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Detection and Adaptation in Parasitic Angiosperm Host Selection

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Abstract

Developmental transitions in some parasitic angiosperms are tied directly to host-derived chemical cues (*xenognosins*). The obligate hemi-parasite *Striga asiatica*, initiates the root apical meristem population (germination), development of the host attachment organ (the haustorium), and shoot apical meristem initiation (seed coat shedding) in response to specific xengonosins. These checkpoints synchronize spatial and temporal tissue development. We have now exploited the external control over these developmental transitions to trace functional expression in haustorial organogenesis. Genes associated with phytohormone regulation, metabolism, vascular tissue development, and reactive oxygen species (ROS) production identified in this study suggest an elaborate and global response closely tied to plant defense and redox chemistry that may also be components of a more general quorum sensing-type mechanism in plants.

Keywords

Parasitic Plants, Semagenesis, Parasitic Evolution, Xenognosis, Host Detection, Quorum Sensing

1. Introduction

While many adaptive or learned strategies have been acquired through biological evolution, nowhere are the learning processes more acute or dynamic than at mutualistic interfaces [1]. The constant and rapidly changing evolutionary tensions between intimately associated organisms may be most developmentally and metabolically dynamic in the sessile parasitic plants where chemical and tactile cues direct lifecycle phases [2]-[4]. The host-derived cues that mediate both acute and chronic developmental decision-making events are known as *xe-*

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nognosins, from the Greek words for "other" (xenos) and "knowledge" (gnosis).

Studies on *Striga asiatica*, an obligate hemi-parasitic angiosperm commonly known as witchweed, have defined the chemistry underlying host/parasite transition strategies in this noxious weed. Distinct classes of xenognosins provide the necessary and sufficient stimulus to initiate at least three separate developmental transitions, each of which is crucial to the successful development of the parasite [3]-[5] (**Figure 1**). Seeds of *S. asiatica* can remain viable under soil conditions for up to twenty years, germinating only in the presence of an SXSg (Sorghum Xenognosin for Striga germination) which includes strigolactones and/or dihydrosorgoleone derived from the roots of prospective host plants, including agriculturally important crops like maize and sorghum (**Figure 1(a)**) [6] [7]. Subsequent development of the host attachment organ, the haustorium, recommits the root apical meristem in a process known as *semagenesis* [8]-[10]. Reactive oxygen species (ROS) are actively exuded at the parasite root tip such that contact with host cell walls oxidatively liberates *p*-benzoquinone (pBQ) products. These pBQs are necessary and sufficient to initiate haustorial development; arrest of vegetative growth, induction of radial swelling of the root tip, and initiation of the ectopic root (haustorial) hairs (**Figure 1(b)**). Given the limited resources of the parasite for vegetative growth (3 - 5 d), this process, which takes about 24 h, prevents premature commitment. Following attachment, additional xenognosins appear necessary to initiate the cells of the shoot apical meristem (SAM). While specific endogenous factors that initiate this transition have yet to be defined formally, the exogenous addition of cytokinins (**Figure 1(c)**) can initiate SAM development [11]. Endogenous hormones derived by tapping the host vascular system [12] may allow the plant to complete its lifecycle, transitioning back to vegetative growth to reach seed set and, ultimately, senescence (**Figure 1(d)** & **Figure 1(e)**).

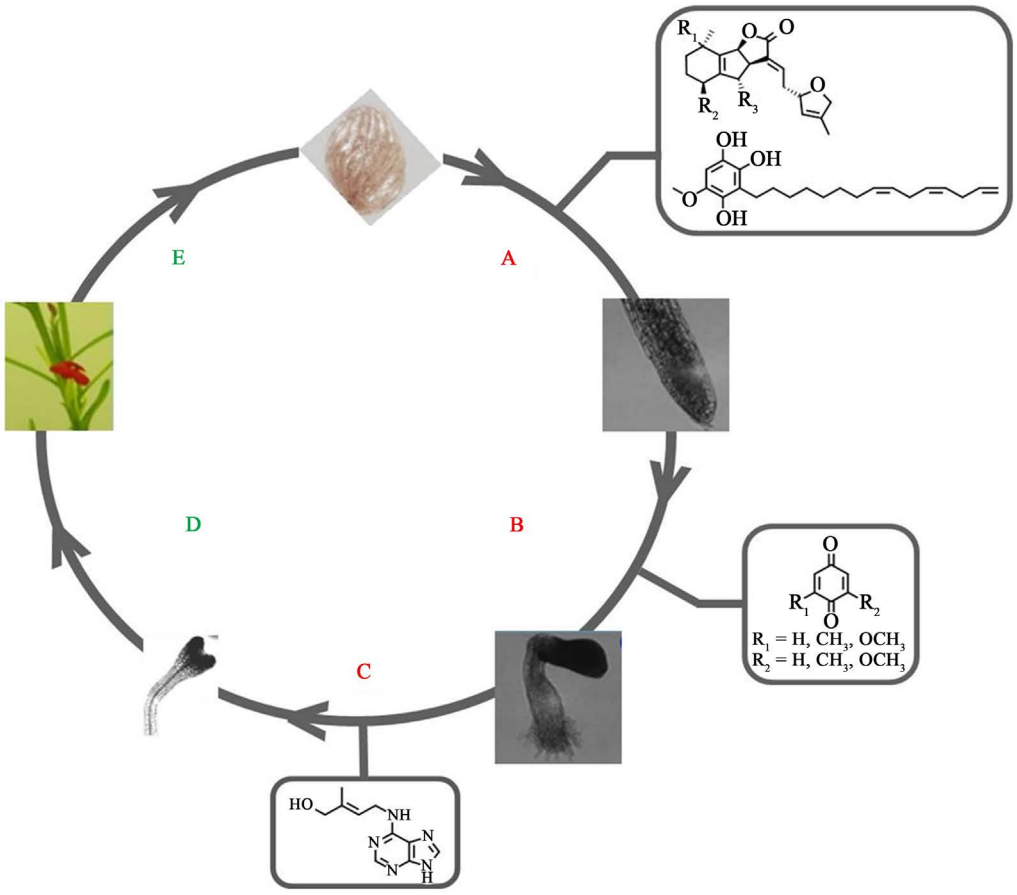

Figure 1. The life-cycle of *S. asiatica* is divided into stages that are either dependent ((a)-(c), red letters) or independent ((d) and (e), green letters) of xenognosins: (a) germination is initiated by strigolactones or SXSg; (b) haustorial organogenesis by *p*-benzoquinones; (c) shoot apical meristem development by cytokinins; (d) & (e) Flowering, seed set, and seed dispersal are host independent.

Many of these xenognosins and their analogues are now readily available, so these transitions can be easily and effectively managed under laboratory conditions. Most importantly, these developmental transitions are so highly synchronized across the population that it becomes possible to explore the molecular elements associated with surmounting the checkpoints on development. Not only will an analysis of the molecular events inform downstream regulatory and functional elements associated with specific plant developmental transitions [9] [13] [14] but the mechanisms of xenognosin regulation and how these cues impact existing machinery will provide insight into the functional evolution and successful strategies of host and parasite adaptation.

Here we combine this xenognosin-directed control over development with transcriptional profiling to identify specific elements associated with the commitment to haustorial organogenesis in *S. asiatica*. The identified genes suggest that semagenesis and haustorial organogenesis are connected to a quorum sensing-like process which facilities the synthesis and integration of biotic signals derived from neighboring plants to manage general growth and development. We also present evidence in support of existing models that the functional origins of these processes lie in the existing defense strategies of plants, most notably the oxidative burst response [15].

2. Materials and Methods

CM-H_2DCFDA was purchased from Molecular Probes (Carlsbad, CA). Murashige and Skoog (MS) media was purchased from Caisson Labs (North Logan, UT). All other chemicals were obtained from Sigma Aldrich (St. Louis, MO). Columbia-0 (Col-0) wild-type *Arabidopsis thaliana* seeds were purchased from Lehle Seeds (Round Rock, TX). *Striga asiatica* seeds were obtained from the U.S. Department of Agriculture (Beltsville, MD) and all experiments were conducted under quarantine conditions for noxious plants. These experiments were conducted under the auspices of the USDA quarantine license awarded to Emory University.

2.1. *S. asiatica* Germination and Haustorium Development

Seeds of *Striga asiatica* were pre-treated and germinated as previously described using 10^{-9} M Strigol [2]. Germination was evaluated after 36 - 48 hours and at this point experiments were initiated. Assays for haustorium development were performed as previously described [9].

2.2. *S. asiatica* Plant Cultures

Plant cultures were obtained by transferring one day old seedlings of *Striga asiatica* to Magenta boxes containing 1xMS medium supplemented with either: 1 mg/L 6-BA and 0.1 mg/L IAA or 1 mg/L 6-BA and 0.5 mg/L IAA. Seedlings were grown at 23˚C with a 16 h photoperiod/day. Regenerated shoots were visible after one week, and the resulting Striga plants typically flowered within 4 - 6 weeks under these conditions.

2.3. SSH Libraries of *S. asiatica* Expression in Response to DMBQ

Forward and reverse subtracted cDNA libraries were generated from mRNA extracted from day-old *S. asiatica* seedlings either treated with 10 μM DMBQ for 6 h (pre-haustorium formation) or untreated. Suppression Subtractive Hybridization (SSH) was performed using a Clontech SSH Kit. These two cDNA libraries were used as both TESTER and DRIVER to generate a complete library of targets both up and down regulated by DMBQ exposure. Sequenced hits were identified by BLAST analysis and added to the NCBI database. These hits were utilized as the source of primers for subsequent relative transcription-PCR analyses at subsequent time points in haustorial organogenesis using actin as an internal standard for amplification.

2.4. NADPH Oxidase Cloning

Total RNA was extracted from germinated seedlings of *S. asiatica* with the RNeasy Plant Mini Kit (Qiagen). Integrity of the isolation was analyzed by electrophoresis in a 8% formaldehyde/1.5% agarose gel. cDNA was generated using Superscript ^{TM} III Transcriptase (Invitrogen) at 50˚C. Based on the conserved NADPH and FAD binding domains of the Respiratory burst oxidases from *Arabidopsis thaliana* (RbohA) and *Nicotiana tabacum (*NtRbohA), several degenerate primers were prepared and investigated. For example, the primer pair forward 5'-GGCAYCCITTYTCWATYACITC-3' and reverse: 5'-GGHGTIGCWCCDATICCNARWC-3' as well as several other degenerate primer pairs successfully cloned genes from 1.5-day-old Striga seedling cDNA. PCR products were isolated and three distinct gene sequences (*SaNOX*1, *SaNOX*2, and *SaNOX*3) were cloned with

the help of TOPO TA Cloning Kit (Invitrogen). Whole cDNA sequences of the three genes were obtained with the help of SMART™ RACE cDNA Amplification Kit (Clontech). Based on cDNA sequences, three genomic sequences were obtained from *S. asiatica* genomic DNA, extracted using the DNeasy Plant Mini Kit (Qiagen). The promoter region for each putative SaNOX homologue was identified by the thermal asymmetric interlaced (TAIL)-PCR method [16].

2.5. Evaluating Localization and Regulation of SaNOX Expression

To analyze the expression pattern of these genes, RT-PCR was performed on 3 distinct sets of tissues: 1) roots, 2) shoots, and 3) leaves and flowers. RNA and cDNA were obtained as described above. In order to exclude genomic DNA contamination, RNA was pretreated with DNase. The RT-PCR studies utilized two primers for each gene (*SaNOX*1-3) and two primers for actin as an internal standard. *SaNOX1*, 2, and 3 expression levels were evaluated by 28, 30, and 30 cycles respectively with an annealing temperature of 58°C. The RT-PCR experiments for each gene were repeated in triplicate with different cDNA samples. Gene specific primers used in RT-PCR were: *SaNOX*1 reverse: 5'-ctgcaccggacgatgactatcttagc-3'; forward: 5'-ctgctatatcatccataacgcctttg-3' or reverse: 5'-cagatcttccgagggacgaatccgtaaaat-3'; forward: 5'-gccatgttgaatttgacggtctcggcag-3'. *SaNOX*2 reverse: 5'-ttgcctaagccatttgaccgcctca-3'; forward: 5'-cctaactgccttatgtgaatgctgagg-3'. *SaNOX3* reverse: 5'-cgagctattggcatttcgtgttgagc-3'; forward: 5'-ccttgtggctttgacatgtgcagagcc-3'. Actin reverse: 5'-caggctgttctctccctttat-3'; forward: 5'tccgatccagacactgtactt-3'. Changes in the expression of *SaNOX*1, 2, or *3* in responses to quinone treatments were evaluated in a similar manner. For Northern analysis, Total RNA from specific Striga tissue was extracted as above. Approximately, 10 µg of each RNA sample was transferred to a nylon membrane, and probed with the P^{32} labeled cDNA sequence for *SaNOX1*. Samples are washed 3 times and exposed to X-ray film (Kodak).

2.6. Cloning SaNOX Promoters and Transient Arabidopsis Transformation

Isolated promoters for *SaNOX*1-3 were cut by SalI and XbaI, and ligated into the PBI101 vector to generate the promoter-directed *β*-glucuronidase (GUS) reporters. Transient expression of the reporter constructs in *Arabidopsis thaliana* Columbia-0 (wildtype) seedlings was facilitated via vacuum infiltration of *Agrobacterium tumefaciens* GV3101 strains bearing the appropriate promoter:reporter construct. Transformed seedlings were selected on 1xMS plates with Kanamycin (50 µg/ml) and subjected to GUS staining [17].

2.7. GUS Assay

Arabidopsis transformants are vacuum infiltrated for 5 minutes in the staining solution (50 mM sodium phosphate buffer, pH 7.0, 0.2% triton-X-100, 1 mM X-Gluc), incubated at 37˚C, and fixed in 75% ethyl alcohol. The pictures were taken with a Canon digital camera.

3. Results

3.1. Developmental Phases Can Be Synchronized by Xenognosins in *S. asiatica* Seedlings

Our approach depends on the synchronized population-wide response of *S. asiatica* to xenognosin exposure. We therefore began by considering exposure times and conditions for each of the xenognosin-mediated developmental transitions: root apical meristem initiation (germination), haustorial organogenesis, and shoot apical meristem (SAM) initiation. Previous studies established that a 6 h exposure to sub-micromolar concentrations of SXSg results in near quantitative germination of *S. asiatica* [6] [11]. Similarly a 6 h exposure to 10 µM 2,6-dimethoxy-*p*-benzoquinone (DMBQ), a previously established haustorial inducer, induces population-wide terminal commitment to haustorial organogenesis [13].

A specific xenognosin for shoot apical meristem initiation has yet to be identified. However, exogenous cytokinins have been shown to induce germination, haustorial organogenesis, and shoot apical meristem development in *S. asiatica* [11]. In these prior studies, the arrested dormancy of the shoot apical meristem was observed by the shedding of the seed coat, which we used as a simple reporter. In *S. asiatica* seedlings in which haustoria were pre-formed, a 4 h exposure to 10 µM trans-zeatin resulted in 50% seed coat shedding ($t_{1/2}$) within 24 hours (**Figure 2**). This effect was not isolated to trans-zeatin as 10 µM treatments with 6-benzyl adenine (6-BA) or kinetin also induced population-wide seed coat shedding with $t_{1/2}$ exposures of 6 h and 10 h, respectively.

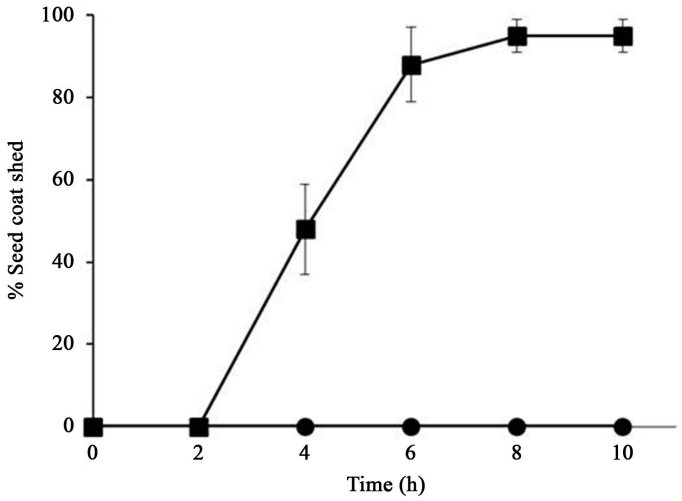

Figure 2. *Cytokinins induce shoot apical meristem development in S. asiatica*. Pre-haustoria are formed on seedlings of *S. asiatica* (See Methods). These seedlings are rinsed in triplicate then placed in buffer either (■) with or (●) without 10 μM trans-zeatin at room temperature. Seedlings are scored for shedding of the seed coat, as an indicator of SAM initiation, at the indicated time point. Results are the average of three trials with error equal to +/− the standard deviation of the mean. n = 50 seedlings.

3.2. Difference Library Construction and Analysis of Regulated Genes

Knowing the exposure time required for developmental commitment allowed us to optimize library construction. Suppression subtractive hybridization (SSH) permits the identification of differential gene expression between two populations and has been exploited for studies ranging from metagenomic analyses to the responses of a single individual to exogenously added molecular signals [18] [19]. In this process, mRNA is isolated from the two populations to be compared and reverse transcribed into cDNA carrying distinct adaptor sequences. dsDNA hybrids formed between members of the two libraries are removed, identifying the differential expression of specific genes.

The phenotypes that score for the initiation of germination and apical meristem initiation (seed coat shedding) provide simple yes/no readouts. The timing of the various events during haustorial organogenesis can be divided into 3 distinct stages: (I) pre-commitment (<2 h), (II) transitioning (2 - 6 h), and (III) post-commitment (8 - 24 h). Stage II is further characterized by an arrest in root elongation and Stage III by (III.a) early commitment/radial swelling, (III.b) haustorial hair initiation (16 h) and (III.c) complete organogenesis. These transitions provide well-defined intermediate time points for library construction, offering improved resolution of the changes in gene expression associated with this developmental transition. We therefore selected haustorial organogenesis as the first xenognosin-regulated transition for evaluation by SSH.

Focusing on Stage II, when mRNA is extracted from day-old *S. asiatica* seedlings treated with 10 μM DMBQ for 6 h and compared with untreated seedlings, 34 unique hits are identified. Estimated fold-change analysis of these products via relative transcription-Polymerase Chain Reaction (rt-PCR), with actin as an internal standard, indicated that 5 of these products show <2-fold change in expression and are therefore excluded from further analysis. Initial assignments of the remaining 29 products is accomplished via BLAST analysis utilizing the NCBI database, and organized into separate groups based on their proposed functions: 1) Cell wall expansion and vascular tissue development; 2) plant defense; 3) nutrient metabolism and transport; and 4) hormone regulation (**Table 1**). In addition, two previously identified expansins of *S. asiatica* [14] as well as a homolog of the quinone oxidoreductase QR2 from *Triphysaria versicolor* [20], a facultative root parasite in the *Orobanche* (broomrape) family, are identified in this library. The observed changes in expression appear specific to xenognostic quinones as the non-inducing di-tert-butyl-benzoquinone has no discernable effect on gene expression and tetrafluorobenzoquinone (TFBQ), a reversible inhibitor of haustorium development, blocks the differential expression mediated by DMBQ [14].

The differential expression of these genes over the 24 h period of haustorium development, relative to un-

Table 1. Sequences obtained from the DMBQ-induced haustorium library.

ID	NCBI ID[a]	Matching sequence[b]	E-value	Putative function	Fold change in expression (h)[c,d]				
					0.5 I	2 II	6[e] II	16 III[b]	24 III[c]
Cell wall Expansion and vascular tissue development									
1	DQ442395	AY144594	6.00E−119	Xyloglucan endotransglycosylase	1	1.5	3	2.5	3
2	DQ442401	AF184233	1.00E−93	Expansin	3	3	3	3	3
3	AF291657	AF512542	2.00E−87	Expansin	2	2	3	2	2
4	AF291658	AB093031	2.00E−85	Expansin	2.5	2.5	2.5	2.5	2
5	DQ442392	AJ223969	9.00E−102	Elongation factor 1	1	1	−10	−10	−10
6	DQ445120	AY231146	5.00E−103	Alpha-tubulin	1	−2	−6	−5	−5
7	DQ445123	AB084381	5.00E−64	Homeobox leucine-zipper	1.5	2.5	3	3	2.5
8	DQ431680	XM_482629	4.00E−39	Putative chorismate mutase	4	2.5	2.5	2.5	2
9	DQ445134	NM_116339	5.00E−68	Unknown	1	−8	−10	−8	−8
Plant defense responses									
10	DQ431679	XM_02532771	2.00e−114	Respiratory burst oxidase	−2.5	−5	−5	−10	−10
11	DQ442383	AB050849	6.00E−68	Calmodulin	1	1	6	5.5	5.5
12	DQ442394	NM_180054	2.00E−27	Calmodulin binding	3	3	3	3	2.5
13	DQ442396	AY206407	2.00E−71	Ascorbate peroxidase	−5	−10	−10	−8	−8
14	DQ442387	ABA93963	2.00E−62	GDSL lipase	1.5	1	−5	−5	−6
15	DQ445121	NM_123583	1.00E−114	Peroxidase	−8	−8	−9	−8	−8
16	DQ442385	AF242491	1.00E−67	PCBER reductase	1	−8	−9	−9	−9
17	DQ442405	AF304462	1.00E−84	Quinone-oxidoreductase QR2	7.5	7.5	8.5	8.5	8.5
Nutrition, metabolism, and transportation									
18	DQ442379	AJ278765	1.00E−62	Sugar transporter	2	2	3	2	2
19	DQ445131	NM_129389	2.00E−27	Antiporter/drug transporter	4.5	4.5	6.5	6	6
20	DQ442390	AK176585	1.00E−32	Carboxypeptidase	1.5	4.5	4.5	5	5
21	DQ445132	AJ849375	5.00E−73	Carbonic anhydrase	2.5	2.5	2.5	2.5	2.5
22	DQ445137	AB183015	1.00E−96	Zinc-binding dehydrogenase	1	2.5	2.5	2.5	2.5
23	DQ445126	AC021199	2.00E−73	Hydroxymethyltransferase	ND	1	4	4	4
24	DQ445129	AY226830	3.00E−18	Desacetoxyvindoline 4-hydroxylase	3	3.5	3.5	3.5	3.5
25	DQ442381	NM_119911	1.00E−92	Catalytic hydrolase	1.0	−8	−8	−8	−7
Hormonal pathways									
26	DQ431687	AF534888	7.00e−54	Type-A RR	1	2.5	3	ND	ND
27	DQ431686	NM_115563	6.00e−59	Type-A RR	1	5	5	ND	ND
28	DQ442403	AY090553	4.00E−100	Auxin-regulated protein	1	1	3	3	3
29	DQ442404	AF373100	2.00E−86	Auxin-regulated protein	3.5	3.5	3.5	3.5	3.5

[a]: NCBI ID of deposited full-length sequence determined from cDNA; [b]: Matching sequence identified through BLAST analysis; [c]: Time seeds were harvested for library construction; [d]: Expression-levels are based on relative transcription PCR to untreated seedlings with actin used as internal standard to normalize results; [e]: Time point for initial SSH library construction. ND = Not Determined.

treated (DMBQ-free) seedlings of similar age by the same rt-PCR analysis method, is summarized in **Table 1**. Specifically, these results are categorized and discussed in terms of the individual developmental stages:

I. Pre-commitment (0.5 h). Of the 29 genes identified in our library, 12 (40%) are differentially expressed within 0.5h after DMBQ exposure. This group includes two known expansins (**3** and **4**) [14], as well as a newly identified expansin (**2**). Up-regulation of these proteins may be correlated with remodeling of the root tip. However, some of the most dramatic changes in expression at this early time point are associated with ROS production. This group includes the down regulation of an NADPH oxidase (**10**) as well as peroxidases **13** and **15**. The significant up-regulation (>7-fold) of a QR2 homologue (**17**) is particularly noteworthy given previous studies in *T. versicolor* that implicate QR2 as a potential receptor for xenognostic *p*-benzoquinones [21].

Also apparent are changes in the expression of genes associated with metabolism and transport of nutrients as well as hormone regulation. A putative auxin regulatory protein, **29**, supports phytohormone regulation early during haustorial organogenesis. A role for auxin at this early time point is also consistent with the arrest of root elongation observed upon DMBQ exposure prior to commitment. Phytohormone studies in *T. versicolor* have also implicated a role for auxin signaling in haustorial organogenesis [22]. As cytokinins induce haustorium development, the developmental transition may also be regulated by both of these often antagonistic hormones [11].

II. Transitioning (2 - 6 h). Differential regulation of the remaining 17 (≈ 60%) genes in the library largely occurs throughout the period of commitment to haustorium development (2 - 6 h). The expansins (**2**-**4**) remain up regulated, while Elongation factor 1 (**5**) and α-tubulin (**6**) are both significantly down regulated, suggesting some relationship between the rate of protein synthesis and microtubule assembly in regulating haustorium development [23] [24]. We note that α-tubulin knockdowns in *A. thaliana* are characterized by radial expansion of the root tip, arrested root elongation, and ectopic root hair formation adopting a structure morphologically similar to the haustorium [23]. Regarding plant defense responses, the NADPH oxidase (**10**), peroxidases (**13** and **15**), and QR2 (**17**) remain differentially regulated throughout this period as well. Calmodulin (**11**) expression increases during this time period implicating Ca^{2+}-mediated signaling events in commitment and/or development. Some genes associated with metabolism and transport of nutrients as well as hormone regulation (**19**, **21**, **24**, and **29**) remains differentially regulated during this time period. Two cytokinin associated response regulators (**26** and **27**) are also upregulated during this period.

III. Post-commitment (16 - 24 h). Finally, the rt-PCR assays of library members at 16 and 24h post-DMBQ exposure show no significant variation in the expression relative to the 6h treatment. These results are consistent with the molecular elements required for haustorium development being largely in place after 6h, coupling expression to the clock for terminal commitment [13].

3.3. Library Guided Identification of Genes for Semagenic ROS Production

The connection to defense and wounding is the most dominant and striking feature of all three stages. ROS production is central to the semagenesis mechanism and likely an early and important element in the functional emergence of parasitic angiosperms. Production is tightly regulated by the presence of xenognostic quinines [9], which may be due to a reduction in the expression of a ROS-producing catalyst. Likely candidates for this catalyst in plants include xanthine oxidases [25], peroxidases [26], lipoxygenases [27], polyamine oxidases [28], mitochondrial NADH dehydrogenases [29], oxalate oxidases [30], and NADPH oxidases [31]-[33]. The SSH library identified two peroxidases (**13** and **15**) and one NADPH oxidase (**10**) down regulated by DMBQ exposure during pre-commitment (0.5 h, stages I) and transition (2 - 6 h, stage II).

Several possible inhibitors for ROS synthesis were evaluated including Salicylhydroxamic acid (SHAM), AEBCF, Apocynin, Phenylarsine oxide (PAO), Diphenylene iodonium (DPI), Oxypurinol, Allopurinol, Rotenone, MDL7257, and Nordihydroguaiartic acid [33]-[41]. Of these, only diphenylene iodonium (DPI) and phenylarsine oxide (PAO) inhibits ROS production in *S. asiatica* seedlings (**Supplementary Figure S1**), most consistent with a superoxide ($O_2^{\bullet -}$) generating NADPH oxidase (NOX), like **10**, as the catalyst [31]-[33]. The putative *Striga asiatica* NADPH oxidase 1 (**10**) is referred to as *SaNOX1* for the remainder of the discussion.

Degenerate oligonucleotide primers for the conserved regions of NOX proteins successfully identified two additional putative NOX homologues: *SaNOX*2 and *SaNOX*3 (accession numbers DQ431678 and DQ431677) [42] [43]. Distinct promoter sites for each of these genes were obtained using thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) [16]. Exon-intron analyses of the full-length genomic sequences, as well

as structural predictions based on the mRNA, are available in the Supplementary Information and summarized below (**Supplementary Figures S2-S4**).

NADPH oxidases in plants are often referred to as respiratory burst oxidase homologue (Rboh) proteins due to their initial discovery as the source of the oxidant in this immune response [42] [43]. Consistent with known Rboh proteins, analysis of the SaNOX1 sequence supports the presence of well-conserved structural features including (from N → C): 1) 2 Ca^{2+}-binding EF-hand domains, 2) 6 transmembrane helices, 3) corresponding histidines for heme-binding on helices III and V, 4) an FAD-binding domain, and 5) an NADPH-binding domain [42] [43]. In contrast, SaNOX2 and SaNOX3 appear to be N-terminal truncations that begin in the loop between transmembrane helix I and II. All three proteins have distinct, homologous sequences with unique exon-intron patterns.

Northern blot and PCR-based expression analysis of *SaNOX*1-3 in *S. asiatica* plants grown in culture confirm *SaNOX*2 & *SaNOX*3 are transcribed throughout the plant, while *SaNOX1* expression is limited to the roots (**Figure 3(a)** and **Figure 3(b)**). In order to further refine *SaNOX*1-3 localization, *Arabidopsis thaliana* was employed as a heterologous host expression system. Transient expression via Agrobacterium-mediated vacuum infiltration was used to identify specific sites of SaNOX expression. *SaNOX*2::*GUS* and *SaNOX3::GUS* transformants accumulate stain throughout the plant (**Figure 3(c)** and **Figure 3(d)**) while in the *SaNOX1::GUS* staining is limited to the root tip (**Figure 3(e)** and **Figure 3(f)**), the corresponding site of ROS production in *S. asiatica*.

Prior to the commitment to haustorial organogenesis, the removal of pBQs restores semagenic ROS production [9], and as shown in **Figure 4**, the expression of *SaNOX1* as well. Additional haustorial inducing pBQs, like the unsubstituted *p*-benzoquinone or methyl-*p*BQ also attenuate ROS production as well as *SaNOX1* expression, further supporting a connection to semagenesis (data not shown).

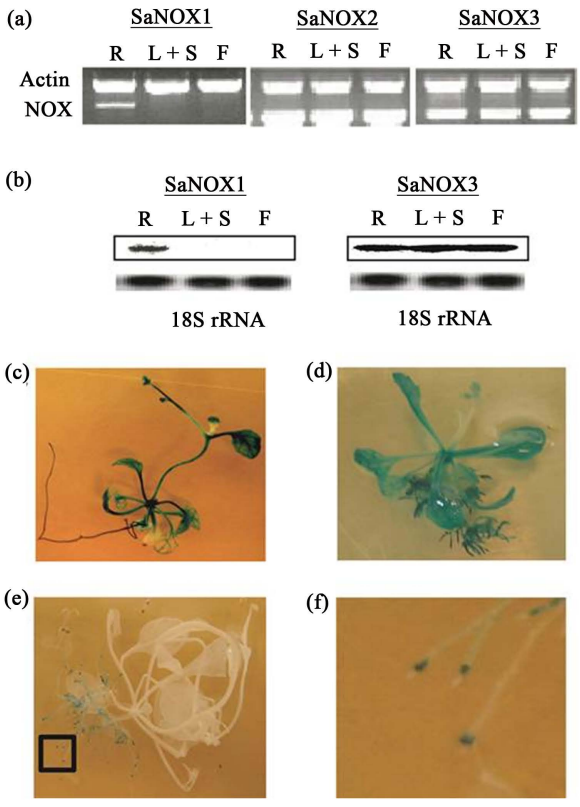

Figure 3. *Tissue localization and expression of SaNOX*1-3. Tissues harvested from germinated seedlings (root) or mature cultured *S. asiatica* plants were (a) screened for *SaNOX*1-3 localization of expression by relative transcription PCR using actin as a control; (b) Expression of *SaNOX1* and *SaNOX3* were confirmed by Northern analyses using 18s rRNA as a control. Lane labels are R: Root, L + S: Leaf + Stem; and F: Flower. (c)-(f) *A. thaliana* mutant lines expressing *β*-glucoronidase downstream of the promoters for *SaNOX*2 (c), *SaNOX*3 (d), and *SaNOX1* (e) & (f) were generated to establish expression localization of each product.

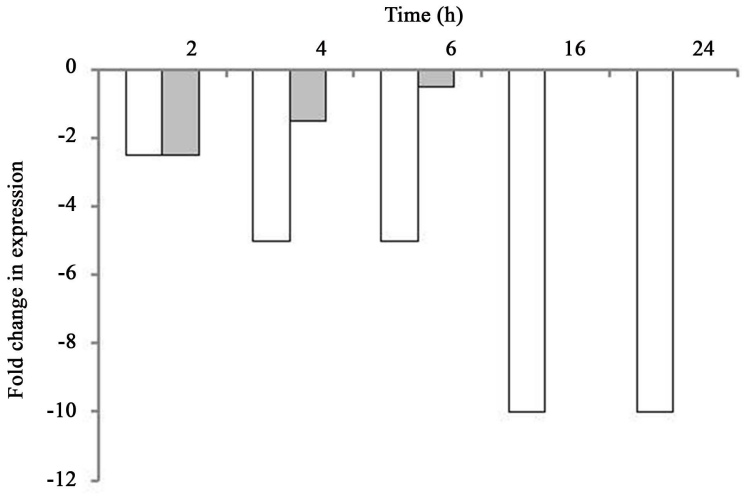

Figure 4. *SaNOX*1 *expression is reversible prior to commitment to haustorial organogenesis*. *SaNOX*1 expression was evaluated via RT-PCR at the indicated time points following the addition of 10 μM DMBQ. After 2 h of DMBQ exposure seedlings were washed in triplicate and placed in either buffer (grey) or returned to a solution of 10 μM DMBQ (white). Auxin was used as an internal standard for normalization.

4. Conclusions

The successful integration of a eukaryotic parasite with its host presents many of the same synchronization challenges faced during multicellular tissue development. The temporal and spatial control over cell fate defines tissue architecture, and the mechanisms underlying these precise responses appear to be similarly constrained between two distinct organisms. Defining the temporal and spatial organization of the chemistry that makes this control possible has significant practical and theoretical consequences for plant development as well as the adaptations necessary for both pathogenic and mutualistic symbioses.

Here we have focused our attention on the haustorium, the host attachment organ whose development is common across the various manifestations of the parasitic strategy in plants [44] [45]. In *S. asiatica*, as well as other parasitic plants, the detection of simple *p*-benzoquinones is both necessary and sufficient to mobilize the genetic elements necessary for the rapid functional transition to the haustorium. Our initial differential expression library identified 29 genes that were regulated during commitment to haustorial organogenesis. These genes fall into the general categories of cell wall expansion and vascular tissue development, nutrient metabolism and transport, hormone regulation, and cellular defense (**Table 1**).

The haustorium provides the vascular conduit between host and parasite in the parasitic plants, and its development begins with the radial swelling and ectopic root hair formation necessary for host attachment. The genes associated with cell wall expansion (e.g., expansins) as well as vascularization (e.g., chorismate mutase) are reasonable candidates for differential expression during haustorial organogenesis. The down-regulation of α-tubulin expression is of particular interest given that *A. thaliana* knockdowns of this protein give root phenotypes similar to that observed in haustorial organogenesis: radial expansion of the root tip, arrested root elongation, and ectopic root hair formation [23] [24]. The general up-regulations of genes associated with nutrient metabolism and transport are consistent with the principal function of the haustorium: tapping host resources. The regulated flux of auxins and cytokinins is probably the most expected of a developmental transition and consistent with previous studies of haustorial organogenesis [22].

Conversely, the changes in defense gene expression seem contradictory. On one hand, the grafting of two tissues through the haustorium must involve the regulation of xenognostic processes, while at the same time constitutive ROS production in the tissue undergoing haustorial organogenesis is central to the chemical mechanism of semagenesis. The SSH library and subsequent PCR analyses confirmed the down regulation of two putative sources of semagenic ROS, a peroxidase (**15**) and an NADPH oxidase (*SaNOX1*) (**10**). Small molecule inhibitors further implicated the NADPH oxidase (SaNOX1) as the source of ROS at the root tip that was regulated in response to DMBQ. Furthermore, heterologous expression in *A. thaliana* confirmed *SaNOX1* expression only at the root tip, the site of haustorial development.

Like other respiratory burst oxidase homologues (Rboh) in plants, structural predictions of SaNOX1 indicate all the features required for calcium-regulated ROS production are conserved in this protein. Based on these predictions, as well as its observed regulation by pBQ concentrations, we suspect that SaNOX1 provides the source of semagenic ROS. Direct support for the redox activity of SaNOX1, however, that may require expression in a heterologous host as knockout/knockdown experiments are currently limited in *S. asiatica*.

As for SaNOX2 and SaNOX3, while they appear to be N-terminal truncations of these Rboh proteins, they retain all of the features associated with ROS production and may be well expressed in their corresponding tissues. The apparent absence of EF-hand domains for calcium regulation is unusual in plants but is not uncommon in other eukaryotes [46]. The variations in their sequence as well as exon-intron utilization argue against their arising simply from recent gene duplication events. Indeed comparative exon-intron analysis between the eight Rboh proteins from *Arabidopsis thaliana* confirm distinct exon-intron patterns for several of those proteins as well, although none display truncations like those in SaNOX2 or SaNOX3 [42] [43]. Such unusual truncations may reflect the accelerated rate at which mutations appear to accumulate among parasitic angiosperms, relative to their non-parasitic relatives [47]. These increased rates of mutation may well provide an adaptive advantage to parasites in dealing with the evolution of resistance in hosts.

In addition to their well-established roles in defense, ROS production has also been implicated in plant growth and development including root hair formation and pollen tube growth [31]-[33]. In this context, the redox events associated with semagenesis may not be unique to haustorial organogenesis, but more generally associated with cell-cell signaling and development. Increasing evidence implicates the semagenic quinones as not only regulating haustorial development in the parasitic plants [15], but more broadly in root meristem growth and development. The addition of semagenic quinones impacts development of both root architecture and notably root hair density in *A. thaliana* and *N. tabaccum* as well as the production of ROS [15] [48].

Root growth and architecture is sensitive to the density and identity of neighboring roots (self and non-self) [49] [50] and the chemistry exploited by *S. asiatica* in semagenesis could serve a more general quorum sensing-type role in plant roots, controlling morphological development (phenotype) based on their proximal neighbors. In this context, understanding the integration of accumulating pBQs via semagenesis in *S. asiatica* may provide greater insight into how plants 'decide' to organize the architecture of their root systems. Indeed, the strigolactones, which were originally discovered as germination stimulants for *Striga spp.* (**Figure 1(a)**), have now been shown to function as regulators of branching in both plants and arbuscular mychorizzal fungi [51] [52]. These findings bolster the model that xenognosin-regulated transitions in parasites likely arose from pre-existing regulatory pathways present among non-parasites. Given the tight regulation of these transitions, these systems now serve as valuable model systems for defining the molecular machinery responsible for signal integration, response to environmental inputs, and decision-making in critical developmental commitments. Such information can be used to regulate the "decision making" processes and drive productive/lethal transitions in plants. This information then is critical to reading and controlling the molecular code of plant environmental sensing and adaptation to an ever-changing environmental landscape.

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Supplemental Information

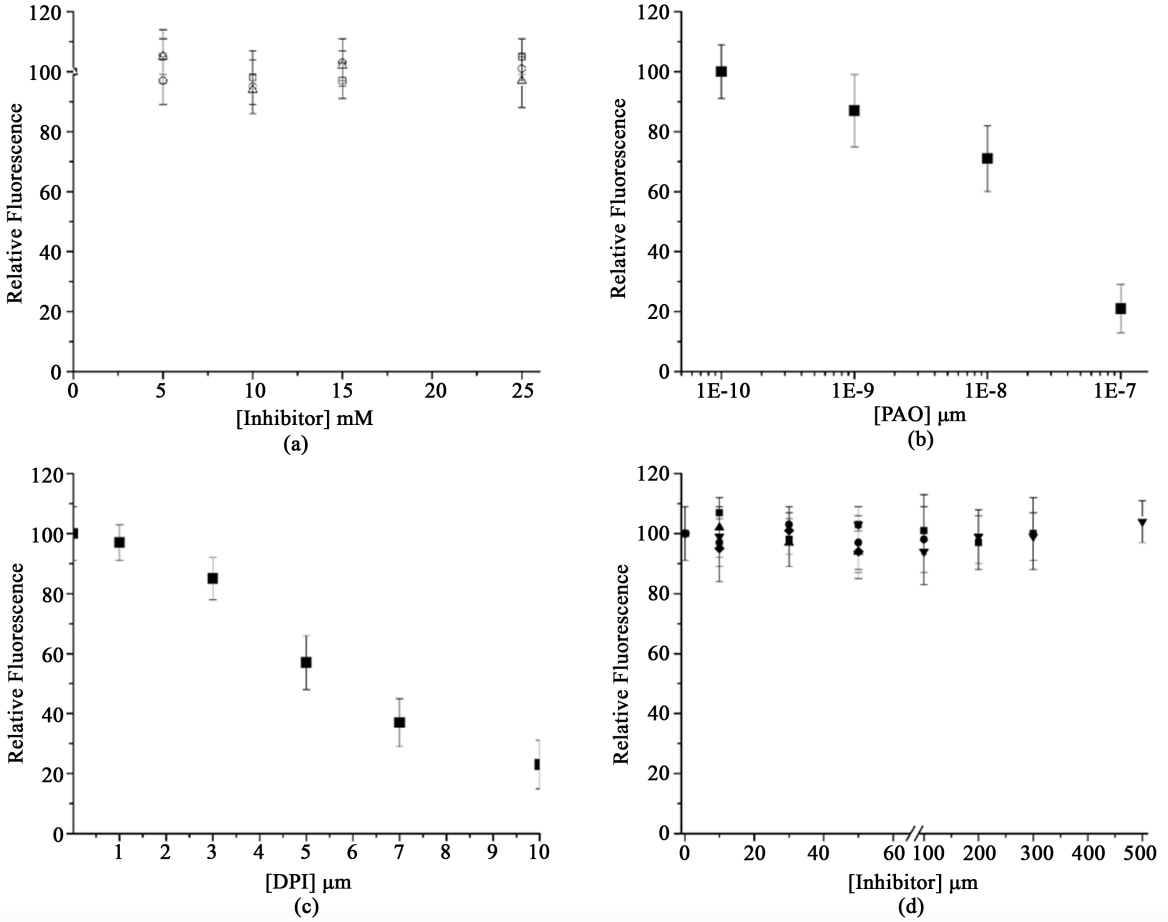

Figure S1. *Effect of ROS inhibitors on CM-H_2DCFDA assay*. Two day old seedlings of *Striga asiatica* are incubated with the indicated concentration of inhibitor for 2 hours then loaded with 10 µM CM-H_2DCFDA for 5 minutes and imaged for ROS production (See Methods). The ROS source for each inhibitor is indicated in parentheses behind each inhibitor. (a) □ Salicylhydroxamic acid (peroxidases) [37], o AEBCF (Animal ***not*** plant NOX proteins), △Apocynin (Animal ***not*** plant NOX) [35]; (b) Phenylarsineoxide (PAO) (NOX) [38]; (c) Diphenyleneiodonium (DPI) (NOX) [41]; (d) ■ Oxypurinol (Xanthine oxidases) [36], ● Allopurinol (Xanthine oxidases) [36], ▲ Rotenone (Mitochondrial complex I) [39], ▼ MDL 72,527 (polyamine oxidase) [33], ♦Nordihydroguaiartic acid (lipoxygenase) [40]. Fluorescence intensity expressed relative to Striga seedlings treated with CM-H_2DCFDA only (set at 100). Results are the average of three trials with error equal to +/− the standard deviation of the mean.

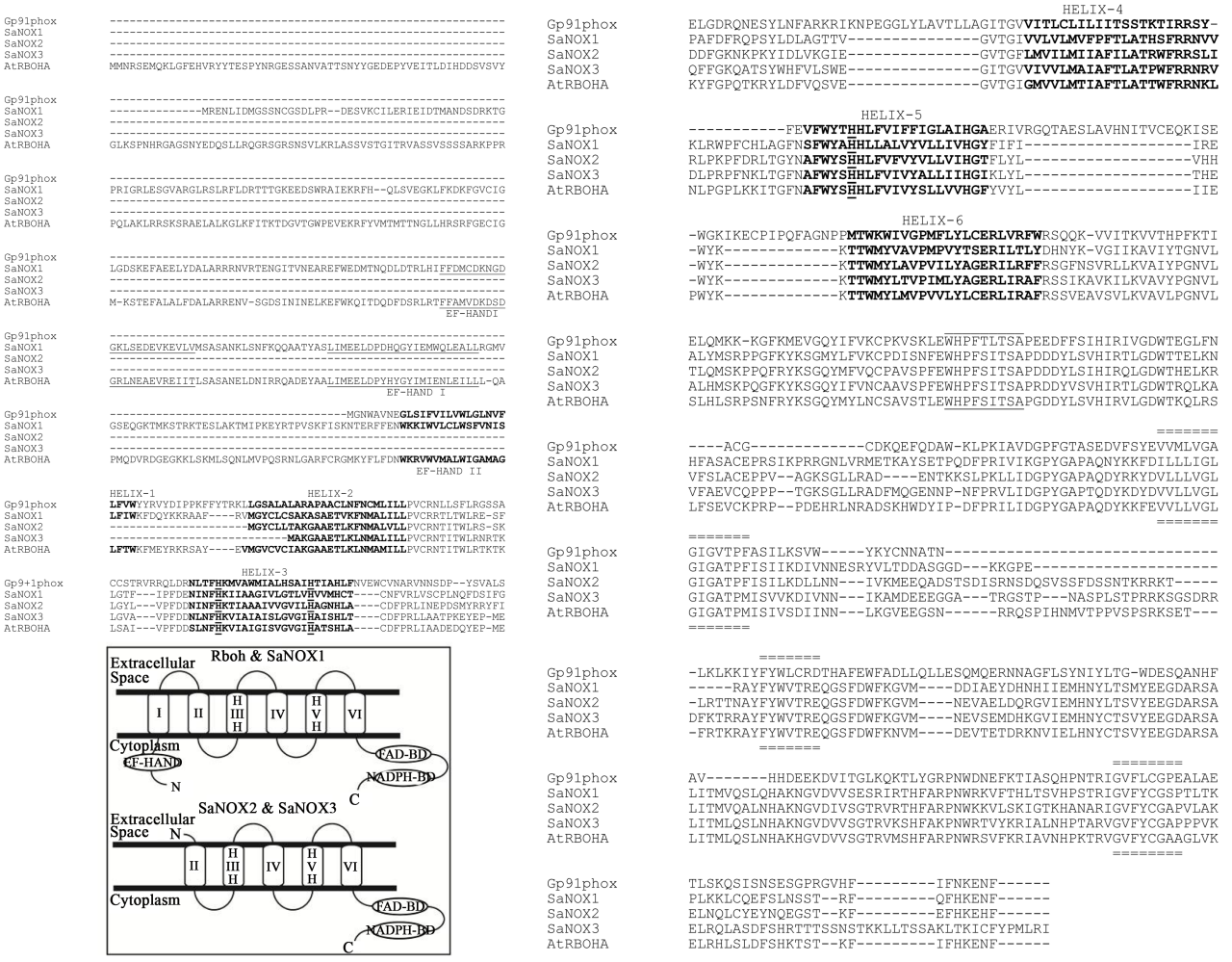

Figure S2. Proposed structure of SaNOX1-3 based on BLAST analysis, CLUSTALW alignments, and published literature on the structure of gp91phox and the *Arabidopsis thaliana* respiratory burst oxidase A (Rboh A). Both of these proteins produce superoxide anions but only Rboh A is regulated by calcium due to the presence of the Ca^{2+}-binding EF-hand domains which are also conserved in SaNOX1 (underlined) but not SaNOX 2 or 3. In addition the first transmembrane helix found in SaNOX1, Rboh A, and gp91phox (H1 and bold text) is absent in SaNOX2 and 3. Near the C-terminus of the proteins are the highly conserved FAD and NADPH binding domains (BD) indicated by the single line or double lines respectively.

SaNOX1

	Genomic coordinates	mRNA coordinates	Exon Length	% Identity
Exon 1	1-304	1-304	304	99.70%
Exon 2	403-565	305-467	163	100.00%
Exon 3	681-729	468-516	49	100.00%
Exon 4	855-968	517-630	114	100.00%
Exon 5	1047-1860	631-1443	813	99.40%
Exon 6	1943-2035	1444-1536	93	98.90%
Exon 7	2120-2235	1537-1652	116	99.10%
Exon 8	2404-2507	1653-1756	104	100.00%
Exon 9	2618-2699	1757-1838	82	100.00%
Exon 10	2810-2957	1839-1986	148	100.00%
Exon 11	3058-3192	1987-2121	135	100.00%
Exon 12	3293-3610	2122-2439	318	100.00%

SaNOX2

	Genomic coordinates	mRNA coordinates	Exon Length	% Identity
Exon 1	1-546	1-546	546	100.00%
Exon 2	654-749	547-642	96	100.00%
Exon 3	849-964	643-758	116	100.00%
Exon 4	1112-1279	759-926	168	100.00%
Exon 5	1362-1521	927-1086	160	100.00%
Exon 6	1627-1809	1087-1269	183	100.00%
Exon 7	1895-2102	1270-1477	208	100.00%
Exon 8	2193-2302	1478-1587	110	100.00%

SaNOX3

	Genomic coordinates	mRNA coordinates	Exon Length	% Identity
Exon 1	1-528	1-528	528	100.00%
Exon 2	621-716	529-624	96	99.00%
Exon 3	908-1023	625-740	116	100.00%
Exon 4	1244-1420	741-917	177	100.00%
Exon 5	2200-2911	918-1629	712	100.00%

Figure S3. Results of SPIDEY analysis using mRNA and genomic sequences for: SaNOX1, 2, and 3 respectively (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/).

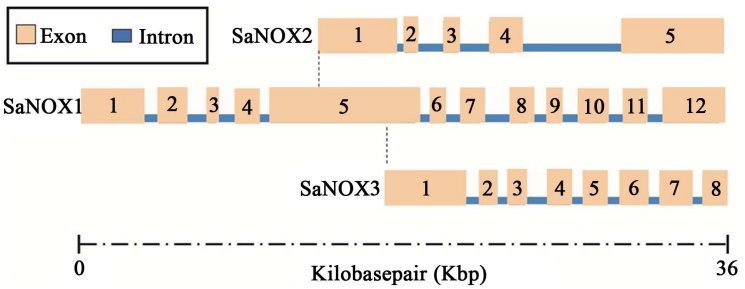

Figure S4. *Exon-intron analysis of SaNOX*1, 2 *and* 3. Genomic sequences of SaNOX1-3 are organized by exons and introns, based on the SPIDEY analysis above (See Supplementary Table 1). The first exons of SaNOX2 and 3 are positioned to reflect the exon of SaNOX1 to which they show the greatest similarity (based on CLUSTALW analysis). Position is indicated by vertical dashed lines Sequences are drawn to scale.