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RNA-Seq analysis of two elite lines of *Withania somnifera* to evaluate potential of heterosis

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Abstract

Metabolite targeted breeding is one of the prime need in Ashwagandha (*Withania somnifera*) to meet its fast growing demand at domestic and international level owing to its vast spectral pharmaceutical potential. Till date only scanty breeding efforts exists majority being introductions or selections from the wilds/landraces through which active bio constituent increase is limited. Hybridization based heterotic positive QTL alleles can be introgressed in a fine quality good agronomic genetic base using marker assisted selection. Investigations under this study generated a large resource of *Withania* specific genomic microsatellite markers and SNP through RNA seq. Using an RNA-seq approach, the expression of 101245 unigenes were quantified in parents .The expression levels of 30% of transcripts differed between parents, a majority of which had more than 1.5 fold-changes.RNA seq will help to decipher the Withanolide biosynthesis pathway and SNP responsible for functional polymorphism in pathway genes and hence predicting the potential of heterosis.

Key word: *Withania somnifera*, heterosis, RNA- seq, withanolides, SNP

Introduction

Withania somnifera (L.) Dunal (Ashwagandha), a pivotal medicinal plant of Indian traditional medicine Ayurveda, is the chief source of Withanolides (Withanolide A, B, D, F, G, Withaferrin A, Withanone and 2-D Withastramonolide etc.). Besides this, Withanosides (Withanosides I, II, III IV V VI and IX), various Withanamides (A, B, C, D, E, F, I, J, K, L, M, N, O, P, Q, R) and alkaloids like withasomine, somniferine, withanine, cuscohygrine, anhygrine, tropine, pseudotrophine, anaferine, choline, tropanol, pseudotropanol, isopelletriene, accounts for its diverse pharmacological potential for which it is a known traditional medicine since 300 years [1]. Withanolides have multiple bioactivities and great potential in drug research because of their antitumour, anti-stress, cytotoxic, immunosuppressive and anti-inflammatory and anti-geriatric activities and have been proven to reverse the Alzheimer's disease and inhibit breast cancer cell growth [2-4]. Withanamides present in berries has recently been proved to have lipid peroxidation inhibitory activity, which is more potent than the commercial antioxidants [5,6]. To meet the growing demands of pharmaceutical industries for bioactive withanolides, withanosides and withanamides through *in planta* production, withanolides targeted breeding programme is being undertaken at CSIR-CIMAP. This study was undertaken to understand the molecular basis of heterosis in withanolide yields. Three classical models suffice for partitioning of heterosis into genetic and molecular interactions effects. The dominance model proposed that distinct set of deleterious recessive alleles undergo genome wide complementation in hybrids; where the over-dominance model states that intra locus allelic interactions at one or more heterozygous genes leads to increased vigour, third model illustrates pseudo-over dominance which is dominance that mimics over-dominance because the mutations involved are linked [7]. Genes showing over-dominance are rare and most sought as single heterozygous gene is needed for heterosis which could be possible if it is a master regulator of biosynthetic pathway [8].

Variation in the levels of metabolites within a species or population is largely quantitative, moderately heritable and shows polygenic inheritance controlled by

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the interaction of environmental and genetic factors. Gene expression is a complex trait determined by various genetic and non-genetic factors. Among the genetic factors, allelic difference may play a critical role in gene regulation. Intraspecific allelic variation is often attributed to qualitative changes that affect the nature of the gene products and quantitative changes that alter the amount of the gene product produced. Quantitative changes in gene expression may be the result of cis or trans variations in gene regulation. Cis-regulation is due to variation that is genetically linked to the locus with differential expression. Alternatively, trans-regulation is due to variation at unlinked loci affecting the level of gene expression. Such differences can arise from cis-regulatory changes that affect transcription initiation, transcription rate and/or transcript stability in an allele-specific manner, or from trans-regulatory changes that modify the activity or expression of factors that interact with cis-regulatory sequences [9]. Both cis and trans regulatory changes contribute to divergent gene expression, but their respective contributions remain largely unknown. These data indicate that interspecific expression differences are not caused by select trans-regulatory changes with widespread effects, but rather by many cis-acting changes spread throughout the genome. Sequence polymorphisms from the whole genome and within gene regulatory elements can affect transcription rate or transcript stability of the associated allele, while trans-genetic polymorphisms cause variation in transcript abundance of both target alleles.

The genetic basis of inter-generic, inter-specific, intra-specific diversity and the consequences of selection on genetic variation in the different wild and cultivated groups have not yet been studied on a genome wide basis in *Withania somnifera*. The genomic abundance and amenability to cost-effective high throughput genotyping make microsatellites and single-nucleotide polymorphisms (SNPs) the most-used markers for genome-wide surveys of genetic diversity. Single-nucleotide polymorphisms (SNPs) represent the most abundant type of variation present in DNA followed by microsatellites. SNPs are mostly biallelic, co-dominantly inherited and occur at high density within genomes. They are thus amenable to the development of genetic molecular markers at low cost, which can provide sufficiently dense genome coverage for the dissection of complex traits [10-12]. SNP identification using NGS technologies essentially has become cheaper and faster. RNA-Seq of *Withania somnifera* using Illumina sequencing with de novo assembly will provide both differential gene expression, alleles specific expression and genetic polymorphism (single nucleotide polymorphisms and microsatellite) [13]. In *Withania somnifera* limited genetic and genomic information is available. Conventional breeding based on phenotypic selection resulted into limited metabolite yield productivity. Current study was undertaken to evaluate the potential of heterosis for biomass, root yield, yields of total alkaloids and Withanolides through RNA seq.

Materials and methods

Plant material and growth condition and field trial

For strategic introgression of metabolite QTLs, two diverse chemotypes, a well-adapted, good root quality, Withaferrin A rich recipient accession from Nagore and high Withanolide A content, biomass and biotic stress tolerant donor accession collected from Patna were selected as potential parental stocks on the basis of genetic diversity scan and high withanolides content. 300 F1 (CWS11xCWS7) hybrids and 100 F1 (CWS7 x CWS11) reciprocal crosses were attempted in 2011-12. The elite lines used as parents and hybrids were phenotyped for following traits in the kharif season, plant growth habit (PGH), leaf by breadth ratio (L/B), days to maturity (DTM), plant height (PH), berries in a cluster (BN), berry colour (BC), primary branching (PB) ,secondary branching (SB), calyx length (CL), root length (RL), root diameter (RD), root fracture (RF), fresh root weight (FRW) and calyx inflation (INF) were evaluated in a randomized block design with three replications at the research farm of the CSIR-Central Institute of Medicinal and Aromatic Plants located within longitude 80^{o} 59' E and latitude 20^{o} 55'N in subtropical climate region. Each hybrid was transplanted in 3m long row with 30x20 spacing row-row and plant to plant, respectively in August 2012-13. Normal cultural practices were followed throughout the crop season.

RNA-seq analysis

Fourth and fifth leaf were sampled from 3 week old glass house grown plants of both elite lines in October 2013, flash frozen in liquid nitrogen and stored at –80°C until used for RNA extraction. Strand specific transcriptome library for sequencing was constructed according to the NEXTflex directional RNA-Seq (dUTP based) (Bioo Scientific) manufacturer's protocol. The library was amplified using 15 cycles of PCR for enrichment of adapter ligated fragments. The prepared library was quantified using Nanodrop and validated for quality by running an aliquot on high sensitivity bioanalyzer chip (Agilent) and subjected to paired end 100bp HiSeq 2000

Illumina sequencing. Reads were *denovo* assembled by Trinity (K mer=25). The assembled transcript isoforms were clustered (cd-hit-v4.5.4 at 95% identity) and searched (blastx, 1E-10) against the Uniprot-tremble and tomato (http://solgenomics.net/ organism/ Solanum_ lycopersicum/ genome; version ITAG2.4) and potato (http://solgenomics.net/organism/*Solanum_ tuberosum*/genome ver 3.4). For the functional classification, matches were compared to the GO association .Differential gene expression profiling was carried out using DE Seq [14]. All the unigenes were subjected to MISA (http://pgrc.ipk-gatersleben.de/misa/) for identification of SSR motifs. Alignment of sequence data from both the parents to the pseudo reference created from pooled assembly of parents was used the Bowtie/SAMtools variant detection pipeline for SNP discovery.

Extraction of withanolides and withanosides and RP-HPLC analysis

The six standards i.e., Withaferrin A, Withanolide A, Withanoside IV, Withanoside V, Withanone, 12 deoxy-withastramonolide were obtained from Chromadex (Irvine, CA) and used for quantification. *n*-Hexane and chloroform and distilled water used for sample preparation were of analytical grade from Merck (Darmstadt, Germany). Methanol and acetic acid used were of HPLC grade from Merck and Sigma Aldrich, respectively. Total leaf and root alkaloids and withanolides were extracted from freshly collected 4 g leaf and root tissue, finely powdered in liquid nitrogen and extracted. A reversed-phase HPLC analysis was performed for the simultaneous analysis of four withanolides, and two withanosides as per standard procedure [15]. The withanolide content of parental lines was estimated in the *kharif* season of years for three years 2006-2008 and along with the hybrids in 2012-13.

Statistical analysis

Descriptive statistics, ANOVA and correlations between traits were calculated using packages Hmisc [16] and Pastecs [17,18] in R. Heterosis was estimated as the percentage of the superiority of the hybrid over its mid-parent value (MP) or better-parent value (BP) or maternal parent as given by [19].

Results and discussion

Based on molecular, phenomic and phytochemical characterization two distinct accessions were selected for intra-specific hybridization [20]. CSW11 selected as female parent is short (30cm) with small slightly branched whitish cream, non-separable rind, fine powderable root with soft, tuberous, starchy texture which breaks with even fracture (preferred by pharmaceutical industry), 5-7 berries at a node which turns yellow on maturity, small light green sub-coriaceous leaves with wavy margin, inconspicuous veins and lax branching from Nagore (Madya Pradesh) with high withaferin A content. Donor parent CWS 7 is a accession from Patna, rich in withanolide A content, tall (1.0-1.5 m), heavily branched, high biomass, dark green sharply acute leaves, 12-15 berries at a node which turns red at maturity and long brown root with prominent lateral branching woody texture, easily separable rind and cannot be easily powdered.

Surveliance of heterosis in agronomic and root quality F_1 hybrids

Heterosis is the superior performance of F_1 hybrid progeny relative to the parental phenotypes. Eighteen phenotypic character i.e., plant height, plant growth habit, days to maturity, berry colour, calyx length, inflation, berry number/involucre, berry colour, primary branch, secondary branch, lamina length and breadth, lamina margin, lamina shape, root length, root diameter, root fracture, root fresh weight was taken on 271 F1 hybrids and 70 reciprocals derived from two different elite genetic stock, CWS11Y and CWS7. Phenotypic variance was observed in hybrids for all agronomic traits

Table 1. Phenotypic variance in 271 F_1hybrids for major phenomic traits

	Mean	SE.of mean	CI. mean	Variance	SD	CV	Kurtosis
PH	46.83	1.06	2.09	342.05	18.49	0.39	0.75
DTM	231.31	1.32	2.59	545.42	23.35	0.10	-0.69
BS	6.97	0.05	0.10	0.66	0.82	0.12	-0.31
CL	2.05	0.02	0.04	0.12	0.35	0.17	-0.15
INF	1.45	0.02	0.05	0.17	0.41	0.28	5.35
BN	6.79	0.16	0.32	6.44	2.54	0.37	1.45
PB	3.67	0.14	0.27	5.75	2.40	0.65	8.46
SB	11.44	0.52	1.03	82.31	9.07	0.79	0.99
L.B	1.91	0.03	0.06	0.24	0.49	0.26	81.27
RL	18.13	0.33	0.66	31.28	5.59	0.31	1.11
RD	1.31	0.03	0.07	0.30	0.55	0.42	18.78
SECR	3.22	0.22	0.43	13.00	3.61	1.12	67.69
RFW	17.54	0.93	1.83	232.99	15.26	0.87	24.95

SE = Standardar Errorl SD = Standard deviation; CV = Coefficient of variability

(Table 1). Large variation in the values of phenotypic traits was recorded in the hybrids. Days to maturity, plant height and root fresh weight trait showed maximum variance which was expected from the contrast phenotype of the parents i.e., early maturing, good quality with even fracture, short root, yellow berry recipient with late flowering tall long root with moderate root quality and uneven fracture red berry CWS7 donor and is an indicator of introgression of QTL alleles from the donor.

Heterosis based evaluation of hybrids provided realistic estimate of degree of dominance for this cross (Table 2). Majority of the morphological traits showed promising and typically positive breeding response to intraspecific hybridization. A range of positive heterosis for better parent, mid-parent as well over maternal parent for both the reciprocal crosses occurred for the trait secondary branching which is a direct contributor of biomass and total alkaloids from leaf in the hybrids. Trait primary branching showed no enhancement but may segregate further to yield desirable recombinations of withanolide content partitioning between root and leaf sinks. Five different potential patterns of hybrid expression can be possible, below-low parent expression; low parent-like expression; mid-parent expression; high parent-like expression P); or above high parent expression. Only mid-parent expression is classified as additive and rest as non-additive expression. Gene expression influence heterosis, by the relative frequencies of additive and non-additive expression levels in the hybrid. Additive expression occurs when the hybrid expression level is equivalent to the mid-parent values while non-additive expression occurs whenever the hybrid expression level deviates from the mid-parent level. Non-additive expression phenotypes can include expression levels between the mid-parent and parental values, expression levels equivalent to one of the parents or expression levels outside the parental range. The identity and frequency of genes exhibiting hybrid gene expression levels outside of the parental range is the interest in the current study.

Root fresh weight and root length with a proportionate increase in root diameter showed positive heterosis which directly contributing to root yield and total alkaloids and withanolides from root. For plant height selection was carried in negative direction. Root fresh weight was found to be significantly highly correlated with Plant height, root length, root diameter, primary and secondary branching which indicates plant biomass increase also will increase root weight (Table 3). Calyx inflation was negatively correlated with days to maturity and positively correlated with berry size. Primary branching was highly correlated with root length, berry number. Secondary branching was also positively correlated with root length, root diameter, secondary root and contributing to root fresh weight. Root length and root fresh weight was highly positively correlated.

Table 2. Surveillance of heterosis in 271 F1 (CWS11xCWS7) hybrids and 70 F1 (CWS7 x CWS11Y)

Trait	Best parent heterosis		Mid parent heterosis		Maternal parent heterosis (CWS11Y)		Maternal parent heterosis (CWS7)	
	Range (%)	No. of hybrids	Range (%)	No. of hybrids	Range (%)	No. of hybrids	Range (%)	No. of hybrids
PH	20.91-137.07	97	21.08-45.89	6	20.905-137.07	96	(-26.25)-(-76.83)	86
BS	20.90-30.63	16	21.71-36.92	40	20.90-30.63	16	22.53-27.86	20
CL	20.04-71.49	107	20.52-84.06	145	20.04-71.49	107	21.73-77.21	59
INF	21.03-27.40	9	21.67-40.66	65	20.35-57.01	39	(-25.47)-(-42.68)	16
BN	37.15	1	28.89-71.85	19	29.42-30.07	25	(-22.86)-(-48.58)	48
PB	(-33.86)-(-90.56)	240	(-11.701)-(-87.39)	40	35.76	2	(-33.86)-(-90.56)	88
SB	29.73-397.30	140	20.75-594.34	180	25-1050	212	(29.73)- (278.38)	46
L/B	31.17-359.83	3	20.49-288.75	10	20.02-322.73	40	(-20.12)-(-35.51)	16
RL	20.472-48.819	4	20.163-80.25	12	25.13-128.50	43	(-22.05)-(-78.75)	64
RD	23.29-310.96	48	25.36-112.15	124	30.30- 87.20	218	23.16 - 41.6	10
RFW	20-492	30	23.75-732.50	63	23.16-1302	142	20- 492	11
DTM	20.54- 34.61	59	20.54-21.38	6	20.55- 34.62	59	(-20.68)-(-21.06)	2

*Desired heterosis: >Atleast 20% in the ideotype direction over the better parent/midparent/maternal parent

Table 3. Correlations of the total alkaloids and six major withanolides content in the hybrids of *Withania somnifera*

	RF	PH	DTM	BS	CL	INF	BN	PB	SB	RL	RD	SECR	RFW
RF	1												
PH	0.42***	1											
DTM	0.03	–0.08	1										
BS	0.08	0.23	–0.14#	1									
CL	–0.09	0.15#	–0.07	0.49***	1								
INF	0.05	0.36***	–0.32***	0.48***	0.46***	1							
BN	0.24	0.48***	0.03	0.03	0.11	0.18*	1						
PB	0.2	0.43***	–0.04	–0.15#	–0.19*	0.05	0.53***	1					
SB	0.35***	0.58***	–0.14#	0.25***	0.33***	0.26***	0.15#	0.01	1				
RL	0.22	0.54***	0.02	0.06	0.09	0.11	0.30#***	0.31***	0.36***	1			
RD	0.22	0.36***	-0.04	0.17#	0.08	0.20*	0.06	0.16*	0.37***	0.23**	1		
SECR	0.15	0.19	0.04	0.00	-0.08	0.06	0.11	0.12#	0.31***	0.2**	0.14#	1	
RFW	0.2	0.61***	-0.04	0.12	0.07	0.15#	0.13	0.42***	0.42***	0.5***	0.34***	0.12#	1

Significance codes= at P= 0 '***' P=0.001 '**' P= 0.01 '*' P=0.05 '#'

This was also reported earlier [21] while studying root morphometric trait in a natural population of *Withania somnifera*. Plant height was used as primary indicators of introgressed donor alleles. Heterosis on quantitative traits of all the F_1 hybrids was calculated individually as per Barth *et al*. [19]. Hybrid performance over the its mid-parent value (MP) or better-parent value (BP) or maternal parent at least above 20% threshold was considered as desired heterosis in consideration of direction of selection. Heterosis has been observed for earliness, short height, good root quality, berry size along with calyx inflation, leaf length /breadth ratio, root length and content of Withaferrin A, Withanolide A, 12-deoxywithastramonolide,Withanoside V, Withanoside IV, Withanone, total leaf alkaloids, and total root alkaloids in various recombination's. The unique gene present in either parent might have complemented to answer the presence of heterotic Withanolide QTLs in *Withania somnifera* hybrids in the desirable combinations for Withania genetic improvement programme.

Some 40 major bioactive alkaloids and sterol lactones based phyto-chemicals such as sitoindosides (saponins), diversely functionalized withanolides, withanamides and glycowithanolides have been isolated from leaf, root and fruits [22]. The current study have been conducted in reference to six standards that is withaferrin A, withanolide A, withanoside IV, withanoside V, withanone, 12 deoxy-withastramonolide which are present in significant amount in root and leaf of the Withania plant. Leaf and root extract of both parents and selected hybrids was subjected to LCMS to confirm the known reference as well as to have an indication

Table 4. Correlations of the total alkaloids and six major withanolides content in the hybrids of *Withania somnifera*

	TALK	WFA	WSIV	12DWS	WA	WN	WSV
TALK	1.00						
WFA	–0.12	1.00					
WSIV	–0.08	0.07	1.00				
12DWS	–0.09	–0.03	0.23**	1.00			
WA	–0.13	–0.06	0.07	0.49***	1.00		
WN	–0.08	0.01	0.41***	0.09	0.45***	1.00	
WSV	–0.11	0.04	0.48***	0.46***	0.29***	0.2**	1.00

Significance codes= at P= 0 '***' P=0.001 '**' P= 0.01 '*' P=0.05 '.'

from mass spectra of other chief constituent metabolites in the parent and hybrid methanolic extracts. A library of metabolite present in *Withania* from the published literature was prepared and verified using pubchem database. The study is still limited by the need of pure compound to be used as standards else then the six committed in this study. One hundred and twenty one field grown hybrids with good agronomic performance at maturity (eight month from days to sowing) was subjected to reverse phase HPLC. Correlation between total alkaloids, four withanolides and two withanosides and their component were documented. Total alkaloid content showed negative correlations with majority of withanolides and withanosides studied indicating they might be present in fractions other than chroroform. A high positive correlation was observed between Withanoside IV and Withanoside V, 12-deoxy withastramonolide with Withanolide A and Withanoside V. Withanolide was found significantly correlated with withanone and withanoside V (Table 4).

RNA-seq analysis

RNA-seq can show the repertoire of expressed sequences found in a particular tissue at a specific time point, even rare transcripts, due to the great depth of sequencing to characterize genes, reveal information on novel transcripts, and look at gene expression, single-nucleotide polymorphisms (SNPs), alternative splicing and structural variation [10]. Single Nucleotide Polymorphisms (SNPs) which are responsible for the functional polymorphism are required to assist in identifying biosynthetic pathway genes that underlie genetic and metabolic variation. RNA-Seq of *Withania somnifera* using Illumina sequencing with de novo assembly which will provide both differential gene expression, alleles specific expression and genetic polymorphism (single nucleotide polymorphisms and microsatellites). Total RNA was isolated from flash frozen leaf tissues of CWS11 and CWS7 to yield mRNA from which directional Nextflex libraries were prepared for Illumina PE Hiseq2000 sequencing. CWS 11Y and CWS7 resulted in 46257608 and 45888610 reads

Table 5. Annotations of Unigenes derived from denovo assembly which contribute to secondary metabolic pathways in *Withania somifera*

Biosynthetic Pathway	CWS11Y	CWS7
Caffeine metabolism [PATH:ath00232]	2	3
Indole alkaloid biosynthesis [PATH:ath00901]	2	2
Phenylpropanoid biosynthesis [PATH:ath00940]	52	56
Flavonoid biosynthesis [PATH:ath00941]	21	13
Anthocyanin biosynthesis [PATH:ath00942]	1	
Flavone and flavonol biosynthesis [PATH:ath00944]	1	6
Stilbenoid, diarylheptanoid and gingerol biosynthesis [PATH:ath00945]	17	18
Isoquinoline alkaloid biosynthesis [PATH:ath00950]	10	12
Tropane, piperidine and pyridine alkaloid biosynthesis [PATH:ath00960]	12	14
Glucosinolate biosynthesis [PATH:ath00966]	7	6
Glycan biosynthesis and metabolism	105	148
Cutin, suberine and wax biosynthesis [PATH:ath00073]	4	4
Steroid biosynthesis [PATH:ath00100]	25	32
Ubiquinone and other terpenoid-quinone biosynthesis [PATH:ath00130]	31	31
Polyketide sugar unit biosynthesis	1	2
Terpenoid backbone biosynthesis [PATH:ath00900]	55	49
Monoterpenoid biosynthesis	3	4
Limonene and pinene degradation [PATH:ath00903]	15	16
Diterpenoid biosynthesis [PATH:ath00904]	7	6
Brassinosteroid biosynthesis [PATH:ath00905]	3	6
Carotenoid biosynthesis [PATH:ath00906]	30	25
Zeatin biosynthesis [PATH:ath00908]	13	20
Sesquiterpenoid and triterpenoid biosynthesis [PATH:ath00909]	9	7
Nicotinate and nicotinamide metabolism [PATH:ath00760]	30	17

respectively. A total of 374053 transcripts were generated comprising 102718 unigenes from the combined assembly which were subjected to annotation and SSR and SNP discovery. An earlier report transcriptome sequencing using Roche 454 platform have also yielded a similar unigenes 89, 548 from the leaf tissue [23]. An insight into the secondary metabolites biosynthetic pathway reported previously [23] and the present study (Table 5) shows all the pathways were included and the N50 of both the studies can shed more light for a better understanding. In this study N50 value of the pseudo reference assembly 2097 was obtained from the pooling of the parental reads of the individual parent CWS11Y (2011) and CWS7 (2028). SSR mining resulted in 1890 uniques SSR in recipient CWS 11Y and 2905 in the donor CWS7 and of which 1897 SSR were found common to both of them. SNPs discovered in the recipient and donor were 97951 and 104287 respectively of which 15874 was common to both parents (Table 6). This resource needs validation and further assigning of functional polymorphism. Differential gene expression analysis in the recipient parent CWS11Y of total 162322 unigenes generated and 67622 annotated; 29269 were involved in biosynthetic pathways including primary and secondary metabolic pathways and 7747 was unique present only in the recipient. Donor CWS7 on sequencing got 147660 unigenes generated of which only 62867 could be annotated. Of these 22880 unigenes were involved in primary and secondary metabolic pathways and 7296 were present exclusively in the donor. There were 3202 genes which were p significant, 1686 upregulated and 1586 down regulated. There were 65 genes which were Q significant such as pseudo-uridine synthases, tropinone reductase, sterol glucosyl transferase and stress related protein1,WRKY-a,UDP-glucosyl transferases, superoxide dismutase, sucrose synthase and lipoxygenases.Using an RNA-seq approach, the expression of 101245 unigenes was quantified in parents .The expression levels of 30% of transcripts differed between parents, a majority of which had more than 1.5 fold-changes.

We conclude heterosis is not a consequence of high level of additive or non-additive expression, but related to transcriptional variation between parents. The lack of correlation between better parent heterosis levels for different traits suggests differential transcriptional diversity at specific set of genes for different traits.RNA seq will help to decipher the withanolide biosynthesis pathway and SNP responsible for functional polymorphism in pathway genes. Understanding the genetic basis of accumulation of withanolides in the leaves, roots and berries and mapping of the quantitative trait loci (QTLs) through transcriptome based markers will provide the basis for devising the withanolides targeted breeding strategies and for improving their content through marker-assisted selection. Marker-aided selection for pathway will accelerate the *Withania* improvement breeding programme for broadening the genetic base of *Withania* cultivars and its stabilization

Table 6. Feature of SSR and SNP mined in the denovo assembly of *Withania somnifera*

Feature	CWS7L	CWS11L
Total number of sequences examined:	62867	67622
Total size of examined sequences (bp):	85803326	90568275
Total number of identified SSRs:	12516	15014
Number of SSR containing sequences:	10745	12685
Number of sequences containing more than 1 SSR:	1495	1940
Number of SSRs present in complex/compound formation:	750	921
Number of SSRs with 1 unit:	7321	9233
Number of SSRs with 2 units:	2373	2531
Number of SSRs with 3 units:	1984	2196
Number of SSRs with 4 units:	74	102
Number of SSRs with 5 units:	14	31
Number of SSRs with 6 units:	0	0
Unique SSRs	1810	2905
Common SSRs	1897	1897
Total Homozygous SNPs	97951	104287
Total Heterozygous SNPs	1475	1542
Common Homozygous SNPs	15874	15874
Common Heterozygous SNPs	229	229

against abiotic and biotic stresses leading to improved withanolides yield which will consequently have a direct impact on fetching high revenue for *Withania somnifera* growing farmer community

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