538
YM-28	4	50	380 – 440	0.5834
DprF3F12	6	55	155 – 200	0.7836
Dpr3D06	8	56	140 – 180	0.7206
Mean	7	-	-	0.7141

In the cluster analysis (Figure 1), the Jaccard's similarity coefficient varied from 0.29 to 1.0, indicating the existence of a significant genetic variability among the accessions. This genetic variability is divided in two main groups: the first group represented, in its majority, the genetic material of IAC collection, presenting two distinct sub-groups: sub-group 1, including varieties Mimoso, Sorocaba, Branco Viçosa, Bira and Leno Dandino, the two last accessions originating from Belgian Congo; sub-group 2, which includes cultivar Angola II, also originating from Belgian Congo, grouped with 99.8% similarity with two commercial varieties obtained in markets in the state of São Paulo (in the cities of Matão and Piracicaba), indicating that, most probably, these commercial varieties originated from the Angola II cultivar. Sub-group 2 was also constituted by varieties Cova Campinas (mutant variety), and Cará do Mato (originating from Mato Grosso do Sul), both from the IAC germplasm bank, besides a commercial variety collected in Cuiabá.

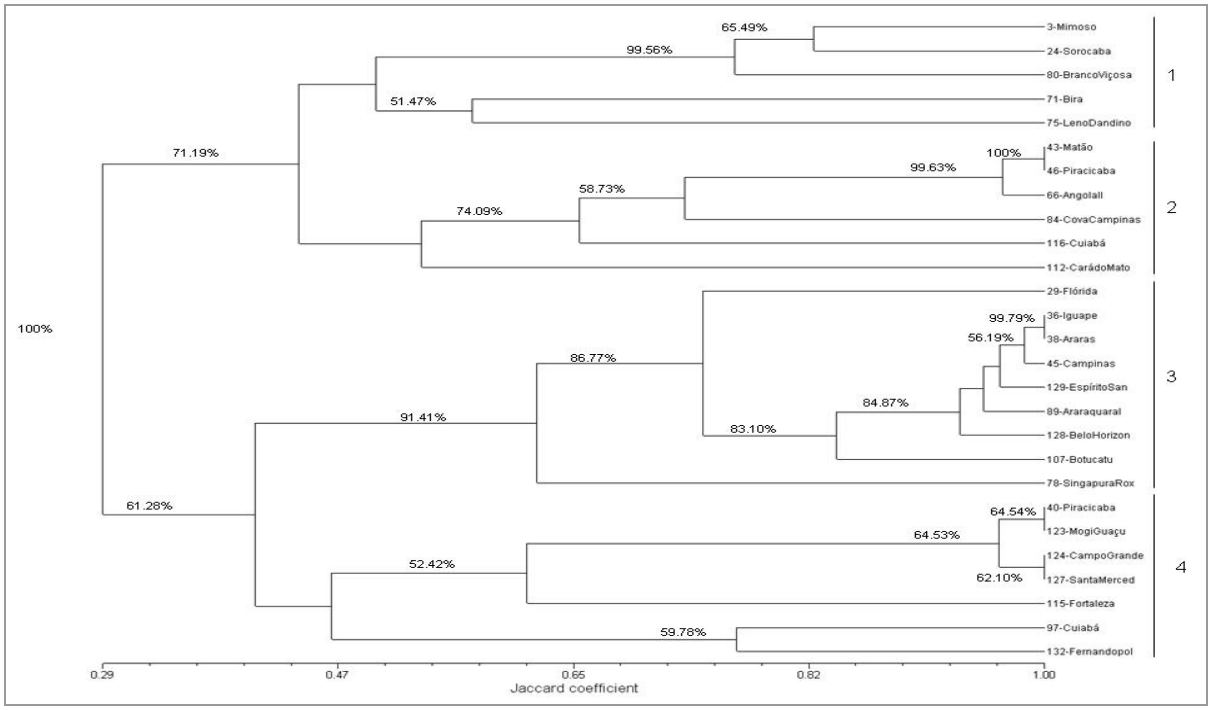

Figure 1. Dendrogram obtained by UPGMA method, Jaccard similarity coefficient and the Bootstrap method (percentages) for 27 *Dioscorea alata* accessions

The second main group, including two sub-groups (sub-group 3 and sub-group 4), is represented in its majority by commercial varieties obtained in Brazilian markets, with the exception of two genetic materials from IAC collection (SRT 29 from Puerto Rico and SRT 78 from Singapore). Sub-group 3 includes Florida variety, introduced in the 1950s and currently the most accepted commercially in São Paulo State (Monteiro and Peressin, 2002), which showed around 70% similarity to a group of commercial varieties obtained in markets in the cities of Iguape, Araras, Campinas, Araraquara, Botucatu, Rio de Janeiro and Belo Horizonte. Therefore, the results indicate that these varieties were probably originated from Florida variety, originally derived from Puerto Rico (Abramo, 1990). This sub-group also includes, although with lower similarity (<65%), the Purple Singapore variety (from IAC), introduced from Singapore in 1949.

Sub-group 4 includes accessions with high similarity level, obtained in Piracicaba, Mogi Guaçu (São Paulo), Campo Grande (Mato Grosso do Sul) and Santa Mercedes (São Paulo), which were grouped, with around 60% similarity, with a variety collected in Fortaleza, Ceará State. This group includes two other commercial varieties, one collected in Cuiabá, Mato Grosso and another in Fernandópolis, São Paulo. Similarly, the principal coordinates scatter graph (Figure 2) agrees with the data presented in the dendrogram, allowing the visualization of the two main groups and the four sub-groups previously mentioned.

Accessions with high similarity level, including materials from distinct regions, were observed in this study, showing the cultivation of the same variety in diverse cities of Brazil or the marketing of the same variety in several places, most probably through CEASA (market supply and distribution center) located in several municipalities of several Brazilian states. Also, it was possible to evidence that materials deriving from other countries, such as Puerto Rico, Singapore and Africa, including Belgian Congo, formed independent groups, which can lead to the hypothesis of distinct ancestry for the varieties commercialized today in Brazil.

High levels of polymorphism were presented by *D. alata* in this study, considering that each allele is considered a unique character and, being a tetraploid species, each individual can present one to four different alleles in each loci. Additionally, the high levels of polymorphism suggest that this molecular marker can be a useful tool with a high precision in the detection of genetic differences between cultivars. However, additional studies, involving a

higher number of accessions, shall be accomplished aiming to a better understanding on how this species germplasm is structured and organized, and on the origins of the cultivated commercial varieties in Brazil.

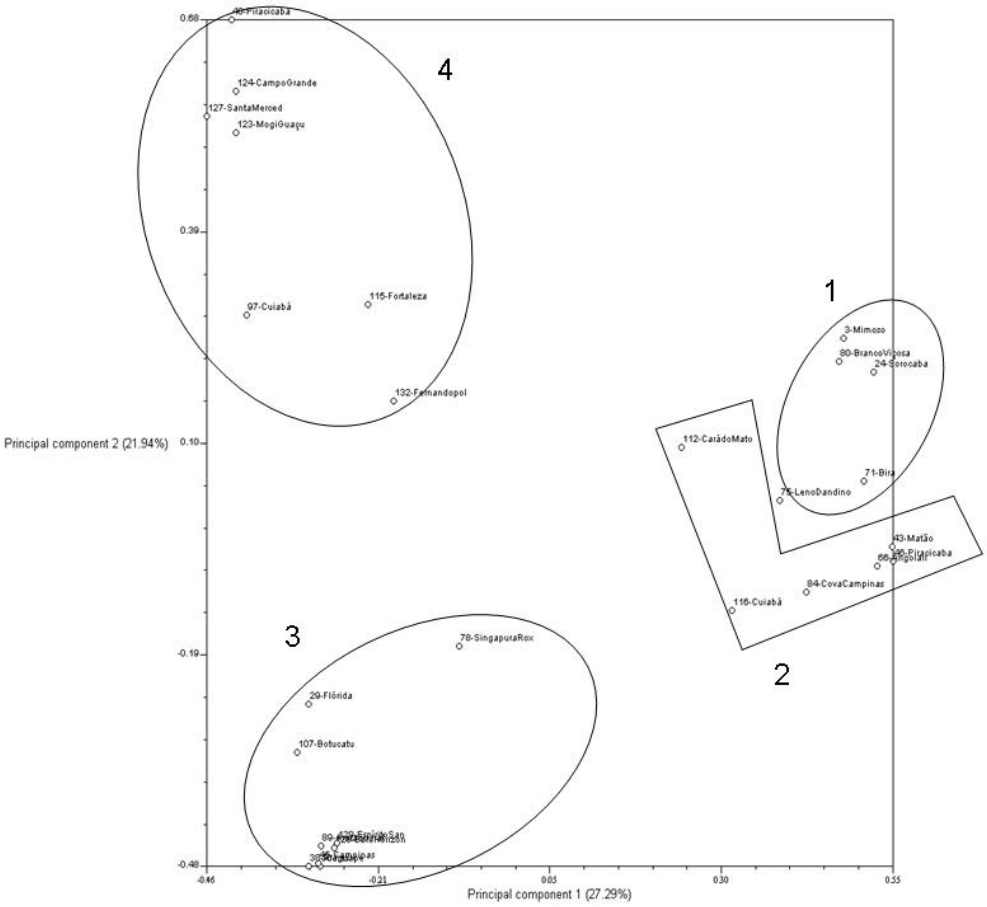

Figure 2. Scatter graph of 27 yam (*Dioscorea alata* L.) accessions obtained from a principal coordinate analysis

Conclusions

Microsatellite markers allowed the identification of high genetic variability in the yam (*D. alata*) accessions originated from two germplasm collections. These markers represent an important tool for the construction of genetic profiles of yam cultivars, showing potential to be used in plant breeding programs and in *ex situ* yam conservation programs as well. The results allowed to infer on the genetic origin of the commercial varieties currently cultivated in Brazil, and on its market distribution.

Acknowledgements

The authors wish to thank FAPESP and CAPES for the financial support and grants given for this research.

References

ABRAMO, M.A. 1990. Taioba, cará, inhame: o grande potencial inexplorado. São Paulo: Ícone. 80p.

AYRES. M.; AYRES,-JR. M.; AYRES, D. L.; SANTOS, A. S. 2005. BioEstat 4.0: Aplicações estatísticas nas áreas das ciências biológicas e médicas. Ed. Sociedade Civil Mamirauá / MCT / Imprensa Oficial do Estado do Pará. Belém. 323p.

Bassam, B.J.; Caetano-Anolles, G.; Gresshoff, P.M. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal Biochem 196, 80-83.

Bousalem, M.; Arnau, G.; Hochu, I.; Arnolin, R.; Viader, V.; Santoni, S.; David, J. 2006. Microsatellite segregation analysis and cytogenetic evidence for tetrasomic inheritance in the American yam *Dioscorea trifida* and a new basic chromosome number in the Dioscoreae. Theor Appl Genet 113, 439–451.

Caixeta, E.T.; Oliveira, A.C.B. de; Brito, G.G. de; Sakiyama, N.S. 2006. Tipos de marcadores moleculares. In: Borém, A.; Caixeta, E.T. (Ed.). Marcadores moleculares. Viçosa: UFV.

Coelho, A.S.G. 2001. BOOD: avaliação de dendrogramas baseados em estimativas de distâncias/similaridades genéticas através do procedimento de bootstrap, v. 2.0. Goiânia: Universidade Federal de Goiás, Instituto de Ciências Biológicas, Departamento de Biologia Geral.

Fao. 2005. Food And Agriculture Organization Of The United Nations. Disponível em: <www.fao.org>. Acesso em: 28 de Março de 2009.

Hochu, I.; Santoni, S.; Bousalem, M. 2006. Isolation, characterization and cross-species amplification of microsatellite DNA loci in the tropical American yam *Dioscorea trifida.* Mol Ecol Notes, 6, 137–140.

Jatasra, D.S. Paroda, R.S. 1983. Genetic divergence in wheat. Indian J Genet Plant Breed, v. 43, p. 63-67.

Mascarenhas, M.H.T.; Resende, L.M.A. 2002. Situação atual e perspectivas das culturas do taro e do inhame no estado de Minas Gerais**.** In: Carmo, C.A.S. (ed). *Inhame e taro: sistemas de produção familiar*. Vitória: Incaper. p. 61-71.

Mesquita, A.S. 2001. Inhame na Bahia: a produção no caminho da competitividade. Bahia Agrícola, v.4, n.2, p.39-48.

Mignouna, H.D.; Abang, M.M.; Fagbemi, S.A. 2003a. A comparative assessment of molecular marker assays (AFLP, RAPD and SSR) for white yam (*Dioscorea rotundata*) germplasm characterization. Ann Appl Biol 142, 269-276.

Mignouna, H.D; Abang, M.M.; Asiedu, R. 2003b. Harnessing modern biotechnology for tropical tuber crop improvement: Yam (Dioscorea spp.) molecular breeding. Afr J Biotechnol 2, 478-485.

MIGNOUNA, H. D.; ABANG, M.M.; WANYERA, N.W.; CHIKALEKE, V.A.; ASIEDU, R.; THOTTAPPILLY, G. 2005. PCR marker-based analysis of wild and cultivated yams (*Dioscorea* spp.) in Nigeria: genetic relationships and implications for *ex situ* conservation. Genet. Resour. Crop Ev, v. 52, p. 755-763.

Monteiro, D.A.; Peressin, V.A. 2002. Cultura do inhame. In: Cereda, M.P. (Ed.). Agricultura: tuberosas amiláceas Latino Americanas. São Paulo: Fundação Cargill, p. 511-522.

MONTALDO, A. (1991). Cultivo de raíces y tubérculos tropicales. Lima: Instituto Interamericano de Ciências Agrícolas de la OEA, p. 91-127.

PEDRALLI, G. 2002. Distribuição geográfica e taxonomia das famílias Araceae e Dioscoreaceae no Brasil. In: Carmo, C.A.S. (Ed.) Inhame e taro: sistemas de produção familiar. Vitória, ES: Incaper. p.15-26.

Rafalski, D.J.A.; Vogel, J.M.; Morgante, M.; Powell, W.; Andre, S.; Tingey, S.V. 1996. Generating and using DNA markers in plant. In: Birren, B.; Lai, E. Non mammalian genomic analysis: a pratical guide. New York: p. 75-134.

Scarcelli, N.; Daïnou, O.; Agbangla, C.; Tostain, S.; Pham J.L. 2005. Segregation patterns of isozyme loci and microsatellite markers show the diploidy of African yam *Dioscorea rotundata* (2n=40). Theor Appl Genet 111, 226-232.

Siqueira, M.V.B.M., Queiroz-Silva, J.R., Bressan, E.A., Borges, A., Pereira, K.J.C., Pinto, J.G.; Veasey, E.A. 2009. Genetic characterization of cassava (*Manihot esculenta*) landraces in Brazil assessed with simple sequence repeats. Genet Mol Biol, v.32, p.104-110.

SNEATH P.H.A; SOKAL R.R. 1973. Numerical Taxonomy*.* San Francisco: Freeman. 573 pp.

Terauchi, R.; Konuma, A. 1994. Microsatellite polymorphism in *Dioscorea tokoro*, a wild yam species. Genome, 37, 794–801.

TOSTEIN, S.; SCARCELLI, N.; BROTTIER, P.; MARCHAND, J.L.; PHAM, J.L.; NOYER, J.L. 2006. Development of DNA microsatellite markers in tropical yam (*Dioscorea* sp.). Mol Ecol Notes, v. 6, p. 173-175.