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Characterizing sweet potato RDR, AGO and DCL genes and potential involvement in defense against virus infections in sweet potato

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Characterizing anti-virus genes in sweetpotato is a vital step in mitigating yield loss due to virus infections. This work lays an insight into the structure and expression of key anti-virus genes. Related plant-based anti-virus genes were used as reference to mine key sweetpotato genes from various databases. BLASTN and BLASTP for transcripts was done for evaluation of phylogenetic relationship. Eight genes were identified: RNA dependent RNA polymerases (RDR) 1, 2, 5 and 6; Argonaute 1; and Dicer-like 1, 2 and 4, with more variants for RDR1 transcripts. Phylogenetically, RDR defense genes evolved more recently than other genes. Given the big number of variants and recent evolution of RDRs, further analysis for DLDGD or DFDGD catalytic domains, organization of coding sequences and gene expression were done on RDRs. DLDGD or DFDGD were observed in RDRs with the exception of IbRDR1c_Ch1_1623 and RDR2_Chr2_1059. RDR1 variants revealed varying exon-intron organization, and the IbRDR1c_Ch1_1623 transcript had no introns. High titres for IbRDR1a_Chr8_3068, IbRDR1b_Chr8_3014 and IbRDR1d_Chr8_1149 were observed in SPVC-infected plants suggesting these RDRI variants are involved in resistance against virus infection. The titre of IbRDR1c_Chr1_1623 was not affected. This study offers an opportunity for molecular breeding and selection of cultivars for distribution to farmers.

Key words: Virus defense genes, gene silencing, RNA dependent RNA polymerases (RDR), catalytic domain, titre.

INTRODUCTION

Studies on sweetpotato virus defense genes have received limited attention. This is against the fact that a wide range of viruses attack sweetpotato. For instance, in East Africa, several sweetpotato viral infections have been identified. These include *Sweet potato feathery mottle virus* (SPFMV: *Potyvirus*; Potyviridae), *Sweet*

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potato chlorotic stunt virus (SPCSV: *Crinivirus*; Closteroviridae), Sweet potato leaf curl sweepoviruses (SPLC: *Begomovirus*; geminiviridae), *Sweet potato mild mottle virus* (SPMMV: *Ipomovirus*; Potyviridae), *Sweet potato chlorotic fleck virus* (*Carlavirus*; Flexiviridae) and *Sweet potato caulimo-like virus* (SPCaV: *Caulimovirus*; Caulimoviridae) (Carey et al., 1998; Mukasa et al., 2003; Aritua et al., 2007; Wasswa et al., 2011). East Africa being a tropical environment with high vector populations lays fertile grounds for infections of newly established gardens/farms, therefore potentially undermining the efforts of increased sweetpotato production. These viral infections cause yield loss of up to 50% in single infections (Adikini et al., 2015). The yield loss may increase up to 98% when two or more viruses co-infect, as in the case of Sweetpotato virus disease that is due co-infection of SPFMV and SPCSV (Gibson et al., 1998).

Some East African varieties which have been in the field for decades, have never gone through any anti-viral therapy but are generally virus-free and have yields which have not apparently declined. For instance, in Tanzania, Tairo et al. (2004) reported that 38 of 73 (52%) asymptomatic field plants were sero-negative to viruses. In Kenya, Ateka et al. (2004) showed that 477 of 638 (75%) asymptomatic field plants were virus-free. In Uganda, of the 200 symptomless plants, only 9 (4.5%) were found to be infected with SPFMV and 5 (2.5%) with SPCSV (Aritua et al., 2007). In Rwanda, Njeru et al. (2008) reported that 71 of 103 (69%) asymptomatic field plants were virus-free. This rarity in infection appears to be due to host resistance. Several studies show that the crop tends to 'heal' itself where it fights off the virus, becoming asymptomatic and eventually virus free (Gibson et al., 2014; Gibson and Kreuze, 2015; Ssamula et al., 2019A).

In plants, viral resistance has been envisaged to be due to basal, innate immunity and RNA silencing (RS) or RNA interference (RNAi) (Muhammad et al., 2019). In sweetpotato, Gibson and Krauze (2015) further postulated that viral resistance is due to gene/RNA silencing. Gene or RNA silencing is a virus surveillance system present in all plants that involves small interfering (si)RNAs which are produced through the coordinated function of RDR-DCL-AGO genes (Borges and Martienssen, 2015; Bologna and Voinnet, 2015). The siRNAs join a RISC (RNase Induced Silencing Complex) that bind to cognate sections of foreign RNA such as plant viruses, 'chopping them up' at each binding position to produce yet more siRNAs (Incarbone and Dunoyer, 2013).

Interestingly, plant genome-encoded RDRs have been reported to be involved across several resistance responses (Rakhshandehroo et al., 2017; Leibman et al., 2022). Plants encode six RDR variants, where RDR1 is the most dominant in gene silencing (Donaire et al., 2008; Garcia-Ruiz et al., 2010). RDR1occurs in all investigated plant species and production of small-RNA during RNA virus infection is activated by RDR1 activity (Cao et al., 2014). Apart from virus defense, RDR1 is involved with responses to other pathogen defense, abiotic stress and defense against insect herbivores (Pandey et al., 2008; Matsui et al., 2017; Polydore and Axtell, 2018). RDR1 is also known to regulate microRNA levels and plays a role in regulating important endogenous genes via mRNA-mediated DNA methylation, and is associated with the biotic and abiotic stress response (Polydore and and Axtell, 2018). Understanding the nature/characteristics of viral defense genes, especially RDRs, opens avenues of mitigating the potential sweetpotato production challenges caused by viruses. In this paper, we report on the potential virus resistance genes and amino acid motifs, structural organization and expression of RDR1 upon virus infection in sweetpotato.

MATERIALS AND METHODS

Mining genes associated with virus resistance

Virus resistance gene sequences for model plants *Arabidopsis thaliana* and *Nicotiana benthamiana* were used as reference to conduct the NCBI searches for related plants with similar genes. These reference sequences were used to construct partial sequences for sweetpotato. Partial sequences were obtained using a blind search and query from RNA seq data previously deposited in NCBI (SRA-database). RNA seq data of virus infected sweetpotato was run on a virtual linux platform through command line. The seq data was *de novo* assembled using de Bruijn graph approach (Roumpeka et al., 2017) into contigs while using previously mined sequences as reference. For quality assurance, a scaffold length of 50% was used (N50) according to Mäkinen et al. (2012) procedure using MetaQUAST and MetaVelvet software (Namiki et al., 2012). These helped to detect putative structural variants and misassemblies which were appropriately trimmed. The obtained sequences were re-matched to reference sequences in NCBI alignment tool for validation. The yielded short reads and assemblies (partial sequences) were also mapped and analysed for expression profiles using the Gene Expression Omnibus in NCBI to validate expressions of these genes. Also MetaGene gene finding software (Noguchi et al., 2008) and FragGeneScan (Rho et al., 2010) were used to validate the reference sequences to the genes. The RNA short reads were reverse transcribed to DNA sequences using platform/tool on biomodel.uah.es. The DNA sequences were re-checked by transcribing back to RNA and homology validated using NCBI. A homology level >95% was used. This validated that the reverse transcribed product (that is DNA) was highly identical to RNA and thus could be used for further evaluations.

Partial DNA gene sequences were BLAST searched on the sweetpotato genome sequence and genome sequences of sweetpotato wild relatives available at http://sweetpotato.uga.edu/. This yielded homologous genomic DNA sequence that contained coding and non-coding sequences. Coding sequences were used in downstream work because they are translated to RNA/protein that potentially code for virus resistance. These coding sequences were used as reference sequences for BLAST searching against a database created using FASTA files. The FASTA files were created by downloading and saving different chromosomal genomic DNA sequences (of sweetpotato) derived from FASTQ sequences from sweetpotato genome database (Yang et al., 2017). The FASTA files were used to create a reference database in BIOEDIT (Hall, 1999) (www.qiagenbioinformatics.com/products/clc-main-workbench) platform and CLC workbench validated using Unipro UGENE

(Okonechnikov, 2012). The partial genes were run at stringency of E^{-10} and E^{-6}; these yielded partial hits of increased sequence lengths (of the initial RNA/nucleotide sequences). Generated partial genes sequences from this process were allocated identifier names and numbers, respectively.

Phylogenetic analysis of sweetpotato anti-virus genes and related genes in other plant species

Phylogenetic analysis was done using translated protein sequences of sweetpotato, its relatives and plants where related viral resistance genes have been reported. During the phylogenetic analysis, random plants were selected as roots. The phylogenetic/evolutionary analysis was done using Phylip Neighbour joining tree building method following the model by Jones et al. (1992) and Bootstrapped with 100 replicates in Unipro UGENE software (Okonechnikov, 2012) and validated in the CLC workbench. This process was done to establish if the predicted proteins were associated or similar to those involved in the processes associated with viral gene silencing.

Amino acid-protein sequence properties of RDRs

Given the big number of variants for RDRs (Table 2) and their recent evolution particularly RDR1 (Appendix: Figure 1), further analysis was done on these RDRs. Our decision to further analyse RDRs was also based on the findings by Leibman et al. (2022) who found melon (*Cucumis melo*) to encode variants of RDR1 genes (CmRDR1a, c1 and c2) whose expression levels variously increased upon infection with various geminiviruses and potyviruses. RDR1 was also reported by various researchers as one of the main enzyme of all RDRs involved in RNA silencing (Qi et al., 2009; Garcia-Ruiz et al., 2010).

In order to establish whether an amino acid derived from RDR coding sequences is potentially involved in viral defense, reference was made to Wassenegger and Krczal (2006) and Hua et al. (2021). The presence of C-terminal canonical DLDGD or DFDGD amino acid motifs was analysed for sweetpotato RDR based coding sequences (genes). Here, the nucleotide sequences of different coding sequences were translated to amino acid (protein) sequences using the *ExPASy* (Gasteiger et al., 2003) translation tools and verified using Open Reading Frame Finder on NCBI platform. The amino acid sequences were aligned in Unipro UGENE and verified in CLC workbench and BIOEDIT platforms. This alignment was also against related RDRs from other plants. Further structural amino acid-protein validations were made against sampled RDRs, to compare the relation with other plants. This was done using the Protein Data bank (https://www.rcsb.org/) and ORION (Ghouzam et al., 2016). These processes led to validation for presence of the canonical DLDGD or DFDGD amino acid motifs in sweetpotato RDRs. Further to this, a conserved domain search for the amino acids/proteins was made in NCBI's conserved domain resource (https://www.ncbi.nlm.nih.gov/Structure/cdd). This was aimed at establishing if the RDR gene family possessed those conserved sites that are historically involved in viral resistance.

Predicting the structure/orientation of DNA coding sequence of RDR1

To analyse sweetpotato RDR1 orientation, a BLAST search was done against the sweetpotato relatives *Ipomoea triloba* and *Ipomoea trifida* available at the genome site http://sweetpotato.uga.edu/. Reference sequences used were the coding sequences of some key genes (variants) of RDRI where significant variations had been observed. The BLAST search revealed homologous sequences for the sweetpotato relatives against particular coding sequences of the potential viral resistance genes. A phylogenetic analysis using coding sequences was performed for the said sequences, to obtain the closest relative of sweetpotato. This was done using Unipro UGENE platform and validated using CLC-workbench and NCBI tree construction options. The nucleotide sequences of the closest relative was aligned to the coding sequence of sweetpotato in BIOEDIT and validated in Unipro UGENE platform. This revealed the structural organization of sweetpotato coding sequences of key RDR1 variants. It also revealed variational arrangements/deviations from the closest relative. Validations of different sequence compositions of exons and introns were run in NCBI to cross check their functionalities. Structural annotational representations/predictions of nature of the coding sequences were then constructed.

Quantification of RDR1 in *Sweet potato virus* C-infected cultivar Beauregard plants

Quantification of RDR1 was done at Agricultural Research Organisation (ARO) – The Volcani Center, Israel in 2019. *Sweet potato virus* C (SPVC) potyvirus was used as it was the only potyvirus that could be accessed at ARO. Single SPVC infections were established by side grafting naturally infected Beauregard shoot tips from field to 10 healthy plants of cultivar Beauregard in a screen house. Plants were established from cuttings taken from the inoculated Beauregard plants, one week after inoculation, to avoid any effects from the virus infector cuttings. Composite leaf samples from top (3rd leaf from top), middle (5th leaf from top) and bottom parts (most bottom leaf) of the stem of the 3 successfully inoculated plants were separately collected for each plant at the end of 2 weeks. Three healthy mock-inoculated Beauregard plants were included as control. The collected samples were temporarily stored in a cold chamber with freezers at -80°C until they were needed for RNA extraction.

RNA was extracted from healthy and SPVC-infected plants using the TRI Reagent protocol, following the supplier's manual (Bio Labs, Jerusalem, Israel). Nucleic acids were quantified using a NanoDrop-ND-1000 spectrophotometer (Thermo Scientific; Bargal Analytical Instruments, Airport City, Israel). The samples were standardised to 500 ng μl^{-1} using molecular grade water. Complementary DNA (cDNA) was synthesised using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Tamar, Israel), following the manufacturer's manual.

Plants were confirmed for infection (Figure 1) using RT-PCR procedure described by Ssamula et al. (2019A). The remaining cDNA was used for determining RDR1 titre (IbRDR1a_Chr8_3068, IbRDR1b_Chr8_3014, IbRDR1c_Chr1_1623 and IbRDR1d_Chr8_1149) using SYBR green method of RT-qPCR. The 25 µl SYBR green qPCR reaction mixture consisted of 12.5 µl of SYBR, 8.5 µl of molecular grade water, 0.75 µl of 5 mM of each primer (Table 1) and 2.5 µl of cDNA. A negative control (molecular grade water) and a housekeeping gene [cytochrome oxidase (cox)] (Weller et al*.,* 2000), were included on the plate (twin.tec PCR plate 96, skirted) and each sample was duplicated to reduce pipetting errors. Plates were sealed with optical adhesive covers (Applied Biosystems). The reaction was performed on Mastercycler® ep realplex Sequence Detection System and qPCR thermal cycler conditions used include 95°C for 15 min (SYBR activation) followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 54°C for 30 s and extension at 72°C for 30 s. Relative RDR1 titre data were analysed from the raw fluorescence data [Ct values at which a change in normalised reporter (ΔRn) crosses the threshold] using the $2^{(\_Delta\ Delta\ C(T))}$ method (Livak and Schmittgen, 2001). The fold change RDR1 titre (target gene) relative to the reference gene (Cox) was determined by the equation: $Cq = 2^{-\Delta\Delta Ct}$: $Cq = 2^{-}[(Cq_{\text{target gene}}) - (Cq_{\text{reference gene}})] - [(\text{mean } Cq_{\text{target gene}}) - (\text{mean } Cq_{\text{reference}}$

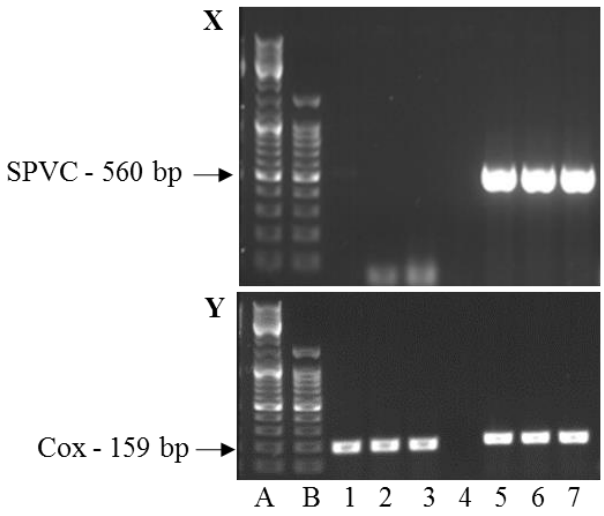

Figure 1. Gel picture showing SPVC amplified products in sweetpotato cultivar Beauregard plants. Plate X depicts SPVC gel picture and plate Y depicts gel picture of the host *Cytochrome C oxidase* reference gene. Lane A = 1kb ladder, B = 100bp ladder (frequently used), 1-3 = healthy plants of cultivar Beauregard, 4 = negative (water) control, and 5-7 SPVC-infected plants of cultivar Beauregard.
Source: Authors

Table 1. Primer sequences used in the study.

Primer name	Sequence (5'-3')	Source
IbRDR1a_Chr8_3068-F	CATCGAGACTTATAGCAGCCG	This study
IbRDR1a_Chr8_3068-R	GGATAATGGCGCAACACACACGATTC	
IbRDR1b_Chr8_3014-F	CTACAAGAAAGCAGAAGGCTCCA	This study
IbRDR1b_Chr8_3014-R	TATCCAGACTAATAGCAGCAGA	
IbRDR1c_Chr1_1623-F	ATCTCATCTGCCTGTAAATA	This study
IbRDR1c_Chr1_1623-R	TTTGATAGAACACCGTACTT	
IbRDR1d_Chr8_1149-F	GTTGGGACCCTGACCTTATT	This study
IbRDR1d_Chr8_1149-R	CTGGAAGCGATTTGGATG	
Cox F	ACTGGAACAGCCAGAGGAGA	Park et al. (2012)
Cox R	ATGCAATCTTCCATGGGTTC	

Source: Authors

$_{gene}$)], where Cq = cycle quantity, ΔΔCq = differences in Cq values between the target gene and reference gene.

RESULTS

Genes associated with virus resistance

According to the gene prediction process, eight genes were obtained. These included four RDRs (RDR 1, 2, 5 and 6); one Agonaute 1 (AGO1); and three Dicer-like (DCL) genes 1, 2 and 4 (Table 2). These sweetpotato genes had variants; RDR1 had four variants, followed by DCL2 with three variants, and RDR5 and DCL1 with two variants each. Other genes had one form (that is, no variant). All the three gene families of sweetpotato had fewer variants than sweetpotato relatives *I. trifida*,

Table 2. Sweetpotato (*Ipomoea batatas*) predicted genes, variants, chromosomal locations and their similarity to *Ipomoea* relatives.

Detected gene	Evaluation on the genome (Yang et al., 2017)			Proposed variant identity	Variants/alternative splice forms/isoforms in *Ipomoea* spp. relatives based on HITS, E-Value and identity			Cumulative no. of variants
	No. of variants	Chromosomal locations	Potential size (nt)		*I. trifida*	*I. triloba*	*I. nil*	
IbRDR1	4	Chr 8	3068	IbRDR1a_Chr8_3068	1	2	1	4
		Chr 8	3014	IbRDR1b_Chr8_3014	1	2	1	4
		Chr 1	1623	IbRDR1c_Chr1_1623	2	4	3	9
		Chr 1	1149	IbRDR1d_Chr8_1149	1	2	1	4
IbRDR2	1	Chr 3	1059	IbRDR2_Chr3_1059	2	2	1	5
IbRDR5	2	Chr 14	2671	IbRDR5a_Chr14_2671	2	5	1	8
		Chr 11	707	IbRDR5b_Chr11_707	2	3	1	6
IbRDR6	1	Chr 10	786	IbRDR6_Chr10_786	1	1	2	4
IbAGO1	1	Chr 3	2201	IbAGO_Chr3_2201	1	1	1	3
IbDCL1	2	Chr 1	3661	IbDCL1a_Chr1_3661	2	1	1	4
		Chr 9	3808	IbDCL1b_Chr9_3808	3	2	1	6
IbDCL2	3	Chr 12	2059	IbDCL2a_Chr12_2059	1	2	4	8
		Chr 13	1685	IbDCL2b_Chr13_1685	5	4	1	10
		Chr 6	1152	IbDCL2c_Chr6_1152	5	5	1	11
IbDCL4	1	Chr 8	2775	IbDCL4_Chr8_2775	1	1	1	3

Ib = *Ipomoea batatas*; RDR = RNA dependent RNA polymerase; AGO = Agonaute; DCL = Dicer-Like; nt = nucleotides. Potential variants/number of variants were obtained by blasting to MSU site for *I. triloba* and *I. trifida*. Considerations were made based on Top Query coverage greater than 80%, Expected value of 0.0 and Identity greater than 80%. For *I. nil* variants with hits greater than 85% identity were considered using NCBI BlastN.
Source: Authors

Ipomoea nil and *I. triloba* (Table 2). In sweetpotato, chromosome 1 and 8 had the highest number of defense genes while other chromosomes had two or one gene. All predicted sweetpotato defense genes had resemblances with genes of wild relatives (Table 2).

Phylogenetic relationship of anti-virus genes in sweetpotato and other plant species

Generally, the virus defense genes of sweetpotato closely related to those of *Ipomoea* species wild relatives, while distantly related to those of other plants. The RDR1 of *Ipomoea* relatives evolved much earlier than those of *Ipomoea batatas*. Of the sweetpotato RDRs, IbRDR1c_Chr1_1623 evolved earlier than the rest. The RDR1 proteins of *Ipomoea* spp. clustered in the same clade. Interestingly IbRDR1d_Chr1_1149 has recently evolved and sequence related to that of Citrus_sinensis_RDR1. (Appendix: Figure 1). The RDR2 protein of most wood-like plants evolved earlier than those of herbaceous plants, of which sweetpotato is part. The *I. batatas* RDR2 protein showed evolutionary lineage from *I. triloba*; with close relationship with *I. trifida* RDR2 protein (Appendix: Figure 2). The RDR5 proteins of *Ipomoea* spp. varied within the phylogram. This is shown by an early evolution of one of the *I. batatas* RDR5 species (IbRDR5a_Chr14_2671) although with a distant relation with that of wild relative *I. trifida* (the isoform). Surprisingly, sweetpotato IbRDR5b_Chr11_707 evolved slightly before the RDR5 of its relatives like *I. triloba* and *I. nil*; although not significant from protein of

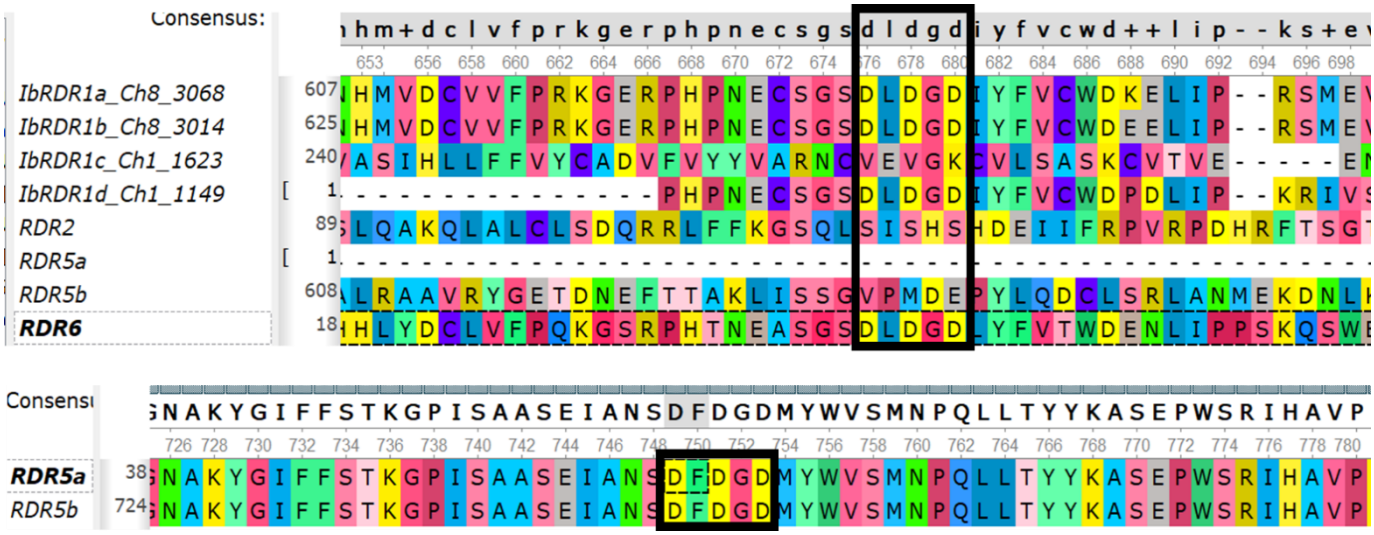

Figure 2. Catalytic domains (in black rectangle) in different sweetpotato amino acids translated from mined defense-gene nucleotide sequences.
Source: Authors

tomatoes (*Solanum lycopersicm*). Other crop RDR5 proteins evolved variously (Appendix: Figure 3). The phylogram showed that sweetpotato RDR6 (IbRDR6_Chr10_786_Protein) evolved earlier than proteins of the relatives *I. nil*, *I. triloba* and *I. trifida*. Of the three sweetpotato relatives, RDR6 of *I. nil* evolved earlier than that of *I. triloba* and *I. trifida*. All these proteins were distantly related to those of other crop plants (Appendix: Figure 4).

AGO 1 protein of sweetpotato (IbAGO 1_Chr3_2201) recently evolved and closely related to *I. trifida* AGO 1 protein, while distantly related to *I. nil* AGO 1. The phylogram showed that sweetpotato AGO1 protein is distantly related to similar proteins from other crop plants like *Nicotiana tabacum*, *Capsicum* species and *Solanum tuberosum* (Appendix: Figure 5). The DCL1 proteins of sweetpotato evolved variously (Appendix: Figure 6). According to the phylogram, IbDCL1a_Chr1_3661 evolved earlier than similar proteins from different crops. However, it was closely related to DCL proteins of tree crops like *Theobroma cacao* and *Vitis vinifera*. Sweetpotato protein IbDCL1b_Chr9_3808 was closely related to DCL1 protein of *I. trifida* and evolved earlier than similar proteins of crops like *Manihot esculenta* and *Jatropha curcas* (Appendix: Figure 6). Sweetpotato IbDCL2c_Chr6_1152 and IbDCL2b_Chr13_1685 evolved earlier than similar proteins of *Ipomoea* relatives (Appendix: Figure 7). IbDCL2a_Chr12_2059 has recently evolved and is related to similar protein of *I. trifida* where they form same clade (Appendix: Figure 7). The IbDCL4_Chr8_2775- protein evolved earlier than DCL4 proteins of different crops like *Coffea eugenioides* and sweetpotato relatives *I. nil*, *I. trifida* and *I. triloba* (Appendix: Figure 8).

Amino acid-protein sequence properties of RDRs

Amino acid catalytic domains/motifs

Catalytic domains of the form DLDGD or DFDGD were observed variously in the RDRs with the exception of IbRDR1c_Ch1_1623 and RDR2_Chr2_1059. The catalytic domain DLDGD was found within IbRDR1a_Ch8_3068, IbRDR1b_Ch8_3014, IbRDR1d_Ch1_1149 and RDR6. On the other hand, catalytic domain DFDGD was found in RDR5a_Chr 14_2671 and RDR5b_Chr11_707 (Figure 2).

Conserved domain evaluation

A conserved domain evaluation for RDRs revealed that all RDRs possessed conserved domains with the exception of IbRDR1c_Ch1_1623. The largest domain was observed in IbRDR1a_Ch8_3068 (of about 572 sequence length, A - coloured blue) and the smallest was in IbRDR2_Chr3_1059 (70 sequence length, D - coloured red) (Figure 3). The domains contained RNA dependent RNA polymerase (RdRP) superfamily for IbRDR1a_Ch8_3068 (A), IbRDR1b_Ch8_3014 (B) and IbRDR5b_Chr11_707 (F); RdRP for IbRDR1d_Ch1_1149 (C), IbRDR5a_Chr14_2671 (E) and IbRDR6_Chr10_786 (G); and RNA-recognition motif (RRM) superfamily for IbRDR2_Chr3_1059 (D) (Figure 3).

Predicted orientation of DNA coding sequence of RDR1

The RDR1 gene family in *I. batatas* was homologous to

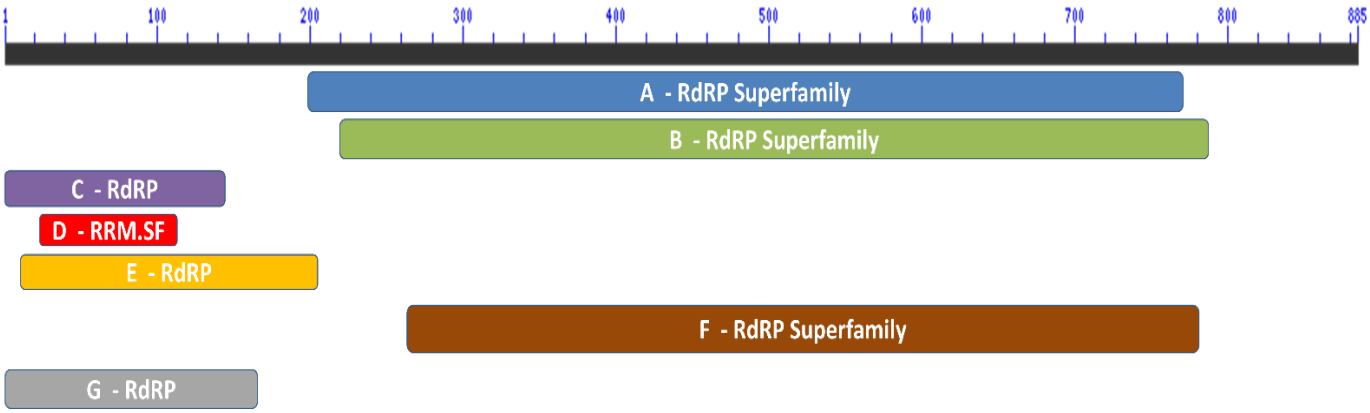

Figure 3. An NCBI architectural coverage of RDR Domains on scale of representative query sequence (for different RDRs). A is RNA dependent RNA Polymerase (RdRP) superfamily domain for IbRDR1a_Ch8_3068; B is RdRP superfamily domain for IbRDR1b_Ch8_3014; C is a RdRP domain for IbRDR1d_Ch1_1149; D is an RNA recognition motif superfamily domain for IbRDR2_Chr3_1059; E is a RdRP domain for IbRDR5a_Chr14_2671; F is a RdRP superfamily domain for IbRDR5b_Chr11_707 and G is a RdRP domain for IbRDR6_Chr10_786.
Source: Authors

those of wild relatives. Analysis of the coding sequences of the RDR1 gene variants revealed differences in orgarnisation of the exons (Figure 4). Four exons were observed in gene transcripts of IbRDR1a_Chr8_3068, IbRDR1b_Chr8_3014 and IbRDR1d_Chr8_1149. IbRDR1c_Chr1_1623 transcript had five exons (Figure 4). The lines between exons showed non-coding regions (introns) (Figure 4); interestingly, the IbRDR1c_Chr1_1623 transcript had no introns. The largest nucleotide sequences were found in IbRDR1a_Chr8_3068 transcript and the least in IbRDR1d_Chr8_1149. A Blast multiple alignment on the different exons revealed that whereas different nucleotide sequences were located within different genes, they were homologous at a level above 95%. This was indicated with similar colouration of exon sequences. Those with different colours were non-homologous (Figure 4).

RDR1 titre in *Sweet potato virus* C-infected cultivar Beauregard plants

In healthy sweetpotato plants, RDR1a, RDR1b, RDR1c and RDR1d were all detectable. RDR1 titre was higher for IbRDR1a_Chr8_3068, IbRDR1b_Chr8_3014 and IbRDR1d_Chr8_1149 in SPVC-infected Beauregard plants than in healthy control plants (Table 3). Highest RDR1 titre was recorded for IbRDR1b_Chr8_3014 followed by IbRDR1a_Chr8_3068 and lowest for IbRDR1d_Chr8_1149. IbRDR1c_Chr1_1623 titre did not vary between SPVC-infected and healthy control plants.

In SPVC-infected plants, IbRDR1b_Chr8_3014, IbRDR1a_Chr8_3068 and IbRDR1d_Chr8_1149 titres were 24.2, 15.7 and 3.3 folds, respectively, compared to healthy control plants (Table 3).

DISCUSSION

Here, we report on sweetpotato genes associated with virus resistance, their phylogenetic relationship, genomic characterization and expression in virus infected plants. This is the first report of RDR genome organization in sweetpotato and RDR expression in healthy and virus-infected sweetpotato plants.

Three gene families, that is, RDR, DCL and AGO were predicted in sweetpotato. These same genes are known to occur in other plant systems where they involve in resistance against various stresses in crop plants (Qin et al., 2018; Cui et al., 2020; Ahmed et al., 2021). Indeed, the RDR, DCL and AGO genes of sweetpotato had resemblance to the defense genes of wild relatives *I. trifida*, *I. nil* and *I. triroba*, and other plants suggesting their involvement in virus resistance in sweetpotato. This resemblance suggests homology during evolutionary development. Coordinated function of RDR-DCL-AGO genes in plants is crucial for processing different classes of small RNAs, which indirectly makes them involved in regulation of diverse biological pathways (Borges and Martienssen, 2015; Bologna and Voinnet, 2015). Members of these three gene families are involved in biogenesis of sRNAs and effective silencing of their targets, viruses inclusive (Bologna and Voinnet, 2015).

Four RDRs 1, 2, 5 and 6; one AGO 1; and three DCLs 1, 2 and 4 resistance genes were predicted, although these three gene families of a hexaploid sweetpotato had fewer variants than in the diploid *I. trifida*, *I. nil* and *I. triroba*. This observation is possible because an organism's genome size does not depend on the number of genes (or chromosomes) it contains (Hou and Lin, 2009). It is, therefore, possible for a more complicated or advanced organism to have less gene variants.

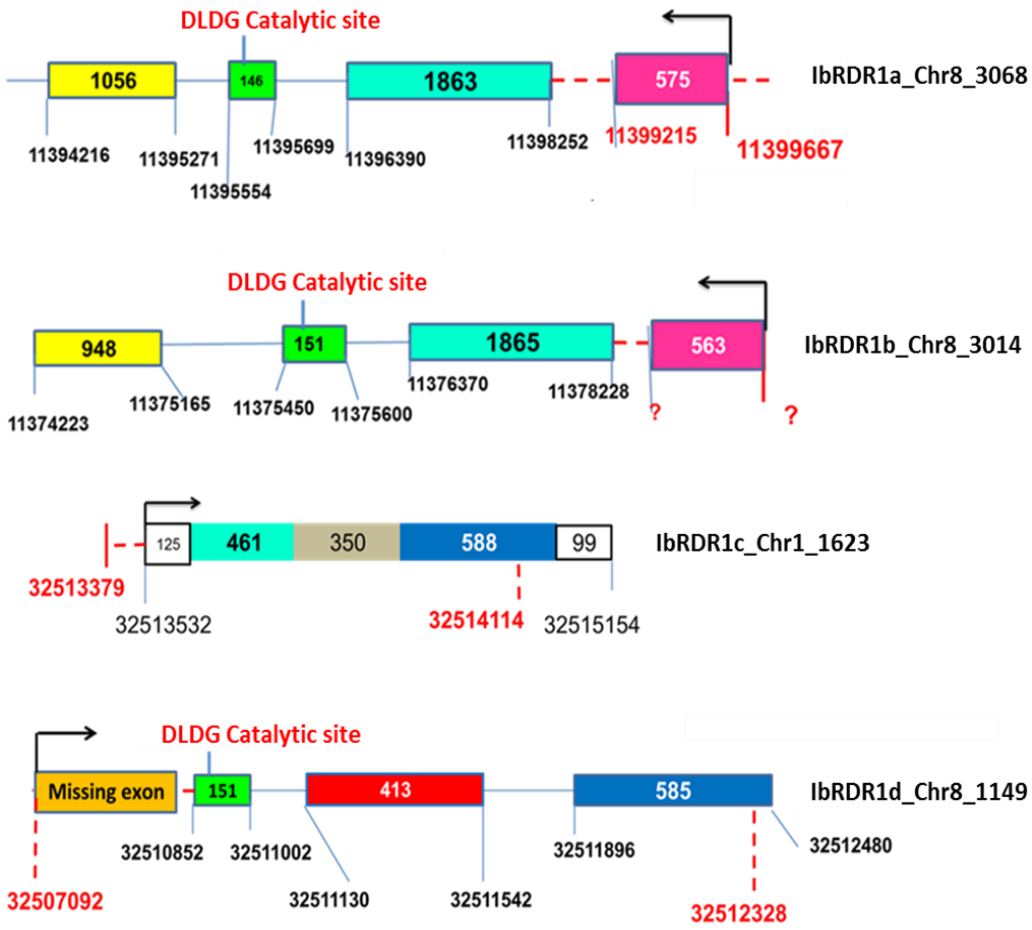

Figure 4. Genome maps of IbRDR1a_Chr8_3068, IbRDR1b_Chr8_3014, IbRDR1c_Chr1_1623 and IbRDR1d_Chr8_1149 genes in sweetpotato. Boxes represent exons and lines indicate introns. The dashed lines represent unknown sequences. Genome maps were based on the relevant databases (http://sweetpotato.uga.edu/ (accessed on 23rd November 2022)). The numbers indicate exon size (nucleotides) and the start codons (ATG) are represented by an arrow. The two numbers at the start and end of the exons indicate start and end points of that exon with reference to sweetpotato chromosomal sequences. For RDR1b and missing exon region in RDR1d sweetpotato chromosomal areas were not yet sequenced hence *I. trifida* sequence was used as reference.
Source: Authors

Table 3. Mean fold change in RDR1 titre (average $2^{-\Delta\Delta Ct}$) at 2 weeks after graft inoculation of cv Beauregard plants with SPVC.

Plant No.	Average $2^{-\Delta\Delta Ct}$			
	IbRDR1a_Chr8_3068	IbRDR1b_Chr8_3014	IbRDR1c_Chr1_1623	IbRDR1d_Chr8_1149
1	23.473	18.572	0.023	2.357
2	27.522	15.927	0.011	4.227
3	21.516	12.706	0.041	3.188
Mean of means	24.2	15.7	0.03	3.3

Source: Authors

Sweetpotato is an autohexaploid species with 90 chromosomes (2n = 6x = 90) and a basic chromosome number of 15 while sweetpotato progenitors *I. trifida*, *I. nil* and *I. triloba* are diploid and with 15 pairs of chromosomes (2*n* = 2*x* = 30) (Isobe et al., 2019). The RDR1 had the highest number of variants. This

observation is in agreement with earlier findings by Ssamula et al. (2019B). The high RDR1 variability probably signifies the dominant role RDR1 plays during viral gene silencing in sweetpotato. Previous research has actually shown that there are six different RDRs; however, RDR1 and RDR6 are the main enzymes that amplify single-stranded RNA from viruses into aberrant dsRNA, which are digested by the host-encoded Dicer-like (DCL) DCL-4 and DCL-2 proteins into 21–22 nts virus-siRNA duplexes (Qi et al., 2009; Garcia-Ruiz et al., 2010).

Each of the chromosomes could have several genes, as was observed in this study, where most defense genes mapped on chromosome 1 and 8. Whereas the largest chromosome of an organism is generally referred to as chromosome 1, the next largest as chromosome 2, and so on, different chromosomes contain different specific genes whereby, each chromosome contains a specific chunk of the genome (Hou and Lin, 2009). Therefore, the variability in gene locations on different chromosomes is not surprising.

The close phylogenetic relationship of the sweetpotato defense genes to the wild relatives is in agreement with findings by Ssamula et al. (2019B), and suggests that these genes acquired their resistance role before separation of the ancestral plants into different taxa. The sustained presence of the defense genes in different plants after divergence into different taxa during evolutionary development further signifies the importance of these genes in plant life of sweetpotato.

The recent evolution of RDR1 protein of *Ipomoea* spp. further points to the significant role RDR1 may be playing in sweetpotato resistance against virus infections. Several earlier studies in other plants have reported an additional role of RDR1 in virus defense (Qi et al., 2009; Garcia-Ruiz et al., 2010). IbRDR1c_Chr1_1623 evolved earlier than the rest of IbRDR1 gene family variants probably indicating this gene variant is less important in virus resistance. Interestingly IbRDR1d_Chr1_1149 has recently evolved and sequence related to that of Citrus_sinensis_RDR1. This is a case of parallel evolution resulting into phenotypic convergence (Nedelcu, 2019). Like sweetpotato, citrus is affected by several viruses some of which are in the same genus e.g, closterovirus (Umer et al., 2019). These viruses, although in different plant species, may require similar defense genes and mechanisms.

The AGO 1 protein of sweetpotato (IbAGO 1_Chr3_2201) recently evolved, yet only IbDCL2a_Chr12_2059 of the DCL variants showed recent evolution. The DCL main role is to digest the aberrant dsRNA from a replicating virus or secondary structure of the virus into 21–22 nts virus-siRNA duplexes (Qi et al., 2009). On the other hand, AGO proteins are catalytic subunits of the silencing complexes that are loaded with small RNAs to execute the sequence specific RNA cleavage by DCLs (Qi et al., 2009; Garcia-Ruiz et al., 2010).

The IbRDR1c_Chr1_1623 variant which was observed to have evolved earlier than the other IbRDR1s did not have catalytic domains of the form DLDGD or DFDGD. This same gene variant did not possess any conserved domain historically relevant to virus resistance. This further suggests IbRDR1c_Chr1_1623 to be of minor or no role in virus defense. The IbRDR2_Chr3_1059 gene variant which also lacked the catalytic domains (DLDGD or DFDGD) possessed a conserved domain but this was only 70 nucleotides long implying that IbRDR2_Chr3_1059 is also of less importance in virus resistance in sweetpotato. The catalytic and conserved domains have been reported to play a positive role in virus resistance functionality (Verlaan et al., 2013). We therefore propose that IbRDR1c_Chr1_1623 and IbRDR2_Chr3_1059 were probably mutated in an ancestor and this probably made sweetpotato susceptible to virus infections. If this hypothesis is correct, these genes could be repaired through techniques like CRISPR/Cas9 and thus improve virus resistance in sweetpotato. However, it could also be true that the other RDRs evolved to enable sweetpotato acquire a certain degree of virus resistance we see today.

The four transcripts of the RDR1 gene family (IbRDR1a, IbRDR1b, IbRDR1c and IbRDR1d) were arranged variously. IbRDR1a, IbRDR1b and IbRDR1d had similar genome organization (intron/exons) with four exons each. This suggests the relatedness in the role these gene transcripts play in virus defense in sweetpotato. The IbRDR1c lacked introns and yet had five exons. As was observed earlier, the IbRDR1c transcript also evolved earlier than the rest of RDRs, and lacked the catalytic and conserved domains. Whereas introns are not expressed, they are important in gene regulation (Shaul, 2017). This, therefore, further confirms that IbRDR1c transcript is generally not functional, despite it having more exons than the IbRDR1a, IbRDR1b and IbRDR1d transcripts.

The RDR1s expressed variously in infected plants. IbRDR1a_Chr8_3068 expressed 24-fold, IbRDR1b_Chr8_3014 increased 16-fold while IbRDR1d_Chr8_1149 was the least increased by 3-fold. IbRDR1c_Chr1_1623 gene expression did not increase upon infection. This confirmed that RDR1c transcript is not functional in sweetpotato virus defense. Observations of increase in expression of RDR1 gene were also made in other crops by previous researchers. For instance, increased expression of RDR1a gene (CmRDR1a) was observed in cucumber upon infection with *Cucumber mosaic virus* where the gene titre increased by 21-fold (Leibman et al., 2022). Interestingly, unlike in our study, in the study by Leibman et al. (2022) CmRDR1a gene titre did not increase in cucumber upon infection with potyviruses *Zucchini yellow mosaic virus* and *Cucumber vein yellowing virus*. This shows differences in the interaction of plants and viruses in different plant species

which subsequently require varying deployment of resistance mechanisms (Yadav and Chhibbar, 2018).

Conclusion

Evaluation of potential genes associated with virus resistance in a commercially important sweetpotato crop certainly helps to contribute to an increase in crop productivity and quality. In the present study, we identified 4 RDRs, 1 AGO and 3 DCLs in sweetpotato genome. Phylogenetic and structural analyses of these gene sequences show differences in arrangement of exons and introns, based on which they can be grouped into distinct clades. Presence of the catalytic domains (DLDGD and DFDGD) indicate the involvement of these genes in virus resistance, and indeed the titre of RDR1a, RDR1b and RDR1d transcripts increased upon infection with SPVC. The genes identified in this study can be used as potential targets for crop improvement for developing virus resistant sweetpotato cultivars.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

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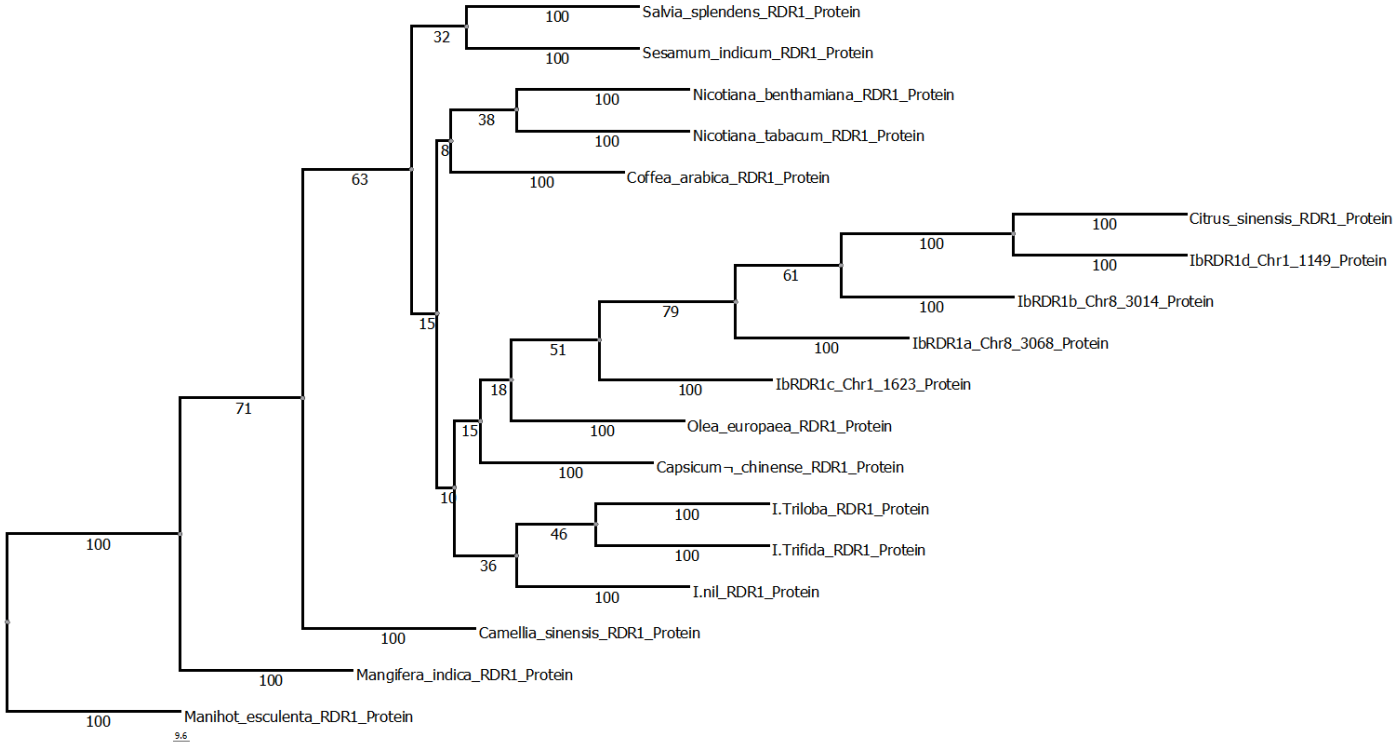
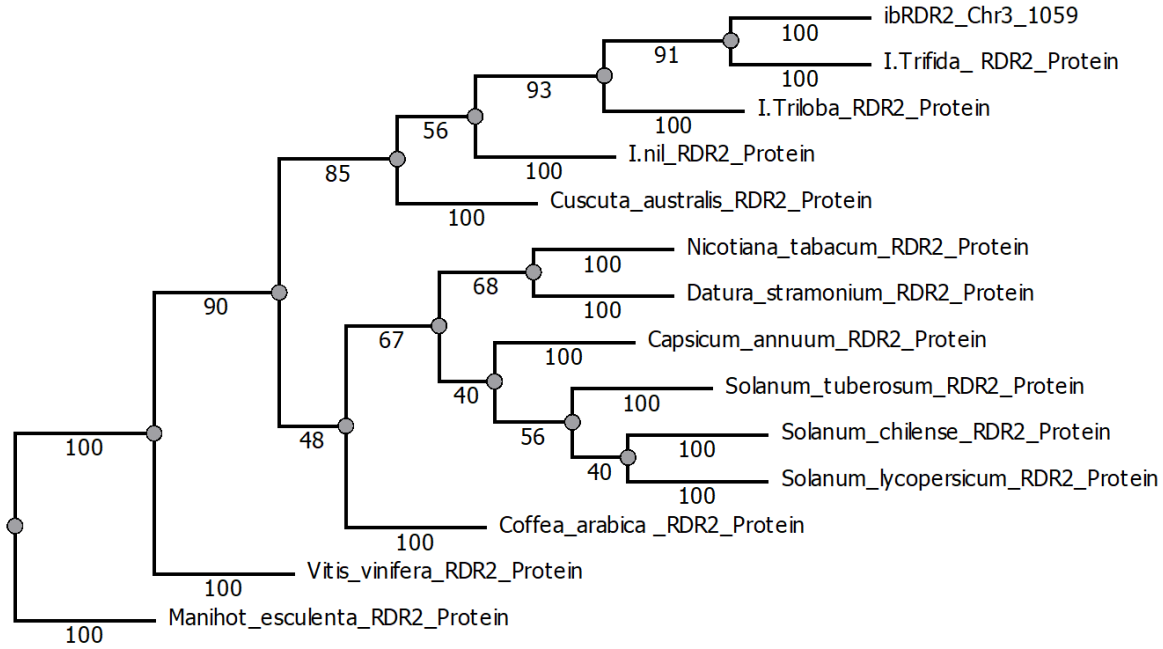
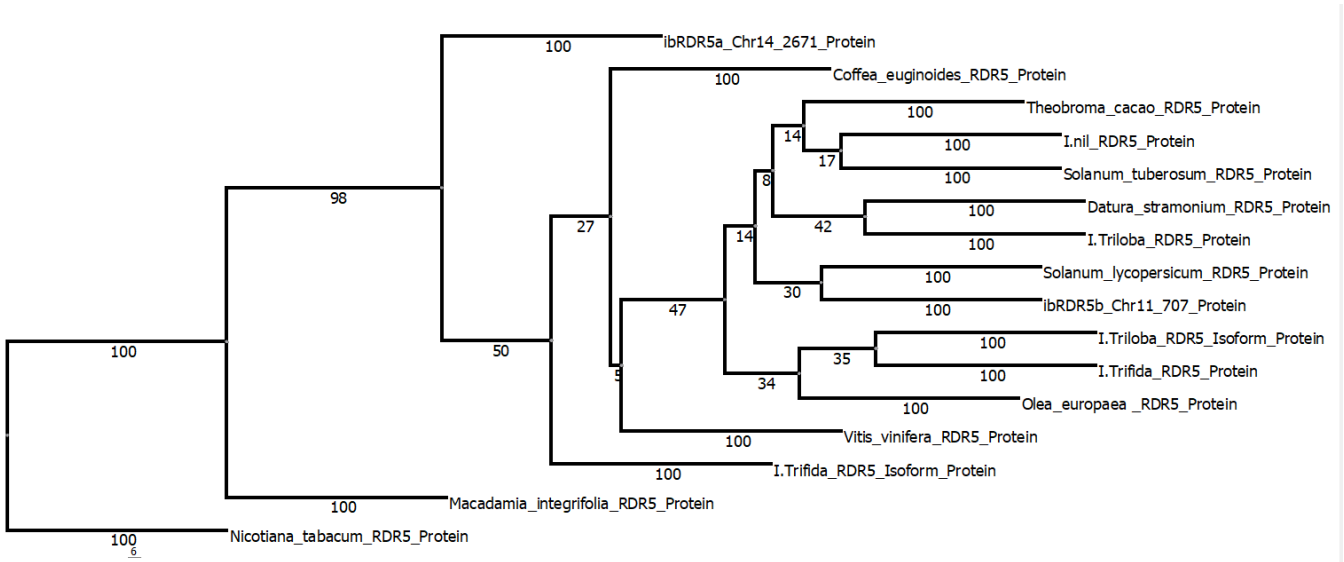
APPENDIX



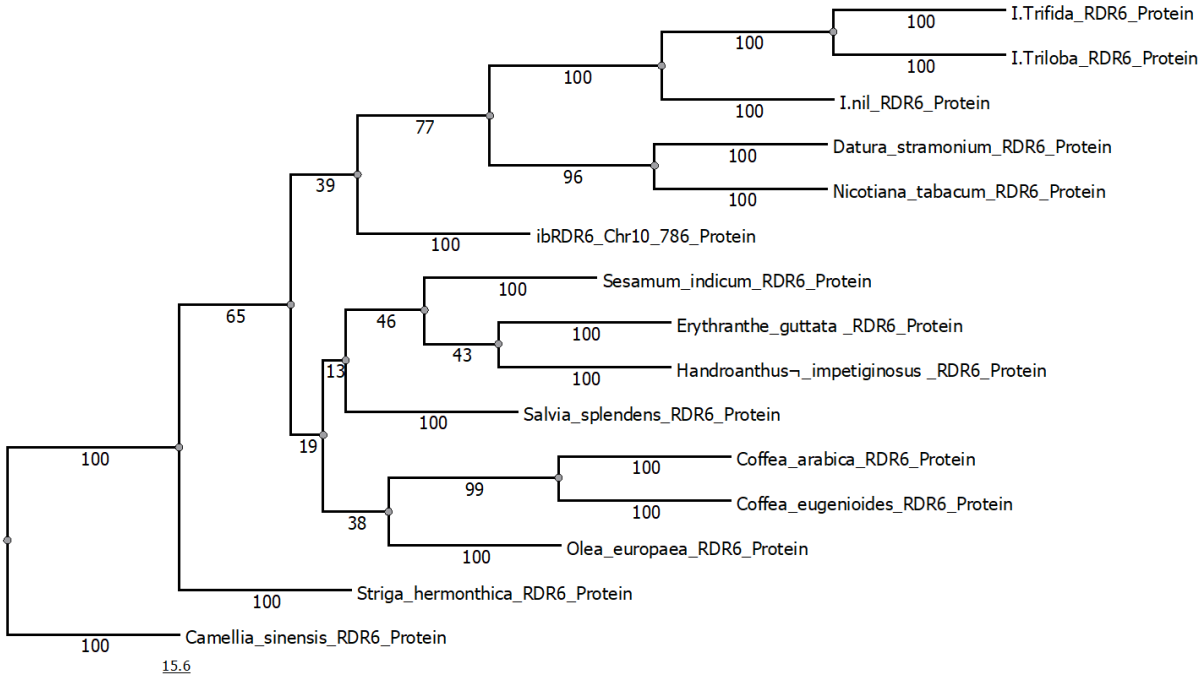
Appendix Figure 1. Phylogenetic tree showing evolutionary relationship of proteins of *Ipomoea batatas* RDR1 and RDR1 from plant species sampled from NCBI and sweetpotato.plantbiology.msu.edu.



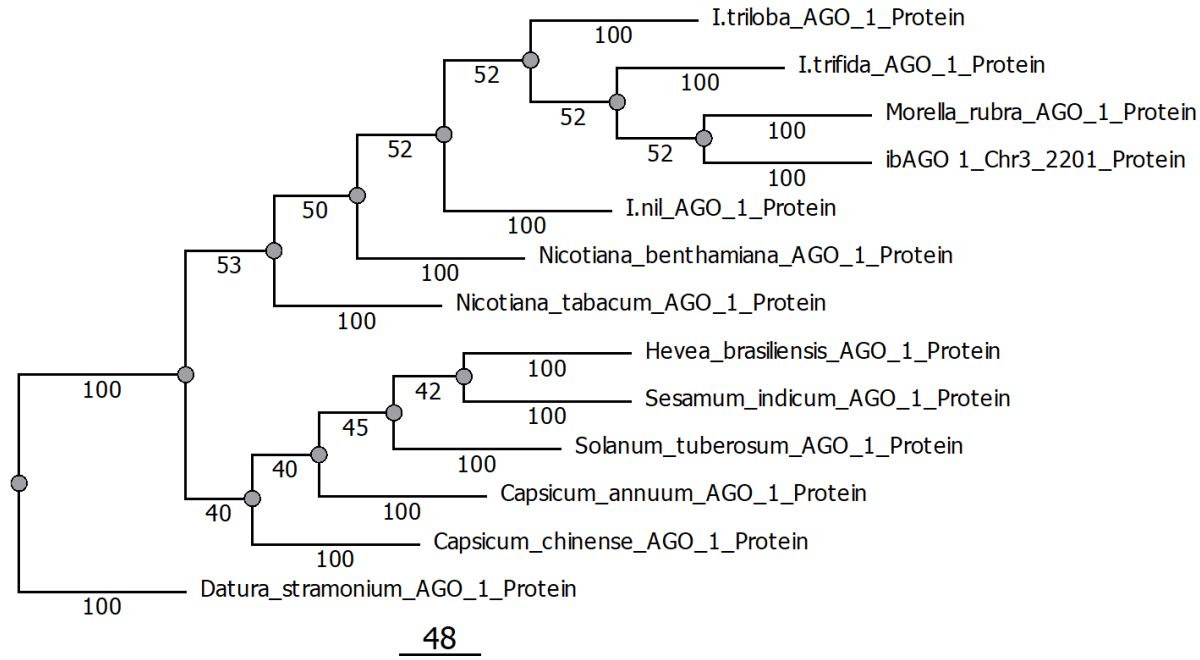
Appendix Figure 2. Phylogenetic tree showing evolutionary relationship of proteins of *Ipomoea batatas* RDR2 and RDR2 from plant species sampled from NCBI and sweetpotato.plantbiology.msu.edu.



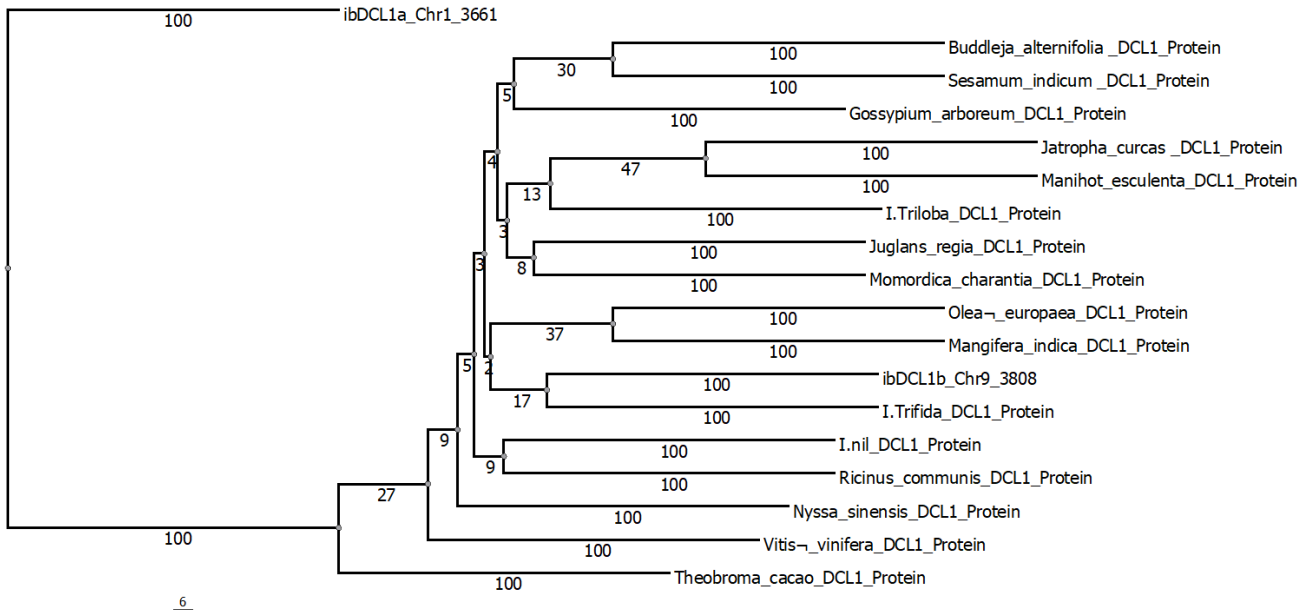
Appendix Figure 3. Phylogenetic tree showing evolutionary relationship of proteins of *Ipomoea batatas* RDR5 and RDR5 from plant species sampled from NCBI and sweetpotato.plantbiology.msu.edu.



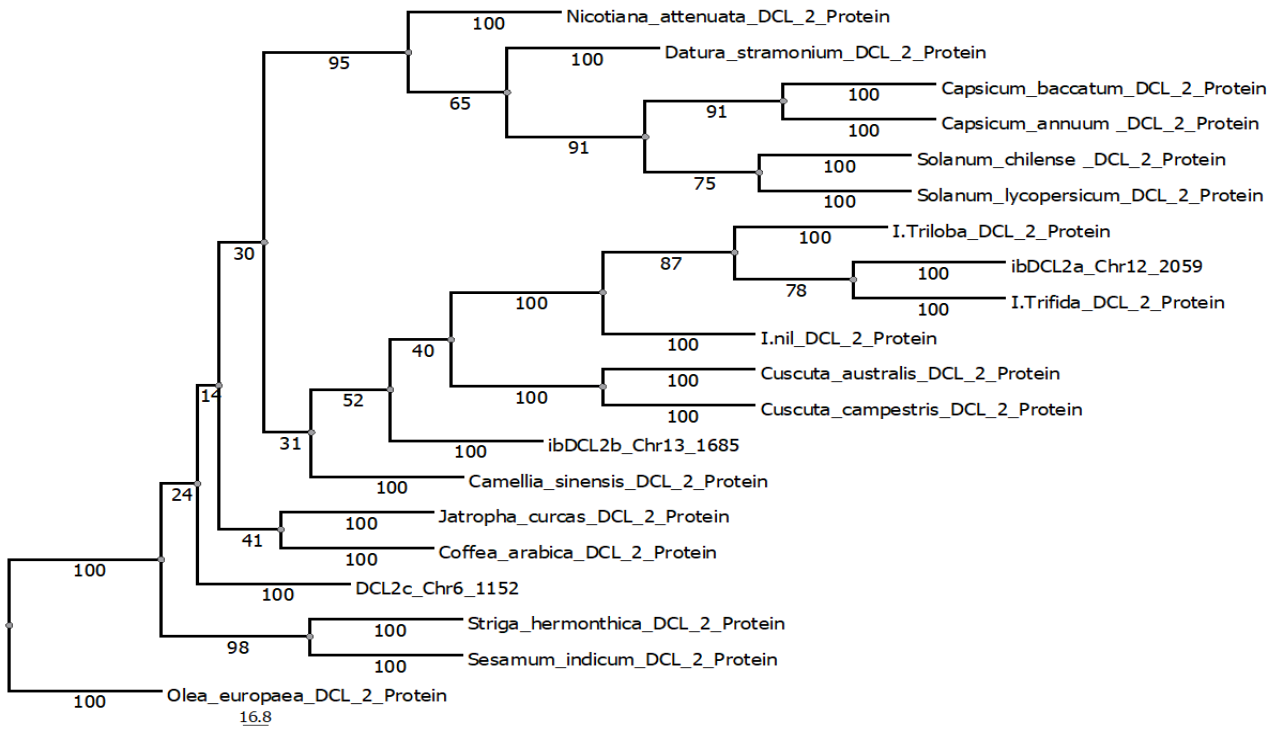
Appendix Figure 4. Phylogenetic tree showing evolutionary relationship of proteins of *Ipomoea batatas* RDR6 and RDR6 from plant species sampled from NCBI and sweetpotato.plantbiology.msu.edu.



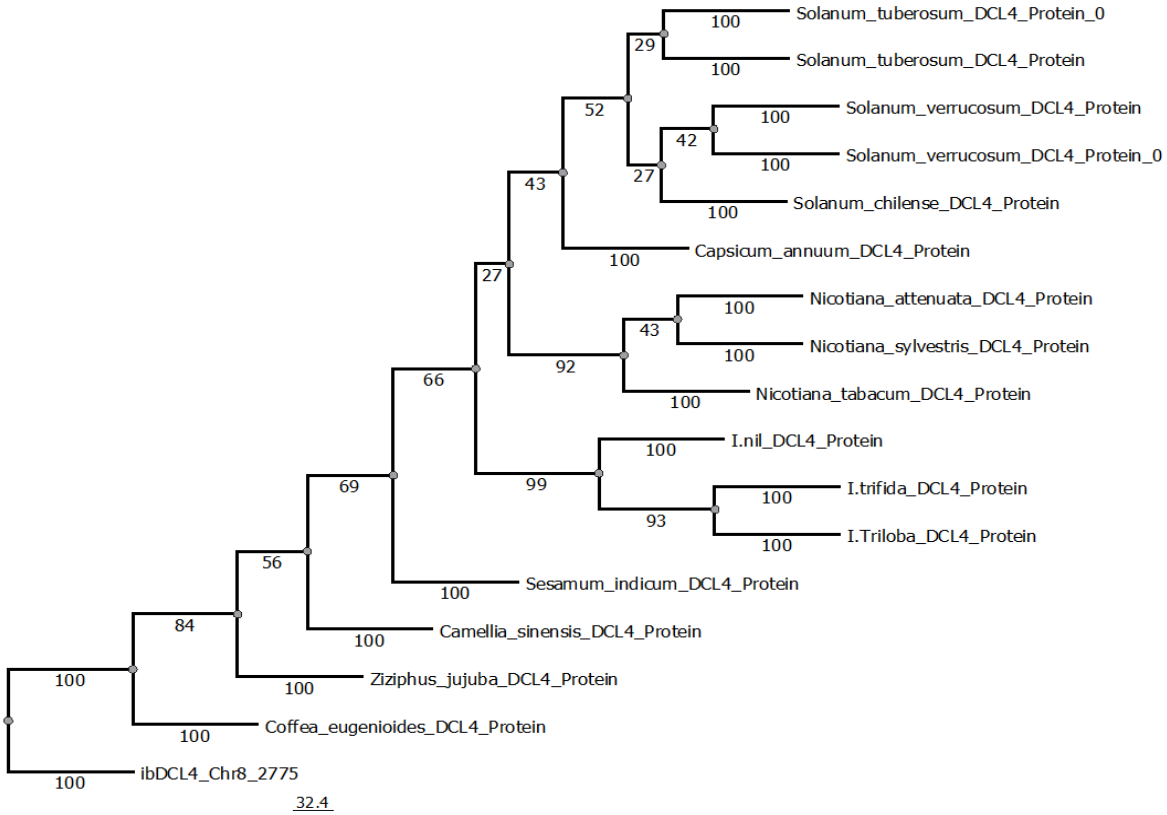
Appendix Figure 5. Phylogenetic tree showing evolutionary relationship of proteins of *Ipomoea batatas* AGO1and AGO1 from plant species sampled from NCBI and sweetpotato.plantbiology.msu.edu.



Appendix Figure 6. Phylogenetic tree showing evolutionary relationship of proteins of *Ipomoea batatas* DCL1 and DCL1 from plant species sampled from NCBI and sweetpotato.plantbiology.msu.edu.



Appendix Figure 7. Phylogenetic tree showing evolutionary relationship of proteins of *Ipomoea batatas* DCL2 and DCL2 from plant species sampled from NCBI and sweetpotato.plantbiology.msu.edu.



Appendix Figure 8. Phylogenetic tree showing evolutionary relationship of proteins of *Ipomoea batatas* DCL4 and DCL4 from plant species sampled from NCBI and sweetpotato.plantbiology.msu.edu.