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Expression Pattern of *in vitro* Organogenesis-associated Genes as Transcriptional Marker in Sandalwood (*Santalum album* L.) Micropropagation

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Abstract

The world's most expensive wood, sandalwood (*Santalum album* L.), requires a stringent mass-propagation technique to prevent future scarcity. Plant tissue culture is an efficient method that regenerates the whole plant from a single cell on a hormone-based growth medium. To efficiently regulate the formation of plants, it is important to understand the developmental organogenesis pathways (i.e., direct and indirect) through gene expression studies. Therefore, an effective protocol for the direct and indirect organogenesis of sandalwood was developed, followed by the characterization of gene expression patterns in investigation. Five *in vitro* organogenesis genes namely, Alternative oxidase (ao), Late embryogenesis abundant (lea), Cytochrome p450 (cyt-p450), ABC transporter (abct), and Serine-threonine phosphatase (stp), were screened from three stages of sandalwood development; (1) inoculated leaf, (2) proliferated leaf shoot/callus formation and (3) shoot formation. The best treatments for plant regeneration in Woody Plant Media (WPM) were SI_{24} (2.5 mg/l 2,4-D) for indirect organogenesis and SD_{14} (2.0 mg/l BAP and 0.4 mg/l^{-1} NAA) for direct organogenesis. During the initial stages of organogenesis, *ao*, *cyt-p450* and *abct* showed no/little change in expression in the direct pathway however up-regulation of *ao* and *abct* and downregulation of *cyt-p450* were observed in the indirect pathway. Expression of *lea* was increased up to 70-fold during direct and dropped to half during indirect organogenesis. The optimization of the sandalwood organogenesis regeneration medium and the identification of distinct gene expression patterns will serve as transcriptional markers for the early prediction of the organogenesis stage, assisting in the sandalwood conservation.

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Introduction

Sandalwood (*Santalum album* L.) is one of the tree species renowned as 'The Royal Tree' of the plant kingdom (Subasinghe 2013). *Santalum album* L. belongs to the family *Santalaceae*, an approximately 12-15 meters tall, evergreen, hemi-root parasitic tree that is highly valued for its fragrant heartwood. In India, sandalwood is more confined to the southern region, especially in Karnataka, Tamil Nadu, and Kerala (Kumar et al. 2012). There are certain traits that hold *S. album* distinctive and inimitable due to its ability to grow under diverse conditions, *viz.* adaptability to very low rainfall and a wide variety of soil types, innate survival capacity, short juvenile phase, and profuse coppicing ability (Singh et al. 2013). However, the major constraints include predominant outcrossing, long seed dormancy period (2-12 months and normally takes 4-8 weeks for the germination), and flowering takes place in 60% of plants only by the end of 3-4 years (Srimathi et al. 1995). Due to overexploitation and a slow regeneration rate of sandalwood trees, there is an immediate need for its conservation. The conventional breeding methods integrate new genetic information which can be an expensive and difficult task because of its long generation time, sexual incompatibility, and heterozygous nature (Rugkhla and Jones 1998). Alternatively, *in vitro* propagation and regeneration techniques are the major approaches for rapid sandalwood propagation that fulfills the scarcity of sandalwood (Mujib 2005). *In vitro* organogenesis requires dedifferentiation of differentiated cells to acquire organogenic competence, re-entry of quiescent cells into the cell cycle, and organization of cell division to form specific organ primordia and meristems. *In vitro* organogenesis depends on the application of exogenous phytohormones, in particular, auxin and cytokinin, and also on the ability of the tissue to respond to these phytohormone changes during culture. Thus, the tissue culture technique can be utilized to induce organogenesis *in vitro* (Thorpe 1990). In contrast to direct organogenesis, callus formation occurs naturally in response to wounding during indirect organogenesis.

Understanding the physiological and molecular basis of regeneration potential in plant cell cultures through gene activity analysis is critical for expanding the genetic pool and improving clonally propagated plantation stocks (Jain and Minocha 2013). Changes in the gene expression pattern are regulated during the developmental switching to direct or indirect organogenesis. It has been postulated that the different hormonal treatments induce different gene responses that are reflected in transcript numbers. Reverse transcription (RT) followed by quantitative polymerase chain reaction (qPCR) represents a powerful tool for the sensitive detection and quantification of low transcripts in tissue (Freeman et al. 1999; Steuerwald et al. 1999; Mackay et al. 2002). Considering the importance of sandalwood conservation, the present investigation for standardization of sandalwood organogenesis tissue culture protocol and the gene expression study was carried out.

Materials and Methods

Tender nodes were collected as explants from approximately 20 years old sandalwood trees cultivated at Anand Agricultural University Campus, Anand, Gujarat, India. After surface sterilization, explants were inoculated on MS medium for the shoot induction response. Leaves that emerged from the nodes were used as the experimental material in the present investigation. For the organogenesis pathways, direct and indirect, *in vitro* leaves were placed on the shoot induction media. The shoot induction media for direct organogenesis was comprised of Woody Plant Medium (WPM) (Lloyd and McCown 1981) supplemented with various combinations of BAP and NAA and 3% w/v sucrose (Table 1) and for indirect organogenesis was comprised of WPM supplemented with various combinations of Glycine, Adenine Sulphate (ADS), Potassium nitrate (KNO_3), NAA, BAP, 2, 4-D and 3% w/v sucrose (Table 2). The media was solidified using 0.9% Agar and the pH of the medium was adjusted to 5.7 ± 0.01. All the cultures were incubated in a growth room maintained at 25 ± 1°C, 40-60% relative humidity, and a 16/8-hour light/dark regime was provided by a cool-white, fluorescent light with 36 $\mu molm^{-2}s^{-1}$ intensity.

For gene expression profiling in sandalwood during organogenesis, five sets of primers, representing five different genes *viz*. Alternative oxidase (ao), Late embryogenesis abundant proteins (lea), Cytochrome P-450 (cytp450), ATP binding cassette transporter proteins (abct), and Serine threonine phosphatase (stp) were designed for the callus and shoot development stage based on Che et al. (2006) on *Arabidopsis* (Table 3). The leaf samples were collected from three stages of direct organogenesis; inoculated leaf (D1), proliferated leaf (D2), and shoot formation (D3), and three stages of indirect organogenesis; inoculated leaf (ID1), proliferated leaf (ID2), and callus formation (ID3). The direct and indirect stages are shown in Fig. 1. RNA extraction was carried out from the samples collected from the different stages of organogenesis using the method described by Ghavana et al. (2011) with minor modification. Preparation of cDNA from total RNA was carried out using a first-strand cDNA synthesis kit (Takara, Japan) as per the manufacturer's instructions. Further, primer screening was carried out with the DNA extracted from sandalwood tissues of different stages. These primers were used for absolute quantification in Real-Time PCR. The standard curve of each gene was carried out using Cq values versus copy number (Table 3; Fig. 2).

Results and Discussion

In direct organogenesis, the relationship between gradient levels of BAP and NAA and the morphogenic potential of plantlets were found which could give the best establishment and multiplication rates. Treatment SD_{14} (2.0 mg/l BAP and 0.4 mg/l NAA) recurred as the best media in terms of bud sprouting frequency, the number of shoots and length of shoots were 60%, 3 and 2 cm, respectively, highest among the other

treatments. The sprouting frequency has increased to 70% after 120 days from the induction phase. Singh et al. (2013) also reported similar results where WPM supplemented with 2.5 mg/l BAP and 0.2 mg/l NAA was used and a bud frequency of 79.16% was obtained. Greenish shoot bud formation in all the treatments has been observed due to the low auxin: cytokinin ratio as explained by Behbahani et al. (2011) and Singh et al. (2013).

Table 1. Treatments for direct organogenesis.

Media code	Basal	BAP (mg/l)	NAA (mg/l)
SD_1	WPM	0	0
SD_2	WPM	1	0
SD_3	WPM	1.5	0
SD_4	WPM	2	0
SD_5	WPM	2.5	0
SD_6	WPM	0	0.2
SD_7	WPM	1	0.2
SD_8	WPM	1.5	0.2
SD_9	WPM	2	0.2
SD_{10}	WPM	2.5	0.2
SD_{11}	WPM	0	0.4
SD_{12}	WPM	1	0.4
SD_{13}	WPM	1.5	0.4
SD_{14}	WPM	2	0.4
SD_{15}	WPM	2.5	0.4

All treatments have been provided 3% sucrose with 0.9% agar.

For indirect organogenesis, Rashmi and Trivedi (2014) reported that 2, 4-D encourage the synthesis of endogenous purines and cytokinins ultimately resulting in higher rates of cell division. The use of 2, 4-D indicated SI_{24} (2.5 mg/l 2, 4-D) as the best treatment for achieving early callus induction response within 35-37 days with 70% callus induction and 50% callus frequency after 80 days. The callus appeared healthy and green in color and friable in nature. This finding is in accordance with the results of Adesoye and Orkpeh (2009) and Behbahani et al. (2011) who also favored WPM over MS. After 120 days, shoot regeneration was first observed in treatment SI_{33} (1 mg/l^{-1} BAP and 0. mg/l 2, 4-D) with 87.50% of callus induction and 75% of callus regeneration frequency.

The relative gene expression pattern of *ao*, an important gene in stress response (Van Aken et al. 2009) was observed (Fig. 2a). During direct organogenesis, gene expression remained nearly consistent in terms of copy number from inoculated leaf to proliferated

leaf, this can be amenable with the report by Millenaar and Lambers (2003) where the excess production of reactive oxygen species free radicals was controlled by *ao* by continuing the citric acid cycle and regulating mitochondrial ubiquitin pool. Whereas during indirect organogenesis, a rise in gene expression was observed from inoculated leaf to the leaf proliferation stage. This suggests that the *ao* capacity increased for carbohydrates storage as mentioned by Steingrover (1981) or a higher concentration of vacuolar solutes was required to maintain osmotic balance (Lambers et al. 1981) due to stress response induced by different doses of 2, 4-D (Ikeuchi 2013 and Tahir et al. 2011). However, the shoot formation through indirect organogenesis showed a reduction in transcript level which is supported by Lambers (1980). The curves indicated that *ao* set the defense equilibrium or threshold in plant cells, in the absence of which plants showed irregularity in growth pattern and responded abruptly to stress as noticed in indirect organogenesis under stress conditions induced by 2, 4-D (Fiorani 2005).

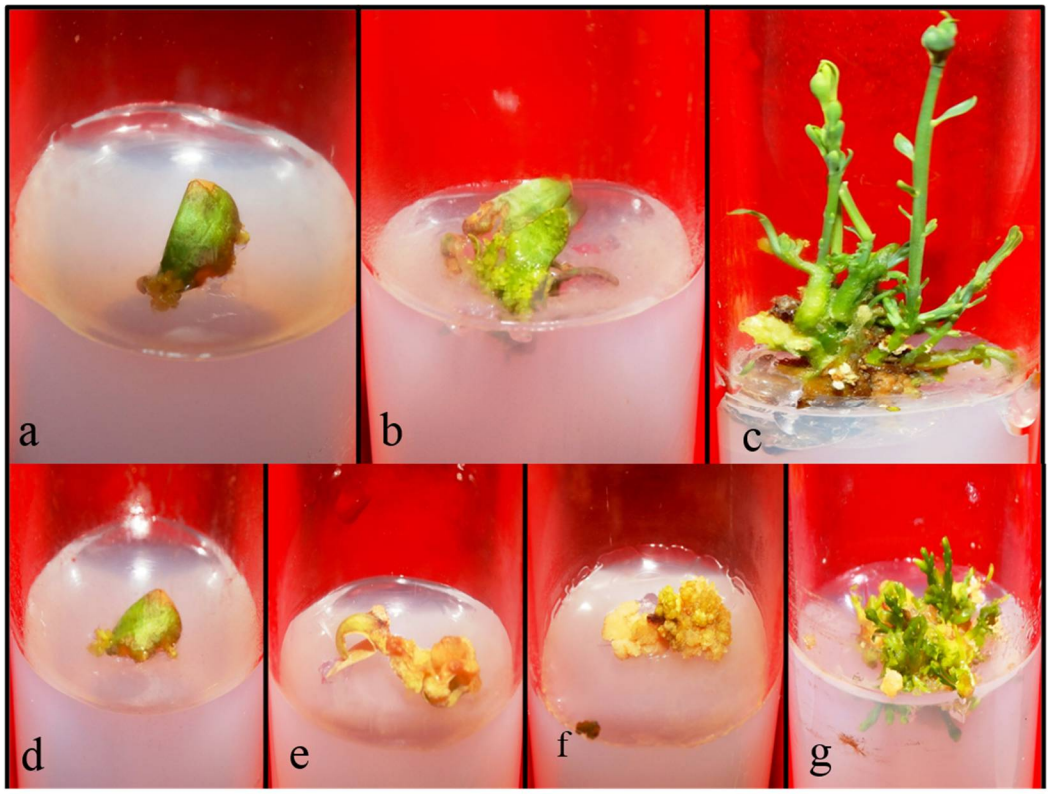

Fig. 1. Various developmental stages of direct (a, b, c) and indirect (d, e, f, g) organogenesis in sandalwood; a & d - Inoculated leaf; b & e - Proliferated leaf; c & g - Shoot formation; and f - Callus formation.

Table 2. Treatments for indirect organogenesis.

Media code	Media composition	BAP (mg/l)	2, 4-D (mg/l)	Glycin (mg/l)	ADS (mg/l)	KNO_3 (mg/l)	NAA (mg/l)
SI_1	WPM	-	-	-	-	-	-
SI_2	Modified WPM	0	1	-	-	-	-
SI_3	Modified WPM	0.5	1	-	-	-	-
SI_4	Modified WPM	1	1	-	-	-	-
SI_5	Modified WPM	0	2	-	-	-	-
SI_6	Modified WPM	0.5	2	-	-	-	-
SI_7	Modified WPM	1	2	-	-	-	-
SI_8	Modified WPM	0.5	2.5	-	-	-	-
SI_9	Modified WPM	0	2.5	-	-	-	-
SI_{10}	Modified WPM	1	2.5	-	-	-	-
SI_{11}	Modified WPM	0	5	-	-	-	-
SI_{12}	Modified WPM	0.5	5	-	-	-	-
SI_{13}	Modified WPM	1	5	-	-	-	-
SI_{14}	WPM	-	-	1	-	-	-
SI_{15}	WPM	-	-	1	25	-	-
SI_{16}	WPM	-	-	1	25	-	-
SI_{17}	WPM	-	2	1	-	-	-
SI_{18}	WPM	-	3	1	25	-	-
SI_{19}	WPM	-	2	-	-	630	-
SI_{20}	WPM	-	3	-	-	-	-
SI_{21}	WPM	-	0.5	-	-	-	-
SI_{22}	WPM	-	1	-	-	-	-
SI_{23}	WPM	-	1.5	-	-	-	-
SI_{24}	WPM	-	2.5	-	-	-	-
SI_{25}	WPM	-	2	-	-	-	-
SI_{26}	WPM	-	3.5	-	-	-	-
SI_{27}	WPM	-	4	-	-	-	-
SI_{28}	WPM	-	4.5	-	-	-	-
SI_{29}	WPM	-	5.0	-	-	-	-
SI_{30}	WPM	-	3	-	-	-	-
SI_{31}	WPM	0	0.5	-	-	-	-
SI_{32}	WPM	1	0	-	-	-	-
SI_{33}	WPM	1	0.5	-	-	-	-
SI_{34}	WPM	2	0	-	-	-	-
SI_{35}	WPM	2	0.5	-	-	-	-
SI_{36}	WPM	4	0	-	-	-	0.4

All treatments have been provided 3% sucrose with 0.9% agar

Table 3. List of genes in development of callus and shoot (from Arabidopsis database) used for the gene expression profiling in micropropagation of *S. album.*

Sr. No.	Gene	Primer name	Sequence (5' - 3')	*In silico* product length (bp)	Stage	Absolute quantity (copy no./µg of total RNA)	Relative quantity
1	Alternative oxidase (*ao*)	SW1 F	TGCCTGCACCG GCTATTG	90	D1	15276.1	1.0
					D2	14721.9	0.9
					D3	41355.4	2.7
		SW1 R	CTTCATCAGCAC GGACCACC		ID1	10470.2	1.0
					ID2	16953.3	1.6
					ID3	16082.7	1.5
2	LEA family protein (*lea*)	SW2 F	GAGAAGGGAAG CGAAGTGGG	145	D1	1067.8	1.0
					D2	5635.8	5.2
					D3	79284.9	74.2
		SW2 R	ACTCCGAAGCA AACTGAGCA		ID1	1994.9	1.0
					ID2	3952.5	1.9
					ID3	951.8	0.4
3	Cytochrome P-450 (*cytp450*)	SW3 F	AAGAGTCGGCT TACGAGCTG	110	D1	39.6	1.0
					D2	41.8	1.0
					D3	81.1	2.0
		SW3 R	CTTGGGGCTGA AGAGATGGG		ID1	73.9	1.0
					ID2	26.9	0.3
					ID3	34.5	0.4
4	ATP Binding Cassette Transporter proteins (*abct*)	SW4 F	GGATGAGCCAA CTTCAGGCT	82	D1	407.7	1.0
					D2	327.2	0.8
					D3	2348.2	5.7
		SW4 R	TGCTAGGCTGGT GTATGGTG		ID1	304.1	1.0
					ID2	674.1	2.2
					ID3	337.0	1.1
5	Serine Threonine Phosphatase (*stp*)	SW6 F	CACACCGTGGTT GATGGCT	150	D1	15749091	1.0
					D2	14435999	0.9
					D3	5599633	0.3
		SW6 R	TGAACGTGACC GGCAAAAAC		ID1	13914516	1.0
					ID2	15238483	1.0
					ID3	ND	ND

ND – not detected

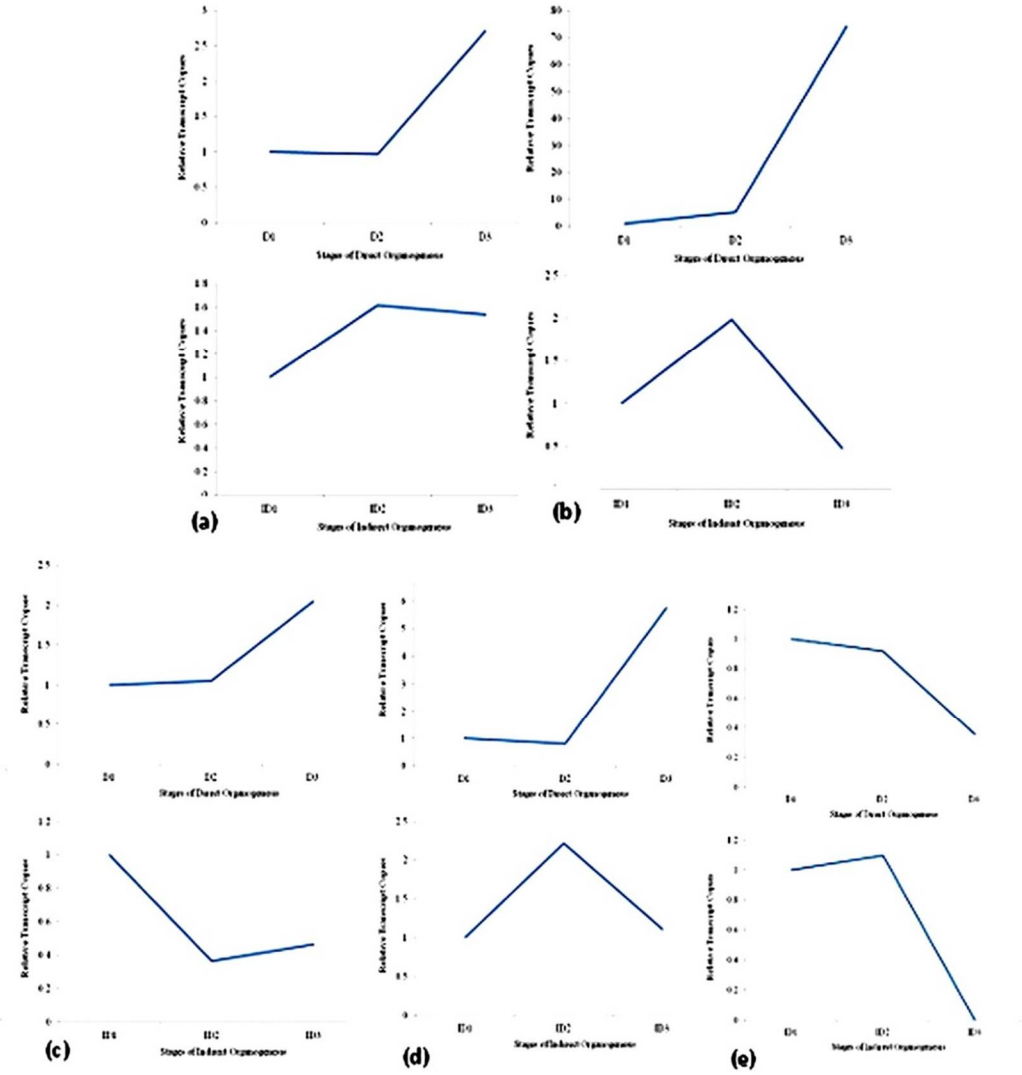

Fig. 2. Relative gene expression pattern of *ao* (a); *lea* (b); *cyt-p450* (c) *abct* (d); and *spt* (e) within three stages of direct and indirect organogenesis. D1 - inoculated leaf, D2 - proliferated leaf and D3 - shoot formation stage, ID1 - inoculated leaf, ID2 - proliferated leaf and ID3 - callus formation.

The expression of the *lea* gene had increased five folds in proliferated stage and sudden up-regulation (74.2-fold) was observed at the shoot formation stage during direct organogenesis. In indirect organogenesis, gene expression increased two-fold in the leaf proliferation stage followed by a sharp decline at the callus formation stage (Fig. 2b) indicating plants' adaptation stage to changing environments. Similar to *ao*, the expression of *cyt-p450* increased two-folds at the shoot formation stage in direct organogenesis showing auxin biosynthesis by *cyt-p450* family proteins which ultimately

affected the growth and development of the plant as explained by Bak and Feyereisen (2001) and Vadassery et al. (2008) in Arabidopsis plant. A continuous down-regulation was observed indirect organogenesis (Fig. 2c). The expression of *cyt-p450* and *ao* pathways varied reciprocally during indirect organogenesis as suggested by Bahr and Bonner (1973) and Theologis and Laties (1978).

Gene *abct* expression downregulated during direct organogenesis from inoculated leaf to leaf proliferation and a six-fold up-regulation was observed at the shoot formation stage. This indicated that as the plant attained maturity, the transcript level had risen. However, in callus-mediated organogenesis two-fold increase in the leaf proliferation stage is followed by downregulation in the next stages (Fig. 2d). A similar result was obtained by Kang et al. (2011) who showed that plants undergo detoxification processes that are necessary for organ growth, plant nutrition, and plant development. An essential gene for controlling the cell cycle, *stp* had a constant downregulation during both direct and indirect pathways (Fig. 2e). Mumby and Walter (1993) explained the downregulation of *stp* due to okadaic acid activation which dephosphorylates and inactivates serine/threonine protein. Whilst, during indirect organogenesis, the transcript copy number sharply delineated to zero at the callusing stage indicating the role of protein serine/threonine phosphatase in the re-entry of quiescent cells into the cell cycle. This re-entry suggested the dedifferentiated phase of callus that may trigger the cell for redifferentiation resulting in an increase in the mRNA level initially which further did not show any expression pattern as described by Villafranca (1996). The available information on the specific functions of different forms of protein *stp* is still severely limited as mentioned by Mumby and Walter (1993).

In conclusion, gene expression could be used as a gene marker to predict the stage identification of sandalwood. To widen the developmental understanding at the molecular level, knowledge of quantitative traits controlled by genes and their impact on environmental, physiological, and developmental factors is required.

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