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Genetic analysis of *Ximenia americana* based on the cross-species DNA-DNA hybridization technique

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Abstract. *Ekandjo A, Naomand E, Kahaka G. 2018. Genetic analysis of* Ximenia americana *based on DNA-DNA based on the cross-species hybridization technique. Cell Biol Dev 2: 1-7.* Genetic analysis of species without the availability of complete genome arrays can be studied using the cross-species hybridization technique. The preliminary microarray studies have created DNA-DNA hybridization of *X. americana* to the GeneChip of *Arabidopsis thaliana* (ATH1). Based on the perfect-match (PM) probe signal, the probe pairs of *A. thaliana* that hybridized with the genomic DNA of *X. americana* were selected and analyzed using a cel file parser script. This selection was to generate new high-density probe mask files. The *X. americana* DNA-DNA hybridization data were effectively represented by this file. In addition, this study carried out gene ontology analysis of hybridization data of *X. americana/A. thaliana*. This gene ontology analysis showed that genes for abiotic stress response were over-expressed in respective comparison to model species of *A. thaliana* under natural conditions. This analysis was confirmed independently with PCR amplification of the orthologous genes using genomic DNA of *X. americana*, such as AT4G15910.1, SAD2, HXK1, ACC, and ERF/AP2. Due to the lack of genomic sequence data in X. americana, the primers for genomic amplification were designed using a genomic sequence of *A. thaliana*. The primers were designed to produce a genomic PCR product of 100 bp. Each selected gene was successfully amplified. Therefore, it gave evidence of homology within primer binding sites. However, the genomic amplification of these crucial abiotic factors in *X. americana* confirms the response type, which supports the adaptation of *X. americana* under natural conditions with stress associated (with heat and drought stress). Although transcript levels of these unique abiotic response factors could not be observed in absolute or relative terms, the study showed the inherent existence of such genes at the genomic level of *X. americana*. Further studies are needed to confirm that some modification of the factors of these abiotic responses or other abiotic responses within the genome of *X. americana* gives the key basis for its adaptation to the comparatively dry and hot climatic conditions.

Keywords: ATH1, cross-species hybridization, gene ontology, abiotic response factors, microarray

INTRODUCTION

Ximenia is also found in other parts of the world, such as South America, although it is primarily indigenous to Africa. *Ximenia* includes in the family of Olacaceae and is widely spread in Africa. *X. americana* and *X. caffra* are the most common species. These two species are distributed in the northern part of Namibia. In this study, we focused on *X. americana*. The *X. americana* has rough dark-grey bark and grows up to 6 meters tall. It thorny branches and has hairless green leaves. Regardless of climate change, *X. americana* bears flowers and fruits annually (Orwa et al. 2009). *X. americana* is a drought-resistant plant where it mostly grows at low altitudes in a wide range of environments, such as savannahs, dry woodlands, dry forests, and along with coastal areas or on river banks (Orwa et al. 2009)

Ximenia americana is of economic importance because of its wood yield. Its fruit is often used for jam and jellies, and its kernel contains a high quantity of oil. *X. americana* fruits are also used for medicinal purposes, treating skin problems, sexually transmitted diseases, headaches, relieving cough, and healing wounds as a topical ointment. X. americana for medicinal purposes due to its free radical scavenging and antioxidant activity (Maikai et al. 2009).

This study analyzed and compared X. americana and Arabidopsis thaliana DNA-DNA hybridization data using gene ontology and independently confirmed ontology results with PCR amplification of orthologous genes within a given biological process. This study requires designing primers in *A.thaliana* background and examining the presence of genomic fragments in *X. americana* genomic DNA.

MATERIALS AND METHODS

Sample collection

The samples (*X. americana* leaves) were collected from Waterberg, Otjiwarongo, in the Otjozondjupa region. The sample collection was conducted during the early summer (September) when the average daytime temperature reached 30˚C, and no rainfall was recorded during that month. The leaves samples were kept on ice from the field to the laboratory. The samples were subsequently stored in the freezer at -20 ˚C until the analysis time.

DNA extraction

DNA was extracted from the leaves of *X. americana* using a DNeasy Plant mini kit (Qiagen). First, 20 mg of *X.*

americana leaves was ground buffer using a mortar and pestle in 800 µL API to get a homogeneous solution. Next, the buffer mixture of *X. americana* - was transferred to two 1.5 mL microcentrifuge tubes (400 µL). Then, 4 µL RNase was added to each tube. The mixture of the samples was incubated at 65˚C for 10 minutes. During the incubation, it was mixed 3 times by inverting the tubes. First, a 130 µL was added to the lysate and then mixed and incubated for 5 minutes on ice. The lysate was centrifuged at 14,000 rpm for 5 minutes. Then the lysate was transferred to a QIAshredder Mini spin column (lilac), put in the collection tube of 2 mL, and centrifuged at 14,000 rpm for 2 minutes. The flow-through fraction from the previous step was moved into a new tube without disturbing the cell-debris pellet. The flow-through volume was measured, and sodium acetate (3M, pH5.2) with 0.1 volume was added. A t room temperature, a 0.7 volume of 50% isopropanol was added to the mixture. This mixture (650µL), including precipitate, was moved to the DNeasy Mini spin column within a 2 mL collection tube, and then it was centrifuged at 13,400 rpm for 1 minute. For the remaining sample, the previous step was repeated. The DNeasy Mini spin column was put into a new 2 mL collection tube, and Buffer AW of 500 µL was added, and it was centrifuged at 13,400 rpm for 1 minute. The flow-through was discarded and centrifuged for 2 minutes at 14,000 rpm. Next, the DNeasy Mini spin column was moved to a 1.5 mL microcentrifuge tube. As much as 50 µL Buffer AE (warmed at 65˚C for 2 minutes) was added directly onto the DNeasy membrane. It was incubated for 5 minutes at room temperature and then centrifuged at 14,000 rpm for 1 minute to elute. The extracted DNA was stored at -20 ˚C.

Quantification of DNA

The quantity of DNA of *X. americana* leave samples (1.5 µL) was analyzed using an ND-NanoDrop 2000c spectrophotometer. The results of the quantification were recorded and tabulated.

Hybridization

DNA-DNA hybridization was proceeded by a commercial service of The Nottingham NASC arrays in the United Kingdom. NASC arrays processed about 50 µL DNA samples. The pre-defined experimental conditions were needed before the DNA was sent to The Nottingham NASC arrays. First, the DNA was labeled with the Invitrogen BIoPrime kit. Then, about 100 ng DNA was dissolved in a microcentrifuge tube with 20 µL of the dilute buffer, and 20 µL of 2.5X random primers solution were added. Denaturation was done by heating in a boiling water bath for 5 minutes; then, it was cooled immediately on ice. Five µL of 10X dNTP mixture and distilled water to a volume of 49 µL were added to the solution on ice. The solution was mixed quickly, and 1 µL of Klenow fragment was added. The solution was mixed softly but thoroughly and centrifuged for 30 seconds. After that, the solution was incubated for 60 minutes at 37˚C, and 5 µL of a stop buffer was added. When incubating, the array was loaded with 200 µL 1X hybe buffer, and later prehybe the array in the oven at 45˚C, at 60,000 rpm for 10 minutes. Next, the probe was incubated at 45˚C for 5 minutes. The prehybe buffer was discarded from the array, 200 µL probes were added, and the debris at the bottom of the tube was eluded. Next, to prevent leakage, the septa were covered with tight spots, and then the arrays were put in the oven for 20 hours at 45˚C, 60,000 rpm. Finally, using the GeneChip operating software, the arrays were washed. After washing, CEL files data were transferred and scanned.

The homologous A. thaliana GeneChip array PM probes were selected by selecting the probe pairs from each probe-set on the *A. thaliana* GeneChip array derived from the hybridization efficiency of genomic DNA from *X. americana* (Hammond et al. 2005). First, biotin-labeled was applied to genomic DNA from *X. americana*, and it was hybridized to the *A. thaliana* ATH1 GeneChip array. Then, selecting a probe-set using Perfect Match probes with the intensities of gDNA hybridization above a set threshold was done to analyze if the probe-set was represented. The selection was carried out using a .cel file parser script written in the programming language of Perl (Xspecies Version 1.1, http: //affymetrix.*Arabidopsis*.info/xspecies/).

The overlapped regions (defined by probe sets) during cross-hybridization were chosen. Then, the primers were designed from those regions' probe sets (Hammond 2005).

Primer design

Using the ProbeFinder Assay Design software, designing primers were performed (www.lifescience.roche.com). Sequences of genomic DNA of *A. thaliana* genes (putative *X. americana* orthologous) as templates for primer design were retrieved from a database of Gene Bank (from The *Arabidopsis* Information Resources-TAIR website). The designed primers were 15-30 bases in length, and the region of interest was designed without any other nonspecific binding possibility. When primers were designed, the Promega kit was followed as per manufacturer instruction to contain 40-60% (G+C). To avoid the production of primer-dimers, the 3'-ends of the primers were not complimentary.

Furthermore, to avoid nonspecific primer annealing, none of the primers contained three G or C nucleotides in a row near the 3'-end. Primers were designed to produce similar melting temperatures and a PCR product of between 70 and 100 bp. The advantage of the fragments of this size is that they are suitable for analysis using multiple platforms such as standard PCR or Real-Time PCR.

Gene ontology classification

Analysis of gene ontology was done to evaluate if particular biological functions were over or under-represented during cross-hybridization. The Gene Ontology terms (Gene Ontology Consortium 2001) were used to perform a classification analysis and to classify the genes based on their putative function. The GO analysis was performed using the TAIR Web site (www.arabidposis.org).

Gene ontology analysis of A. thaliana *genome*

Using the bulk GO annotation, genomic annotations of *A. thaliana* were sub-classified. The retrieval tool is available on the TAIR website. Using the same bulk GO annotation, uploading Data Locus identifiers of more than 11,000 probes (between *A. thaliana* and *X. americana*, the homology was positive) were made. The lists of all GO annotations for a given set of genes using the locus identifiers were obtained. The number of genes per category was noted and tabulated.

Gene ontology analysis of X. americana *PCR confirmed loci*

Uploading locus identifiers of the 5 PCR positive *X. americana* loci (putative orthologous) were performed using the same bulk GO annotation. This analysis retrieved a list of all GO annotations and the number of genes per category.

Independent confirmation using standard PCR

Standard PCR was used to obtain genes and, during cross-hybridization, were analyzed to confirm their presence in *X. americana* (Table 1).

Standard PCR amplification reactions

The isolated DNA of *X. americana* was performed to amplify the targeted amplicon using standard PCR. The reagents (Table 1) were added to the PCR tubes placed on ice according to Fermentas life science protocol, kit no: #K0171. For each tube, there was a 25 µL reagent. For PCR amplification, 10 primers were used (Table 2).

PCR reaction mixtures were thoroughly mixed, and PCR was done using thermal cycling conditions (Table 3). The PCR was running overnight. The PCR conditions were set according to Fermentas life science protocol, kit no: #K0171 (Table 3).

PCR products gel electrophoresis

Approximately 2,200 lg of agarose powder was placed in a 500 mL screw cap reagent bottle. After that, 200 mL of 1×TBE buffer was added. The mixture was dissolved in the microwave until no bubbles appeared in the solution. Under running tap water, the solution was cooled until the bottle could be held for at least 6 seconds. Under the fume hood, 5 µL of ethidium bromide was added to the solution and poured into the gel tray. For 30 minutes, the 1% agarose gel was allowed to stand. Then, the comb was gently removed from the gel. The gel was moved into the electrophoretic tank filled with 1×TBE buffer until the gel was covered with buffer. The mix of PCR products (10 µL) and loading dye (3 µL) was loaded on the gel. In the first well, DNA lader100 bp (8µL) was loaded. At 120V, the gel was run for 60 minutes. On the UV transilluminator, the DNA bands were visualized.

RESULTS AND DISCUSSION

DNA quantification and quality analysis

Finally, to measure the concentration and quality of DNA samples, a NanoDrop 2000c was used at the ratio of 260/280 nm to assess the purity of DNA. The DNA is accepted as "pure" if the ratio is ~1.8-2.2. This ratio is a secondary measure of nucleic acid purity. If the ratio is considerably lower, it may indicate that protein, phenol, or other contaminants are present that strongly absorb at or near 280 nm (www.info@nanodrop.com). For example, the ratio derived from the graph of DNA samples was 2.05, indicating that the DNA was pure with a concentration of 139 ng/µL at 260/280 (Table 4; Figure 1).

Table 1. Reaction master mix for 25 µL (Fermentas life science protocol, kit no: #K0171)

Reagents/components	Final concentration	1× reaction
PCR Master Mix 2×	1×	12.5 µL
Primer forward	0.3µM	1.2 µL
Primer Reverse	0.3µM	1.2 µL
Template DNA	500ng	2.5 µL
Nuclease-free water	Makeup 25 µL	7.6 µL
Total	25 µL	25 µL

Table 3. Standard PCR reaction parameters

Segment	Number of cycles	Target temperature	Hold time
Pre-denaturation	1	94 °C	2 minutes
Denaturation		94 °C	1 minute
Annealing	45	60 °C	1 minute
Extension		72 °C	2 minutes
Final extension	1	72 °C	5 minutes
Hold	0	4 °C	0 minutes

Table 2. The primers IDs and the gene IDs for each primer

Primers ID	Forward primer	Reverse primer	Gene ID	Expected size (bp)
252921	CTTCCATGGGAACTCACGTT	CCCCATACATTGCACATCCT	AT4G39030.1	100
256075	CAAATTCAGAGCGTGAACCA	TTTCTCTCAAAGTCAAATTCAAGC	AT1G18150.2	100
260133	AACCGCTCTTGTCACCAAGT	CCAGTTGGCACGACAAAAA	AT1G66340.1	100
245523	AGGTTTAGATCAAGAGGCAGAGTC	GCACGATTGGAAGGTCTGTAG	AT4G15910.1	100
263448	AACTGCAGAAGTTAGCTGCACA	TCAGAATCGTCGTCATCATCA	AT2G31660.1	100
253705	GGTATTCTGAAGAAACTGGGAAGA	CCATGGCTATAACCGATTTCTG	AT4G29130.1	100
259439	TTCAAGCTCCGACAATTTCA	TGATGGGTTGGTCAAAATCA	AT1G01480.1	100
264415	ACATGGACGATGGGTCATAAG	GCTGAACCGGTAATCAATGG	AT1G43160.1	100
249036	AGCTGCGCCAACGAATTAT	CGATTTTGTCCTCCTTGACC	AT5G44200.1	100
255220	GAAGTTCAATGTTTCGTTTCATGT	GGATTATACAAGGCCCCAAAA	AT4G05320.2	100

A. thaliana/X. americana DNA-DNA hybridization data

The putative orthologous genes of *X. americana* were studied using *A. thaliana* ATH1 GeneChip arrays. A similar technique was applied to investigate ovine and *Brassica oleracea* (Graham et al., 2011; Hammond et al., 2005, 2006). The gDNA of *X. americana* was labeled and then hybridized to the ATH1 GeneChip arrays. The hybridization signals were detected for all probes on the GeneChip for *X. americana* gDNA samples.

In this study for *X. americana*, the average hybridization signal intensities of gDNA for 10 selected putative orthologous genes of the ATH1 GeneChip array was 6.6589 (Table 5). Since gDNA from *X. americana* produced hybridization signals on the ATH1 GeneChip array, this information can be applied in genetic analysis to identify probes and probe sets with good homology between *A. thaliana* and *X. americana*. Only one replication was done in this study since fewer replications are required for experiments incorporating confirmatory testing such as PCR.

Gene ontology analysis of *A. thaliana* genome annotations

The number of genes associated with a particular GO biological process in the genome of *A. thaliana* is shown in Table 6. There were 47 and 46 genes that carried annotations relating to response to abiotic or biotic stimuli and response to stress, respectively. Furthermore, about 66 genes in the *A. thaliana* genome were not ascribed to a biological function. About 5% of genes were assigned to biological function genes that control protein metabolism and transport in the *A. thaliana* genome. Moreover, the least number of genes (3%) was assigned to signal transduction and transcription.

Gene ontology analysis of *A. thaliana/X. americana* hybridization data

Table 7 shows the number of genes related to biological processes that were retrieved from the locus identifiers of the set of the hybridized probe (putative orthologous).

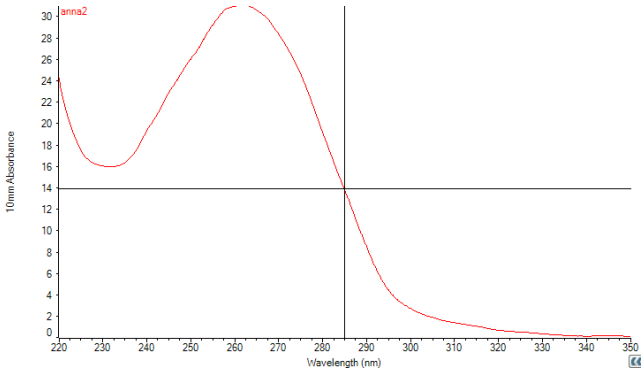

Figure 1. The graph of *X. americana* DNA concentration (139ng/µL) was measured using nanodrop.

Table 6 shows that the number of genes related to response to abiotic or biotic stimuli and stress response were 1,333 and 1,036, respectively. Further, the data indicate that 10,226 probe sets that represented putative orthologous genes of *X. americana* were not assigned to a biological function. The number of this probe set was higher than the A. thaliana because the genetic makeup of X. americana is not yet known. The putative orthologous genes associated with signal transduction had a least 906 probe set.

Table 4. The concentration of *X. americana* DNA samples

Sample ID	Nucleic acid [ng/µL]	A_{260}	A_{280}	$A_{260/280}$	$A_{260/230}$	Sample type
XimeniaDNA	139	2.779	1.356	2.05	1.75	DNA
XimeniaDNA	62.5	1.251	0.611	2.05	1.44	DNA
XimeniaDNA	703	14.061	8.718	1.61	1.87	DNA

Table 5. The genomic DNA hybridization intensity values

Probe-set ID	Corresponding *A. thaliana*	*X. americana* DNA Leaves intensities
252921_at	AT4G39030	8.72618
256075_at	AT1G18150	7.74376
260133_at	AT1G66340	5.6179
245523_at	AT4G15910	6.54669
263448_at	AT2G31660	9.27173
253705_at	AT4G29130	5.6248
259439_at	AT1G01480	5.19156
264415_at	AT1G43160	7.21048
249036_at	AT5G44200	5.54982
255220_at	AT4G05320	5.10657

Table 6. GO biological process in *A. thaliana* genome

GO biological process	Gene count	%
Response to abiotic or biotic stimulus	47	6
Response to stress	46	6
Unknown biological processes	66	6
Protein metabolism	50	5
Transport	34	5
Signal transduction	11	3
Developmental processes	13	6
Transcription	9	3

Table 7. DNA-DNA hybridization GO annotations

GO biological process	Gene count	%
Response to abiotic or biotic stimulus	1,333	9
Response to stress	1,036	12
Unknown biological processes	10,226	1
Protein metabolism	3,961	2
Transport	1,699	3
Signal transduction	906	6
Developmental processes	1,488	2
Transcription	1,701	3

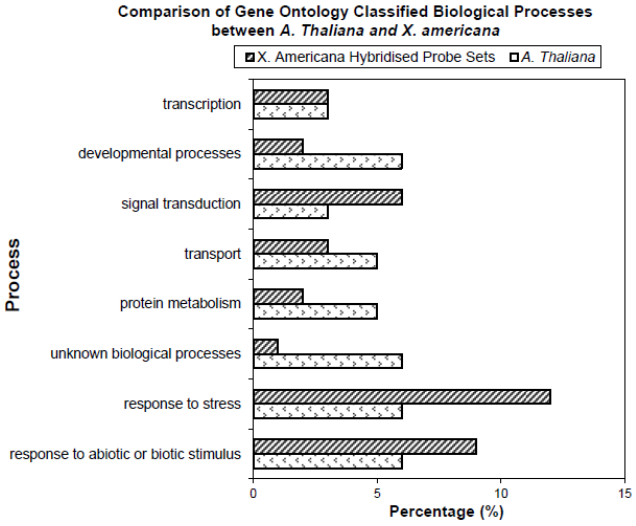

Figure 5. Comparison of gene ontology classified biological processes between *A. thaliana* and *X. americana*

Comparative gene ontology analyses

Between *A. thaliana* and *X. americana*, the gene ontology classifications of biological processes were compared. Figure 5 compares gene-ontology of the biological process presented in *Arabidopsis thaliana* with biological processes represented by hybridized regions (probe set) between *A. thaliana* and *X. americana* (http: //www.geneontology.org/; http: //www.arabidopsis.org).

The results illustrated that only 6% of biological processes in *A. thaliana are* associated with the stress response. The further GO analysis revealed that 6% of the *A. thaliana* genome was not assigned to any specific biological process. The data suggest that 12% of the biological processes were assigned to stress response in the cross-hybridized region. The representation of stress responses increased by 6% in *X. americana*. The over-representation of stress response was shown in *X. americana* under natural conditions. This observation was confirmed by an increased response to abiotic or biotic stimuli from 6% to 9%. Given the significance of the representation, an independent confirmation was required.

Independent confirmation

The DNA-DNA hybridization identified more than 20,000 probes (genes) in this study. In the study of *B. oleracea* with the same techniques, 40,099 genes were identified (Hammond et al. 2005). In this study, putative orthologous genes of X. americana demonstrating abiotic and stress responses were selected for independent confirmation.

DNA quality analysis and quantification

For PCR amplification, the quality of DNA extracted was at a reasonable standard, although it was not pure (ratio of 260/280 was 1.61). On the two extracts, the concentration of DNA samples of *X. americana* were 1546.2 ng/µL and 700 ng/µL, respectively (Table 8).

After the DNA of *X. americana* was quantified using ND-NanoDrop 2000c (Table 4), DNA amplification was performed using primers designed to independently confirm if the ten genes were found on the microarray results presented in *X. americana*. Figure 6 demonstrated that five out of ten genes (AT4G15910.1; AT2G31660.1; AT4G29130.1; AT1G01480.1; AT1G43160.1) of targeted orthologous genes were positive compared to a housekeeping gene (AT4G05320.2) as the positive control. Furthermore, these five genes from the targeted ten genes that are present in *X. americana* were confirmed from PCR results. These genes had sizes around 100 bp.

Independent confirmation of X. americana *biological process*

Gene ontology analysis of *X. americana* GeneChip showed that abiotic stress response genes were over-represented (12%) than *A. thaliana* (6%). The observation was independently confirmed with standard PCR amplification on the orthologous genes of AT4G15910.1; SAD2; HXK1; ACC, and ERF/AP2 (Figure 7). Furthermore, it was confirmed that biological processes responding to abiotic and biotic stimuli, other metabolic processes, other cellular processes, and transport were over-represented. The other biological processes that presented and were active were signal transduction, protein metabolism, transcription, and developmental processes.

The data may reflect the environmental interaction of *X. americana*. All five genes confirmed independently by PCR and GO found in *X. americana* were associated with environmental changes. Moreover, these genes were over-represented in *X. americana* than in *A. thaliana.* It is because *X. americana* grows in dry, rocky places and harsh conditions, while *A. thaliana* grows in a sandy and cold environment (Orwa et al. 2009). For instance, a gene whose transcript level in roots and leaves increases progressive drought stress is encoded by AT4G15910.1. This particular transcript level is also shown to be affected by the changes in three levels of endogenous or exogenous abscisic acid (Kawaguchi et al. 2004).

An important beta-domain family protein is encoded by SAD2 (super sensitive to ABA and drought 2); it is likely involved in the nuclear transport of ABA signaling. The mutation in SAD2 shows higher tolerance for UV stress, higher production of UV protective secondary metabolites, and reduced nuclear localization of MYB4 (a repressor of UV stress response genes) (Verslues et al. 2006; Zhao et al. 2007). ABA is an important mediator in activating the plant's responses to adverse environmental stimuli. Water stress condition during somatic growth increases ABA levels, which can be limited by reducing stomata opening when ABA levels increase (Leung and Giraudat 1998). In other aspects of stress adaptation, ABA is also involved. For example, in cold acclimation, the ABA-deficient mutant in *A. thaliana* is impaired (Mantyla et al. 1995) compared to *X. americana* which grows in dry and hot environments and has a root morphogenetic response to drought conditions. In signaling stress conditions, the role of ABA has also been considerably reported by molecular studies showing that ABA-deficient mutants are influenced in the regulation of several genes by drought, salt, or cold (Leung and Giraudat 1998).

Table 8. The DNA concentration using ND- NanoDrop 2000c

Sample ID	Nucleic acid [ng/µL]	A260	A280	A260/280	A260/230	Sample type
XimeniaDNA	1546.2	30.925	19.226	1.61	1.97	DNA
XimeniaDNA	700	14.061	8.718	1.61	1.87	DNA

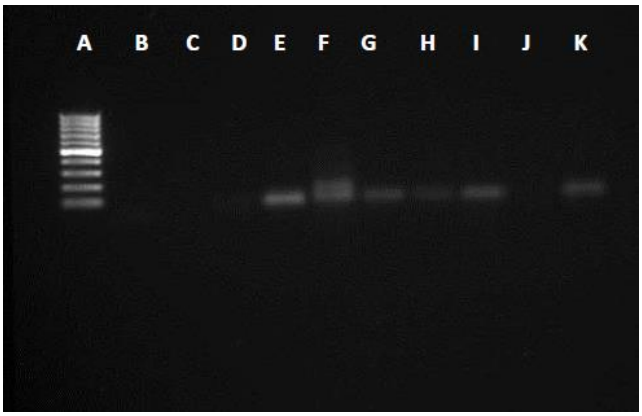

Figure 6. DNA amplifications of *X. americana* with different primers. A) DNA ladder (100bp); B) AT4G39031.1 (not present); C) AT1G18150.2 (not present); D) AT1G66340.1 (not present); E) AT4G15910.1 (present); F) AT2G31660.1 (present); G) AT4G29130.1 (present); H) AT1G01480.1 (present); I) AT1G43160.1 (present); J) AT5G44200.1 (not present) and K) AT4G05320.2 (present, positive control)

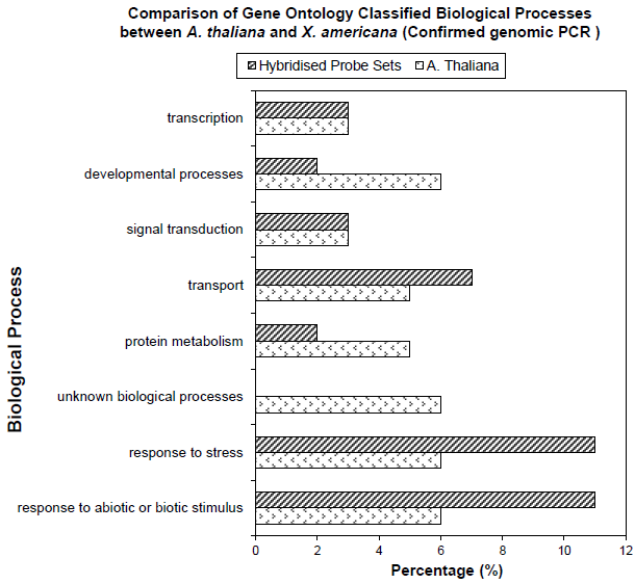

Figure 7. Genomic PCR-based independent confirmation of *X. americana* biological process.

The data of ontology results (Figure 5) suggests that stress response representation in *X. americana* increased by 6%, and over-representation of stress response occurred in *X. americana* under natural conditions, drought, and heat dependently examined using PCR (Figure 6). An increase in response to abiotic or biotic stimuli from 6% to 9% confirmed the observation. According to Jiang and Zhang (2004), the phytohormone abscisic acid (ABA) is one of the most important regulators of plant responses to abiotic stresses. Plants store higher amounts of ABA under drought, cold, or salt stress conditions, where drought stress has the most prominent effect. It elucidates the more abiotic stress genes in *X. americana* compared to *A. thaliana* due to *X. americana* growing in a dry environment.

The gene of AT4G29130.1 encoding a hexokinase (HXK1) in the plant glucose-signaling network roles as a glucose sensor for signaling networks of nutrients, light, and hormones in controlling growth and development responding to the changing environment (Moore et al. 2003; Cho et al. 2006; Karve et al. 2012). Therefore, all metabolism, growth, development, and abiotic and biotic stress responses are controlled, at least partially, by sugars (Harrington and Bush 2003). The changes in hexokinase levels by HXK1/ HXK2 affect plant tolerance to methyl viologen (Sarowar et al. 2008). Methyl viologen is a compound that damages the tissues of green plants by disturbing their photosynthesis. Hence, methyl viologen can be served as an herbicide. The alteration of bipyridyl residues to mono-cation radicals affects its herbicidal property. The radical reacts with oxygen, lead the formation of a superoxide anion, which can initiate the formation of hydroxyl and H_2O_2 radicals. These products cause oxidative destruction to cellular components, including DNA damage, lipid peroxidation, and protein inactivation (Timbrell 2000).

AT1G01480.1 is one of the 1-aminocyclopropane-1-carboxylate (ACC) synthases. It catalyzes the conversion of 5-adenosylmethionine (SAM) to ACC. The oxidation of ACC to ethylene is catalyzed by ACC oxidase. This plant hormone ethylene is produced to respond to various environmental stress types, such as low temperature and chemicals and water stress, wounding, physical load, and disease (Karve et al. 2012; Kato et al. 2000; Li et al. 2012; Liang et al. 1992).

A member of the ERF (ethylene response factor)/AP2 (The APETALA2) is encoded by AT1G43160.1. The essential roles of these AP2/ERF proteins are presented in the transcriptional regulation of a kind of biological processes associated with growth and development and various environmental stimuli responses (Jung et al. 2010; Licausi et al. 2013; Nakano et al. 2006). Ethylene is noticed as a stress hormone because its production is induced by stress signals, such as excess chemicals and metals, mechanical wounding, drought, extreme temperatures, and pathogen infection. In higher plants, ACC is an immediate precursor of ethylene. Endogenous levels of ACC highly influence ethylene production in plants. Hence, in the early phase of plant response to stress, ACC accumulates along with a rapid burst in ethylene synthesis (Bibi et al. 2006).

In this study, transcript levels of these essential abiotic response factors could not be determined in absolute or relative terms. Nevertheless, the study showed the inherent

presence of such genes at the *X. americana* genomic level. Further studies will be needed to show that some kind of modification of the factors of these abiotic responses or other abiotic responses within the genome of *X. americana* gives the key basis for its adaptation to the comparatively dry and hot climatic conditions.

In conclusion, During DNA-DNA cross-hybridization between *X. americana* with *A. thaliana*, gene ontology analysis of perfectly matched probe-pairs showed an increase in ontologies related to the biological process and abiotic stress compared to the ontology database of *A. thaliana*. Such evidence indicates that molecular mechanisms govern its adaptation to the prevailing natural conditions. *X. americana* that grows under harsh conditions leads the evolutionary adaptation to survive under these conditions. This study investigated the presence of 10 probe pairs of more than 20,000 perfectly matched probe pairs. The selected probe pairs confirmed the hybridization data and the presence of stress that related orthologous at the genomic level between *A. thaliana* and *X. americana*. Furthermore, successful amplification of *X. americana* genomic fragments provided evidence of homology within primer binding sites and some biological processes that integrate external stress signals, such as heat stress and drought stress in *X. americana*.

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