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MOLECULAR AND FUNCTIONAL IDENTIFICATION OF DIFFERENTLY EXPRESSED GENES ASSOCIATED WITH FLOWERING IN BAMBARA GROUNDNUT (*VIGNA SUBTERRANEA* (L.) VERDC.)

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Abstract
Bambara groundnut (Vigna subterranea) flowering is a crucial developmental stage at the reproductive growth phase. The timing of flowering is controlled by a number of interrelated genetic pathways that are encoded by proteins (candidate genes). This research work identified markers associated with the flowering in Vigna subterranea and the role of variant identified genes in flowering. Deoxyribonucleic acid (DNA) of selected Bamabara groundnut accessions was extracted from leaf samples of 3 weeks old plants, using Dellaporta Miniprep for Plant DNA Isolation procedure. The high-quality DNA was sequenced using Diversity Arrays Technology (DArT) and single nucleotide polymorphism (SNP) markers associated with flowering. Four markers associated with the flowering of Vigna subterranea were extracted from the sequence and their amino acid sequences were used as a query to search the legume protein database in Vigna radiata. The markers with adequate information associated with flowering were 24385352|F|0-28:T>C-28:T>C, 27641816|F|0-17:C>T-17:C>T, 24384204|F|0-24:C>T-24:C>T and 24346601|F|0-67:T>C-67:T>C, significant at $P < 1.68 \times 10^{-4}$on chromosomes 7, 11, 4, and 5. Twenty(20) functional genes that control flowering in Bambara groundnut were identified. The identified genes included histones, Polyketide, cyclase/dehydrase, Transcription factor MYC/MYB N-terminal, Rhamnogalacturonate lyase, DHHC-type zinc finger protein, Putative S-adenosyl-L-methionine-dependent methyltransferase, Ribosomal protein L2, D-galactoside/L-rhamnose binding SUEL lectin domain, Lipase GDSL, Histone deacetylase superfamily, Basic-leucine zipper domain, TUP1-like enhancer of split, Zinc finger ZZ-type, Homeodomain-like, Phosphatidylethanolamine-binding protein PEBP, Leucine-rich repeat which are veritable tools in controlling flowering in Bambara groundnut. This study revealed that flowering in Bambara groundnut is controlled by the interplay of genes.

Keywords: Identification, Functional, Candidate genes, Flowering and Bambara groundnut.

Introduction
Diversity has been established in Bambara groundnut (*Vigna subterranea* [L.] Verdcourt, Syn: *Voandzeia subterranea* [L.] Thouars) of Nigerian origin (Osundare *et al*., 2022). Studies based on morphological characterization of some Nigerian accessions of Bambara groundnut have shown differently expressed phenological and agronomic traits (Mayes *et al*., 2013; Goli, 1997). However, research on candidate genes associated with flowering of Bambara groundnut, and understanding this concept remains a gap in Bambara groundnut

research. Flowers are reproductive structures in Bambara groundnut that transform to seeds, which successfully pass genetic material to the next generation. Flowering in Bambara groundnut raises hope for anticipated yield. The emergence of flowers in plants is a gene-environment activity (Miguel *et al*., 2001). Differential gene expression analysis research has also shown that Bambara groundnut flowering responds to different sets of genes under certain environmental conditions, (Khan *et al*., 2017; Chai *et al*., 2015; Shareef *et al*., 2013; Mabhaudhi *et al*., 2013; Berchie *et al*., 2012; Mwale *et al*., 2007). The need to understand the differentially expressed genes associated with flowering in underutilized legumes is imperative; it is an insight to cell responses due to protein presence or absence. Differentially expressed genes are essentials of plant growth regulators and development, most especially at the flowering stage.

Gene presence and activity are different in plants, and some are present at specific times of plant development to complete a cycle. The functions of the genes are not limited to the formation, emergence and colour of flowers, formation of leaves but also elongation of cells, all these determining the traits associated with plants (Ojolo *et al*., 2018). The identified traits are passed from the mother cells to the daughter cells through encoding genes which form the basis of heredity. Although, advances in molecular biology and quantitative data have developed large reference data with associated gene effects to reveal phenotypic differences in plants. These resources are online to tap, but geneticists are more concerned about accurate identification and interaction of loci affecting phenotypic variation. This study identified the differently expressed genes associated with flowering in Bambara groundnut and proteins encoding the genes. The findings of this study will guide researchers in the identification of candidate genes of desired traits in molecular research of underutilized legumes.

Materials and methods

Conventional (field) evaluation was carried out at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria and International Institute of Tropical Agriculture, research station, located at the Institute of Agricultural Research and Training (IAR&T) Ikenne, Nigeria for three years. Seeds of one hundred accessions of Bambara groundnut were collected from the Genetic Resources Centre (GRC), of IITA in 2017. The accessions can be found in Osundare *et al*., 2022.

DNA Extraction and Genotyping

Morphological data and accession grouping of the Bambara groundnut germplasm used in this study have been previously described by Osundare *et al*., 2022. Three weeks after planting, leaf samples of each accession were collected and the plant stands from which the samples were collected were tagged. DNA extraction was done at the Bioscience Centre, International Institute of Tropical Agriculture, Ibadan following the procedure of Dellaporta Miniprep for Plant DNA Isolation (Weigel and Glazebrook, 2009). The high-quality DNA (100 ng/µL) samples were shipped to DArT Pty Ltd., Canberra, Australia, for genotyping using the whole genome profiling service of DArTseq technology. Diversity Arrays Technology (DArT) was used to identify high-throughput single nucleotide polymorphism (SNP) genotyping. The obtained DArT marker set was filtered on the basis of individual marker-related statistics by removing markers with inappropriate quality control parameters with call rate $\leq$ 80 % and missing data $\geq$ 20 % in TASSEL 5.0 software (Bradbury *et al*., 2007). The informativeness of the marker was determined using the polymorphic information content (PIC). A total of 11, 821 DArT seq SNPs were generated, out of which 5, 927 (50.13 %) high quality markers

were retained for data quality at a call rate of 80% and used in the analysis. Four informative markers were associated with flowering out of the 5, 927 DArTseq SNP significant markers. Marker-trait associations (MTAs) was determined for flowering traits (number of days to flowering and number of flowers per peduncle) using the Genome wide association study (GWAS) (Figure 1 and 2). The significance of associations between SNPs and flowering traits was based on the threshold $P < 1.68 \times 10^{-4}$, calculated by dividing 1 by the total number of SNPs (5927) in the analysis (Li *et al*., 2016). In the absence of Bambara groundnut genome, trimmed sequences of filtered SNPs were aligned to the *Vigna radiata* reference genome (available on LIS, https://www.legumeinfo.org accessed 11 November, 2022).

Identification of candidate genes in flowering

The identified significant markers associated with flowering through GWAS were queried with the available protein signature on the available online sequence databases using the legume Information system (LIS) for the identification of candidate expressed protein names, controlling flowering in Bambara groundnut. The sequences were submitted for blast on *Vigna radiata* genome. The blast search was performed for the trimmed nucleotide sequences (60-80bps) of significant Bambara groundnut SNPs on *Vigna radiata* database in the legume information system. After marking the annotated positions in the genome database, the scroll was zoomed to 1Mb to identify surrounding candidate genes. Twenty (20) encoding proteins were identified and existing legume genomic knowledge data bases were searched to determine if the identified encoding proteins were associated with flowering and by extension, determine that they regulate flowering in Bambara groundnut.

Results and Discussion

Marker Traits Association and Protein functions

The extraction of significantly associated markers revealed only four markers were associated with flowering in Bambara groundnut out of 5, 927 significant markers. Table I revealed associated markers with Bambara groundnut flowering, they include 24385352|F|0-28:T>C-28:T>C; 27641816|F|0-17:C>T-17:C>T; 24384204|F|0-24:C>T-24:C>T and 24346601|F|0-67:T>C-67:T>C and were significant at $P < 1.68 \times 10^{-4}$ at chromosomes 11, 7, 4 and 5. Hence, only 0.06 % of the total number of significant markers was associated with flowering in Bambara groundnut.

Marker 24385352|F|0-28:T>C-28:T>C identified candidate protein(variant) on chromosome 7, gene nomenclature was Vradi07g07630 within the region of Vr07:17881455..17882105, with protein name 'Polyketide cyclase/dehydrase' (IPR019587) associated with flowering in Bambara groundnut. Marker 24385352|F|0-28:T>C-28:T>C was also identified as candidate protein(variant) on chromosome 7, gene nomenclature was Vradi07g07680 within the location of Vr07:18234488..18236837, with protein name 'Polyketide synthase, enoylreductase' (IPR020843) involved in plant biosynthesis. Marker 24385352|F|0-28:T>C-28:T>C also identified candidate protein (variant) on chromosome 7, gene nomenclature was Vradi07g07680 and Vradi07g07750 with gene region of Vr07:18234488..18236837 and Vr07:18641936..18645271, with protein name 'Polyketide synthase, enoylreductase' (IPR020843) and 'Transketolase, C-terminal/Pyruvate-ferredoxin oxidoreductase' (IPR009014) respectively, involved in the biosynthesis of a variety of plant which enhances autotrophic growth. Marker 24385352|F|0-28:T>C-28:T>C also identified a candidate protein (variant) on chromosome 7, gene nomenclature was Vradi07g07600 within

the location of Vr07:17644695..17646647, with protein name 'Transcription factor MYC/MYB N-terminal' (IPR025610) involved in biosynthesis of secondary metabolites including flavonols and lignin and morphogenesis of flowers. Marker 24385352|F|0-28:T>C-28:T>C also identified a candidate protein (variant) on chromosome 7, the gene nomenclature was Vradi07g07740 within the region of Vr07:18443116..18446924, with protein name 'Deoxyxylulose-5-phosphate synthase' (IPR009014) involved in the transportation of the molecules across the membrane in the cell. Marker 24385352|F|0-28:T>C-28:T>C also identified a candidate protein (variant)on chromosome 7, gene nomenclature was Vradi07g07700 within the region of Vr07:18282445..18287676, with protein name 'Rhamnogalacturonate lyase' (IPR008979) involved in the slow degradation of the petal cell wall in flowers.

Marker 27641816|F|0-17:C>T-17:C>T identified a candidate protein on chromosome 11, gene nomenclature was Vradi11g06830 within the region of Vr11:6871551..6876657, with the protein name 'DHHC-type zinc finger protein' involved in the regulation of yield increase (Table 1). Marker 27641816|F|0-17:C>T-17:C>T identified a candidate protein (variant) on chromosome 11, gene nomenclature was Vradi11g06000 within the region Vr11:5890615..5896336, with protein name 'Putative S-adenosyl-L-methionine-dependent methyltransferase' (IPR004159) involved in a large group of plant development including flowering in plants. Marker 27641816|F|0-17:C>T-17:C>T identified a candidate (protein on chromosome 11, gene nomenclature was Vradi11g06250 within the region Vr11:6211377..6212072, with protein name 'histone H1-3' (IPR005818) conciliates in chromatin folding, proper stomata functioning, genetic expression, and cellular differentiation. Marker 27641816|F|0-17:C>T-17:C>T identified a candidate protein (variant) on chromosome 11, gene nomenclature was Vradi11g06490 within the region Vr11:6517125..6519306, with protein name 'Ribosomal protein L2' (IPR002171) present in mitochondrial, involved in the flowering of plants. Marker 27641816|F|0-17:C>T-17:C>T identified a candidate (protein variant) on chromosome 07, gene nomenclature was Vradi07g00920 within the region Vr07:1912863..1918248, with protein name 'D-galactoside/L-rhamnose binding SUEL lectin domain' (IPR000922) also present in *Arabidopsis thaliana*, involved in the flowering development (GO:0009908). Marker 27641816|F|0-17:C>T-17:C>T identified a candidate protein (variant) on chromosome 07 gene nomenclature was Vradi07g00940 within the region Vr07:1979503..1989132, with protein name 'Microspherule protein, N-terminal domain' (IPR025999). N-terminal domain synthesizes chloroplast proteins in mitochondria in plants. Marker 27641816|F|0-17:C>T-17:C>T identified a candidate protein(variant) on chromosome 07, gene nomenclature was Vradi07g01680 within the region Vr07:2821523..2823634, with protein name 'Lipase, GDSL' (IPR001087) involved in flower development. Marker 27641816|F|0-17:C>T-17:C>T identified a candidate protein (variant) on chromosome 07 gene nomenclature was Vradi07g01700 within the region Vr07:2841425..2849603, with protein name 'Histone deacetylase superfamily' (IPR000286), regulates flowering and fruit development in plants.

Marker 24384204|F|0-24:C>T-24:C>T identified candidate protein (variant) on chromosome 04, gene nomenclature was Vradi04g03640 within the region Vr04:7184338..7189633, with protein name 'Basic-leucine zipper domain' (IPR004827), delayed flowering. Marker 24384204|F|0-24:C>T-24:C>T identified candidate protein(variant) on chromosome 04, gene nomenclature was Vradi04g03560 within the region Vr04:7074159..7080129, with protein name 'TUP1-like enhancer of split' (IPR011494), mediates repression in

outer whorls of flower. Marker 24384204|F|0-24:C>T-24:C>T identified candidate protein (variant) on chromosome 04, gene nomenclature was Vradi04g03760 within the region Vr04:7635166..7638397, with protein name 'Zinc finger, ZZ-type' (IPR000433), regulates flowering time. Marker 24384204|F|0-24:C>T-24:C>T identified candidate protein (variant) on chromosome 04, gene nomenclature was Vradi04g03850 within the region Vr04:7965793..7996880, with protein name 'Homeodomain-like' (IPR009057). Homeodomain-like regulates flowering time in higher plants. Marker 24384204|F|0-24:C>T-24:C>T was also identified candidate protein (variant)on chromosome 04, gene nomenclature was Vradi04g03610 within the region Vr04:7140389..7141382, with protein name 'Phosphatidylethanolamine-binding protein PEBP' (IPR008914). Phosphatidylethanolamine-binding protein PEBP is a flowering locus protein in plants that controls flowering.

Marker 24346601|F|0-67:T>C-67:T>C also identified as candidate protein on chromosome 05, gene nomenclature was Vradi05g02840 within the region Vr05:3602575..3635224, with protein name 'Leucine-rich repeat' (IPR001611). Leucine-rich repeat is involved in developmental processes, among which arecell proliferations and flower development.

The significant markers revealed proteins controlling flowering in Bambara groundnut and indicated that flowering in Bambara groundnut is controlled by the interplay of multiple genes. The candidate genes found to be associated with flowering in this study agrees with findings from other investigations of plant species. For instance, Vandasue *et al*. (2021), reported Polyketide cyclase/dehydrase as a candidate protein that controls flowering in *Arabidopsis thaliana*. Keith *et al*., 2002, reported that different mitochondrial ribosomal proteins (L2-L4) presence or absence in mitochondrial are involved in the regulation of flowering in soybean, cotton, tomato, and Arabidopsis. Uniprot database confirmed the involvement of a candidate protein 'D-galactoside/L-rhamnose binding SUEL lectin domain' in flower development (GO:0009908) in Arabidopsis thaliana plants. Sukarkarn *et al*., 2020, reported N-terminal domain proteins in *Brassica napus* and identified that terminal flowering gene negatively regulate flowering time in Brassica napus. Yingjie *et al*., 2014, reported Zinc finger, ZZ-type, regulates flowering time in Chrysanthemum. Jensen *et al*., 2001, also reported that over expression of terminal flower1-genes in perennial ryegrass delays flowering, as it is expressed in inflorescence shoots and vegetative meristems. Michael *et al*., 2006, also reported Basic-leucine zipper domain delayed flowering in maize. Joanne and John (2012) also reported TUP1-like enhancer of split mediated repression in the outer whorls of flower. GDSL Lipase is required for Anther and Pollen Development (Wei 2020; Ishiguro *et al*., 2001). Liu *et al*., 2016, also reported that histone deacetylases are essential for gene expression in plant development and revealed the involvement of histone deacetylases in the transcriptional regulation of multiple developmental processes. This was also confirmed by Kinga *et al*., 2015 that 'histone H1-3' conciliates in chromatin folding, proper stomata functioning, genetic expression and cellular differentiation. Yutong *et al*., 2022 reported 'Histone deacetylase superfamily' regulates flowering and fruit development in *Capsicum annuum*. Ali *et al*., 2017, reported that basic leucine zipper domains are involved in fundamental seed development processes from the flowering stage. Liu and Karmarker, 2008, reported that Tup1 families plays an important roles in developmental processes in floral organ identity specification. Yingjie *et al*., 2014, reported that Zinc Finger Protein Regulates Flowering Time by regulating gibberellin (GA) biosynthesis under both long and short

days. Vijee *et al.,* 2018, revealed that homeodomain-like gene regulates flowering time in pepper. Michael *et al*., 2006, identified Phosphatidylethanolamine-binding protein PEBP in *Arabidopsis FLOWERING LOCUS T* (*FT*) gene, as a controller of flowering in plants. Hossein and Frank, 2019 reported that pentatricopeptide repeat protein affects flowering in *Arabidopsis thaliana*. Yuan *et al*., 2015 reported 'Putative S-adenosyl-L-methionine-dependent methyltransferase' as a large group involved in plant development including flowering in *Lonicera japonica*. Keiko 2004 indicated that leucine-rich repeat receptor kinases (LRR-RKs) regulate a wide variety of developmental and defense-related processes including cell proliferation, stem cell maintenance, hormone perception, host-specific as well as non-host-specific defense responses, wounding responses, and symbiosis. Chia *et al*., 2011, also reported that 'Transketolase' enhances autotrophic growth in *Rhodopseudomonas palustris*. Sujit (2016) also reported Transcription factors MYC/MYB N-terminal are key factors controlling development in plants.

Isveet and Robert, 2009, also reported that several type III PKSs have been found in plants and all of them participate in the biosynthesis of secondary metabolites such as inhibition of flowering. Dongmei *et al*., 2022 reported Type III polyketide synthases (PKSs) are key enzymes involved in the biosynthesis of a variety of plant specialized metabolites, including flavonoids, stilbenes, and sporopollenin. Martin and Paz-Ares, 1997, identified the increasing presence of MYB genes in higher plants, and *Arabidopsis thaliana* is estimated to contain more than a hundred MYB genes in their control of gene expression, especially in cellular proliferation and development. Louwrance *et al.,* 2014, reported the increasing dynamics of molecules with the presence of Deoxyxylulose 5-Phosphate Synthase through Methylerythritol 4-Phosphate Pathway in Arabidopsis. Verónica-Alhelí *et al*., 2018, reported that Rhamnogalacturonan lyase (RGL) enzyme is capable of catalyzing the degradation of cells. Zhou *et al.,* 2017, reported that the flowering and grain yield of transgenic rice increased by DHHC-type zinc finger protein genes. Veronica *et al*., 2018 reported 'Rhamnogalacturonate lyase' to regulate pollen and flower development in higher plants. Anna *et al.,* 2012 reported that methyltransferase (MTases) are a veritable biotechnology tool in crop improvement. Ila *et al.,* 2012, reported that histone dynamics resulted in variants responsible for gene regulation in plants, although different biochemical techniques in the analysis of histones have been developed to identify specific histones and its regulatory roles. Rong *et al*., 2021 reported 'Deoxyxylulose-5-phosphate synthases' are involved in the transportation of molecules through biosynthesis in *Pinus massoniana*. Shashi *et al*., 2006, also reported N-terminal domain synthesizing chloroplast proteins from mitochondria in plants.

Conclusion

This study is the fundamental research which initialized the genetic basis of Bambara groundnut in relation to flowering with four significant markers genetically associated, they include 24385352|F|0-28:T>C-28:T>C; 27641816|F|0-17:C>T-17:C>T; 24384204|F|0-24:C>T-24:C>T and 24346601|F|0-67:T>C-67:T>C found on the *Vigna radiata* genome and should be recognized as candidate markers. The markers also revealed functional genes that control flowering in Bambara groundnut, to include histones, Polyketide cyclase/dehydrase, Transcription factor MYC/MYB N-terminal, Rhamnogalacturonate lyase, DHHC-type zinc finger protein, Putative S-adenosyl-L-methionine-dependent methyltransferase, Ribosomal protein L2, D-galactoside/L-rhamnose binding SUEL lectin domain, Lipase GDSL, Histone deacetylase

superfamily, Basic-leucine zipper domain, TUP1-like enhancer of split, Zinc finger ZZ-type, Homeodomain-like, Phosphatidylethanolamine-binding protein PEBP, Leucine-rich repeat. Further efforts to validate the findings using other high throughput technologies and databases should be targeted in identifying functional genes in Bambara groundnut flowering towards marker assisted selection.

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Data Availability
The data used to support the findings of this research are available from the corresponding author upon request.

Conflict of interest
The authors declare that there is no conflict of interest.

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