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Development of Microsatellite Markers for Anthracnose Resistance in Greater Yam (*Dioscorea alata* L.) from Expressed Sequence Tags of Heterologous Crop Species

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Abstract

Greater yam (*Dioscorea alata* L.), belonging to the family Dioscoreaceae, is a staple food crop of the tropics. It is cultivated for its edible, nutritious, underground tubers. Its productivity declined due to anthracnose disease. There was no sequence information for yam in the public database. Therefore, the current study was designed with an objective to generate simple sequence repeat (SSR) markers for yam from publicly available expressed sequence tag (EST) sequences of crops related to yam. A total of 415801 ESTs collected from 15 different species were used to design 600 SSR primer pairs. Out of 600, only 308 SSR primer pairs were included in the study. Among the 308 screened, 39 (13%) pairs were identified polymorphic for greater yam. The PCR-based polymorphic microsatellites in greater yam revealed 24 markers with distinct gene expression. Out of the 24, five markers (VSU 146, VSU 155, VSU 161, VSU 172 and VSU 182) were informative for use in greater yam anthracnose resistance. These five informative markers indicated resistance to fast-growing salmon (FGS) or slow-growing grey (SGG) strains of *Colletotrichum gloeosporioides* (*C.g.*) that cause anthracnose disease in greater yam. The 24 polymorphic markers could be tried for screening other strains of *Colletotrichum gloeosporioides.*

Key words: *Dioscorea alata* L., simple sequence repeat markers, yam anthracnose

Introduction

Dioscorea alata L. (greater yam/water yam/winged yam) is an important crop species of the tropical and subtropical regions of the world (Abang et. al., 2006). It is the most widely distributed species in the humid and sub-humid tropics and is appreciated for its high yield potential, ease of propagation through production of bulbils or full or cut pieces of tubers, reliability of sprouting, early vigor for weed suppression and long storage life of the tubers. All yams are considered annual or perennial tuber-bearing and climbing plants. The order Dioscoreales belongs to the monocotyledonous group with characteristic parallel venation of leaves and adventitious root system (http://en.wikipedia.org/wiki/Yam). The global yam production is almost 47 million metric tons. Africa alone accounts for about 96% of the world's yam production. Nigeria is the major producer (71%) and *D. alata*, is one of the major species grown in this region (www.iita.org). Abang et al. (2003) reported that 80 to 90 % decline in yam production occurred due to a fungal disease, anthracnose (*Colletotrichum gloeosporioides*), which necessitated the development of advanced crop improvement tools.

Greater yam is highly susceptible to anthracnose resulting

in severe economic yield loss (McDonald et al., 1998; Ano et al., 2002; Abang et al., 2006). There were four strains of *Colletotrichum* associated with greater yam anthracnose disease in Nigeria (Abang et al., 2001; Abang, 2003). These were the slow-growing grey (SGG), the fast-growing salmon (FGS), the fast-growing grey (FGG) and the fast-growing olive (FGO) strains. However, disease resistant germplasm accessions have been identified at IITA against only two strains (FGS and SGG) of anthracnose (Table 1). Molecular differentiation of yam genotypes for virulent SGG and FGS strains of *Colletotrichum* with genetic markers could assist epidemiological studies (Mignouna et al., 2001; 2002a; 2002b) and molecular markers are ideal tools to investigate genetic effects on the resistance or susceptibility to diseases such as anthracnose (Mignouna et al., 2003). Microsatellites or simple sequence repeats (SSRs) are informative molecular genetic markers in many crop species. SSRs are PCR-based, highly polymorphic, abundant, widely distributed throughout the genome and inherited in a co-dominant manner in most cases (Kumpatla and Mukhopadhyay, 2005).

Tostain et al. (2006) developed SSR markers in *D. alata*, *D. abyssinica,* and *D. praehensilis*. They characterized 16 polymorphic loci and concluded that several of the markers were transferable to other *Dioscorea* species. Hochu et al. (2006) identified eight DNA microsatellite markers in *D. trifida* and tested them for polymorphism and amplification patterns on a panel of five *D. trifida* cultivars. Similarly, Aceto et al. (2003) developed seven SSR primers for a heterologous crop species, *Asparagus acutifolius* and identified heterozygosity of the SSR markers in this species.

The current investigation was designed to use the less expensive (Aaronson et al., 1996) computational or bioinformatics approach that takes advantage of the available ESTs of crops related to yam containing SSR sequences at GenBank, National Centre for Biotechnology Information (NCBI). The initial step in the development of SSR markers is identification of EST sequences containing such repeats from published EST data sets, followed by the design of PCR primers flanking the SSR repeat stretch using SSRIT tool. This approach permitted the development of a large number of markers in a short span of time (Morgante and Olivieri, 1993; Kantety et al., 2002; Picoult-Newberg et al., 1999). Identification of polymorphisms in yam germplasm with this approach can eliminate the need for costly library construction and subsequent time consuming bioinformatics analysis of huge sequence information.

Materials and Methods

The fully expanded young leaves from six yam genotypes having differential resistance/susceptibility to FGS and SGG strains of anthracnose (Table 1) were harvested at the International Institute for Tropical Agriculture (IITA), Nigeria. These leaf samples were freeze dried and supplied to Virginia State University (VSU) for the current study.

The total genomic DNA of six genotypes (Table 1) was isolated from 0.2g of freeze dried young leaves using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). The isolated DNA was tested for quality and quantity respectively on 1.2% agarose gel as well as on a spectrophotometer (SmartSpec; BIO-RAD, Hercules, CA).

Bioinformatic data mining of the publicly available database (http://www.ncbi.nlm.nih.gov), resulted in

Table 1. Greater yam germplasm used for the current study

Germplasm accession	Accession code	Genotype response to disease	Disease affected plant part
TDa 95-310	A	Susceptible to the FGS and SGG strains of *C. gloeosporioides*	Leaf, stem
TDa 92-2	B	Susceptible to the FGS and SGG strains of *C. gloeosporioides*	Leaf, stem
TDa 93-36	C	Susceptible to the FGS and SGG strains of *C. gloeosporioides*	Leaf, stem
TDa 95/00328	D	Resistant to the FGS strain of *C. gloeosporioides* but susceptible to the SGG strain	Leaf, stem
TDa 87/01091	E	Resistant to the SGG strain of *C. gloeosporioides*	Leaf, stem
TDa 85/00250	F	Susceptible to FGS and SGG strains of *C. gloeosporioides*	Leaf, stem and tuber

Table 2. Heterologous crop species selected for SSR mining and the number of ESTs used from each species

Sample number	Crop species related to greater yam (*Dioscorea alata* L.)	Common name	Number of ESTs used from GenBank
1	*Solanum tuberosum*	Potato	267259
2	*Manihot esculenta*	Cassava	38411
3	*Colocasia esculenta*	Taro	36215
4	*Allium cepa*	Onion	28193
5	*Ipomoea batatas*	Sweet potato	19493
6	*Beta vulgaris*	Beet root	13496
7	*Asparagus officinalis*	Asparagus	8485
8	*Daucus carota*	Carrot	3309
9	*Lilium*	Lily	688
10	*Raphanus sativus*	Radish	105
11	*Allium sativum*	Garlic	73
12	*Dioscorea* spp.	Yam	35
13	*Tulipa*	Tulip	21
14	*Brassica rapa*	Turnip	17
15	*Dahlia*	Dahlia	1

415801 ESTs from 15 tuber producing species including monocots as well as dicots (Table 2) related to yam. These ESTs were obtained from the portion of the transcript that usually codes for a protein. These transcriptional regions do not change within a gene family as they were conserved across species.

The EST sequences were screened to identify SSRs using SSRIT tool (http://www.gramene.org/db/searches/ssrtool). Only 226 primer pairs were selected for use in the current study out of 600 primers designed using Primer3 (http://primer3.sourceforge.net/releases.php) tool. Eighty-two PCR primers were obtained directly from the published articles selected from GenBank EST downloads. Therefore, a total of 308 SSR primer sequences were used in the current investigation. These primers were synthesized at Ocimum Biosolutions LLC (Indianapolis, Indiana). The oligonucleotide lengths varied between 17-27 nucleotides. The GC content was in the range of 30-60% with a Tm value around 50°C.

Invitrogen Platinum PCR Super Mix (Invitrogen, Carlsbad, CA) was used for the amplification of the total yam genomic DNA (10 ng μl^{-1}). A series of annealing temperatures, 55°C – 60°C, were tested with the Bio-Rad thermocycler iCycler® for PCR reaction. Optimized PCR conditions included one cycle of 94°C for 2 min followed by 30 cycles each with denaturation at 94°C for 30 sec, annealing at 56°C for 45 sec and extension at 72°C for one min and then a final extension at 72°C for 7 min. The PCR products were realized on 2% agarose gel and viewed on UV Transilluminator using handheld device to score the bands manually for each amplified monomorphic and polymorphic reaction for respective genotype and primer combinations. The observed amplification scores for the five polymorphic informative markers are presented (Table 3).

Table 3. SSR polymorphisms among greater yam genotypes with *C. gloeosporioides* phenotypes

Primer code	Informative marker sequence	PCR product size (bp) for the six yam germplasm					
		A	B	C	D	E	F
VSU 146	F CTAACAACACACACACGGG						
	R TGGACTAACGTGGTGTAGG	600	600	600	600	**100**	600
VSU 155	F CCTCCCTCTAAGTGATCAAGG						
	R GAGATATAAGGGTTGAAGTTC	100	100	100	**600**	100	100
VSU 161	F CGAGTCCAATCTGAACCTAATTCATTG						
	R GAGGGTGCATTTGATGCTAAC	100	100	100	100	**200**	100
VSU 172	F GTCAACTATTTTGCTTCATCAC						
	R TTCGATTCTTTGCATCGCTAG	400	400	400	400	**200**	400
VSU 182	F GATACCAATAGGCGAAAGGAG						
	R AGGGGTGGAGTAGCAATGTT	400	400	400	**100**	400	400

F: Forward, R: Reverse

Results and Discussion

The primary objective of the present study was to investigate phenotypic polymorphisms in six different germplasm of *D. alata* L. and their differential expression for anthracnose disease resistance utilizing the information from current microsatellite marker analysis. The 82 primers, selected directly from previously published data for heterologous crop species related to yam, were not useful as there was no observed polymorphism in the yam germplasm tested. The oligonucleotide primers designed by computational mining of GenBank EST data from heterologous crop species had more favorable results towards the goal of the project. Out of the 308 primer sets tested, 80 showed clear amplification, of which 39 were polymorphic and 41 monomorphic.

Out of the 39 polymorphic primers, there were 24 unique primer sets showing differential expression among resistant (D and E) and susceptible (A, B and C) greater yam germplasm and only five were informative among the resistant phenotype (Table 3). These five informative primer sets (VSU 146, VSU 155, VSU 161, VSU 172 and VSU 182) had polymorphisms either in TDa 95/00328 (D) or TDa 87/01091(E), or in both accessions and these two specific germplasm accessions demonstrated resistant phenotypes to the FGS or SGG strains of yam anthracnose disease. The primers from the five informative markers were expressed within the core nucleotide SSR sequences of taro (unpublished), *Amorphophallus* (AB 286184, AB 286189), beet root (AJ 437652) and carrot (AY 530816). These are tuber crops that are related to greater yam as they store carbohydrates, belong to the same class Monocotyledonae, proving that they have evolutionary similarities as supported by previous investigations (Morgante and Olivieri, 1993; Kantety et al., 2002; Picoult-Newberg et al., 1999). Thus, sequences from related species could be utilized to develop markers in yam (Markwith and Scanlon, 2006).

Further, the five primer pairs selected were specific and represented differences in gene sequences that had resistance to the SGG and FGS strains causing anthracnose. An example of this specificity was with primer pair coded 146, which had polymorphism represented in the resistant yam accession, TDa 87/01091 (E). This polymorphism represented the phenotype for resistance to the virulent SGG strain that causes anthracnose disease. A 100 base pair fragment was amplified, compared to a 600 base pair amplification for the other germplasm accessions. This demonstrated that there was a difference in the nucleotide sequence in TDa 87/01091 for resistance to the SGG virulent strain resulting in the polymorphism observed. In TDa 95/00328 (D) the primer coded VSU 155 represented a nucleotide difference associated with the resistant strain FGS. The remaining VSU 161, VSU 172 and VSU 182, were distinct in showing polymorphisms in resistant germplasm accessions (Table 3). These findings are in line with those of Segarra-Moragues et al. (2004) and Morgante and Olivieri (1993).

The markers showed reliable amplification and considerable polymorphism for genetic analysis of commercial yam germplasm as confirmed by preliminary sequencing of amplified PCR products (Table 4). The PCR amplified templates of the polymorphic genotypes were purified and sent for sequencing (Agencourt Biosciences, Beverly, MA). Out of the 24 primer sets selected, only 10 were randomly chosen for sequencing as a pilot study to confirm the polymorphisms that represented resistant strains of the anthracnose disease.

The 120 sequences including forward and reverse, were aligned using NCBI Blast alignment tool (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) to get consensus sequence for each genotype after trimming the individual sequences. The useful consensus sequences showing single nucleotide sequence differences were selected as shown in Table 4. The consensus sequences were screened for SSRs using SSRIT tool (http://www.gramene.org/db/searches/ssrtool). There were di- and tri-nucleotide repeats in sequences from both resistant and susceptible genotypes, which lead to identification of SSRs. As the number of SSRs per sequence were very few, it was very difficult to conclude that these polymorphisms were due to nucleotide repeats. The polymorphism observed could be either due to inversions or due to deletions at those particular loci in the respective consensus sequence. Therefore, these could be used as In-del markers and not as SSR markers for yam germplasm characterization. This study gave results at low cost and in less time in greater yam. The SSR markers, VSU 146, VSU 161 and VSU 172, yielded a unique marker set for anthracnose resistance screening

Table 4. Bidirectional sequencing of PCR product resulted in few SNPs

Primer code	Genotype	Consensus sequence of the amplicon	Repeats
VSU 148	C	ataatcaacgctgccggtgctcttcttaggggcaggactgttcgtgccact gggaacgggagatgcagacactgggttttgcatagggacatagttctctt cgctgtctcctgagtctgtgctggtgatgctctgggtggagatggggcgg cagtactgggaggagctggagttgaa	Trimer
VSU 148	D	ggcctgggtcttctccttgtccacctgaacgttttccaccttctcatcagag gtgacggatgaagtagatggcttgcggtgggggcttggggaatcaacg ctgccggtgctcttcttaggggcaggactgttcgtgccactgggaacgg gagatgcagacactgggttttgcatagggacatagttctcttcgctgtctcc tgagtctgtgctggtgatgctctgggtggagatggggcggcagtactgg gaggagctggagttgaaggtgtggttggccctagacc	Trimer
VSU 155	B	ttttccactctctctcatcagaggtgacggatgaagtagatggcttgcggt gggggcttggggagctcggctggaagtcctgggccagataatcaacgc tgccggtgctcttcttaggggcaggactgttcgtgccactgggaacggg agatgcagacactgggttttgcatagggacatagttctcttcgctgtctcct gagtctgtgctggtgatgctctgggtggagatggggcggcagtactggg aggagctggagttgaaggtgt	Trimer, dimer
VSU 155	D	ggaacgggagatgcagacactgggttttgcatagggacatagcttcttctt cgctgtctcctgagtctgtgctggtgatgctctgggtggagatggggcgg cagcactgggaggagctggagttgaa	Trimer
VSU 167	A	taccactgggaacgggagatgcagacactgggttttgcatagggacata gttctcttcgctgtctcctgagtctgtgc	No repeats
VSU 167	D	cgtgccactgggaacggggcatagggacatagttctcttcgctgggagg agctggagacctagatgcagacactgggttttgtctcctgagtctgtggg cggcagtaccttaggggcatgtggattgggtggagatgtgttgaaataat caacgctgccggtgctcttgtctggtgcagatgtgttgagagataataatc gctgccggtgctcttgtctgagatgtgttgaaataatcaacgctgccggtg ctcttgtctg	Dimer, trimer

against SGG strain and SSR markers, VSU 155 and VSU 182, for resistance against FGS strain.

The present investigations lead to valuable input for yam genomic database. The markers if tested using advanced gene expression technology RT-PCR or Gene mapper would provide more precise information in less time. Further, distinct differences were observed among the germplasm in comparison with genotype F, which was identified by IITA as a greater yam genotype with poor textural quality. Tuber texture is one of the important tuber morphological features that contribute to post-harvest tuber shelf life. For all the genotypes, except for genotype F, only anthracnose disease resistance/susceptibility information was available. If the phenotypic information of all the genotypes were known, useful markers for yam crop improvement could be easily harvested. Thus, the remaining polymorphic primers could be tried for future comparisons of other phenotypic groups such as tuber morphology. The 39 SSR markers developed could be useful tools in screening against the remaining two strains (FGG and FGO) of *Colletotrichum gloeosporiodes* and to find out their potential use for nutritional, morphological and economic quality traits available within the yam germplasm maintained at IITA.

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References

Aaronson, J. S., Eckman, B., Blevins, R. A., Borkowski, J. A., Myerson, J., Imran, S. and Elliston, K. O. 1996. Toward the development of a gene index to the human genome: an assessment of the nature of high-throughput EST sequence data. *Genome Res.*, **6**:829-845.

Abang, M. M., Green, K. R., Wanyera, N. W. and IIoba, C. 2001. Characterization of *Colletotrichum gloeoporioides* Penz. from yam (*Dioscorea* spp.) in Nigeria. In: *Root Crops in the 21st Century*. Akoroda, A.O., Ngeve, J. M. (Eds.). *Proceedings of the 7th Triennial Symposium of the International Society for Tropical Root Crops*, Africa Branch, Cotonou, Genin. pp. 613-615.

Abang, M. M. 2003. Genetic diversity of *Colletotrichum gloeosporioides* Penz. causing anthracnose disease of yam (*Dioscorea* spp.) in Nigeria, Bibliotheca Mycologica Vol. 197. J Cramer in der Gebr. Bomtraeger Science Publishers, Berlin, Stuttgart.

Abang, M. M., Winter, S., Mignouna, H. D., Green, K. R. and Asiedu, R. 2003. Molecular taxonomic, epidemiological and population genetic approaches to understanding yam anthracnose disease *Afr. J. Biotech.*, **2**(12): 486-496.

Abang, M. M., Asiedu, R., Hoffmann, P., Wolf, G. A., Mignouna, H. D. and Winter, S. 2006. Pathogenic and genetic variability among *Colletotrichum gloeosoporioides* isolates from different yam hosts in the agroecological zones in Nigeria. *J. Phytopathol.*, **154**:51-61.

Aceto, S., Sica, M., Gamba, G., Montieri, S., Farina, A. and Gaudio, L. 2003. Isolation and characterization of microsatellite loci from *Asparagus acutifolius* (Liliaceae). *Molecular Ecol. Notes*, **3**:242-243.

Ano, G., Anas, G. and Chidiac, A. 2002. Création et utilisation de variétés résistantes aux maladies, éléments essentiels de la diversification agricole en Guadeloupe. *Phytoma*, **55**:36–37.

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

Kantety, R. V., Mauricio, L. R., David, E. M. and Mark, E. S. 2002. Data mining for simple sequence repeats in expressed sequence tags from barley, maize, rice, sorghum and wheat. *Pl. Mol. Biol.*, **48**: 501-510.

Kumpatla, S. P. and Mukhopadyay, S. 2005. Mining and survey of simple sequence repeats (SSRs) in expressed sequence tags (ESTs) of dicotyledonous plants. *Genome*, **48**:985-998.

Markwith, S. H. and Scanlon, M. J. 2006. Characterization of six polymorphic microsatellite loci isolated from *Hymnenocallis coronaria* (J. LeConte) Kunth (Amaryllidaceae). *Molecular Ecol. Notes*, **6**:72-74.

McDonald, F. D., Alleyne, A. T., Ogarro, L. W. and Delauney, A. J. 1998. Yam anthracnose in the English speaking islands of the eastern Carribbean-successes and research advances in disease management. *Trop. Agric.*, **75**:53-57.

Mignouna, H. D., Abang, M. M., Green, K. R. and Asiedu, R. 2001. Inheritance of resistance in water yam (*Dioscorea alata*) to anthracnose (*Colletotrichum gloesoporioides*). *Theor. Appl. Genet.,* **103**:52-55.

Mignouna, H. D., Abang, M. M., Onasanya, A. and Asiedu, R. 2002a. Identification and application of RAPD markers for anthracnose resistance in water yam (*Dioscorea alata* L.). *Ann. Appl. Biol.*, **141**:61-66.

Mignouna, H. D., Mank, R. A., Ellis, T. H. N., an den Bosch, N., Asiedu, R., Abang, M. M. and Peleman, J. 2002b. A genetic linkage map of water yam (*Dioscorea alata* L.) based on AFLP markers and QTL analysis for anthracnose resistance. *Theor. Appl. Genet.,***105**:726-735.

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

Morgante, M. and Olivieri, A.M. 1993. PCR-amplified microsatellites as markers in plant genetics. *Plant J.,* **3**:175-182.

Picoult-Newberg, L., Ideker, T. E., Pohl, M. G., Taylor, S. L., Donaldson, M. A., Nickerson, D. A. and Jacino, M. B. 1999. Mining SNPs from EST databases. *Genome Res.*, **9**: 167–174.

Segarra-Moragues, J. G., Palop-Esteban, M., Gonzalez-Candelas, F. and Catalan, P. 2004. Characterization of seven (CTT)n microsatellite loci in the pyrenean endemic *Borderea pyrenaica* (Dioscoreaceae): remarks on ploidy level and hybrid origin assessed through allozymes and microsatellite analyses. *J. Heredity*, **95**(2):177-183.

Tostain, S., Scarcelli, N., Brottier, P., Marchand, J. L., Pham, J. L. and Noyer, J. L. 2006. Development of DNA microsatellite markers in tropical yam (*Dioscorea* spp.). *Molecular Ecol. Notes*, **6**:173-175.