Physiologia Plantarum
An International Journal for Plant Biology

Latex-less opium poppy: cause for less latex and reduced peduncle strength

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Received 30 April 2013;
revised 28 May 2013

doi:10.1111/ppl.12086

A genotype 'Sujata' developed earlier at CSIR-CIMAP from its parent 'Sampada' is considered to be the latex-less variety of *Papaver somniferum*. These two genotypes are contrasting in terms of latex and stem strength. Earlier we have carried out microarray analysis to identify differentially expressing genes from the capsules of the two genotypes. In this study, the peduncles of the two genotypes were compared for the anatomy revealing less number of laticifers in the cortex and vascular bundles. One of the important cell wall-related genes (for *laccase*) from the microarray analysis showing significantly higher expression in 'Sampada' capsule was taken up for further characterization in the peduncle here. It was functionally characterized through transient overexpression and RNAi suppression in 'Sujata' and 'Sampada'. The increase in acid insoluble lignin and total lignin in overexpressed tissue of 'Sujata', and comparable decrease in suppressed tissue of 'Sampada', along with corresponding increase and decrease in the transcript abundance of *laccase* confirm the involvement of *laccase* in lignin biosynthesis. Negligible transcript in phloem compared to the xylem tissue localized its expression in xylem tissue. This demonstrates the involvement of *P. somniferum laccase* in lignin biosynthesis of xylem, providing strength to the peduncle/stem and preventing lodging.

Introduction

Opium poppy (*Papaver somniferum*) has long been one of the world's most important medicinal plant because of its unique ability to synthesize the drugs morphine, codeine and thebaine in addition to a variety of other biologically active cyclopentanophenanthrene and benzylisoquinoline alkaloids. The medicinal significance of morphine and codeine makes their biosynthetic pathway an important biotechnological target (Higashi et al. 2010, Higashi et al. 2011). The importance of these pharmacologically significant alkaloids along with the opium-linked evils is well known. The plant *P. somniferum* has articulated laticifers (Wittler and Mauseth 1984) and it is easy lancing the capsule to obtain the latex for narcotic drug, compared to the chemical processing of the straw and the capsule, not oozing latex. Considerable work has been carried out on the identification and morphological characterization of laticifers in numerous plant species. But, the mechanism by which laticifers are generated through invasion is unknown and needs additional investigation (Hagel et al. 2008). These structures expand along their lateral walls, elongate intrusively

Abbreviations – TS, transverse section; RQ, relative quantity; ASL, acid soluble lignin; AIL, acid insoluble lignin; TL, total lignin.

by tip extension, between meristematic cells and move into newly formed organs through branching (Lev-Yadun 2001, Evert 2006). Although the events of cell wall degradation and damage during the laticifer development are debatable (Da Cunha et al. 1998, Lee and Mahlberg 1999, Hagel et al. 2008), an equilibrium between disassembly and formation of new cell wall cannot be ruled out as pectinases and transcript of polygalacturonases are detected in the latex of milkweed and para rubber tree, respectively (Wilson et al. 1976, Ko et al. 2003). Invasive laticifer development has not yet been reported in opium poppy (Hagel et al. 2008), but pectin-modifying enzymes, including pectin methylesterases, have been described (Pilatzke-Wunderlich and Nessler 2001), which are also indicated in intrusive fiber cell growth in *Populus spp*. (Siedlecka et al. 2008) and *Arabidopsis* pollen tube growth (Jiang et al. 2005). Hence, participation of similar enzymes is being predicted by Hagel et al. (2008) in the laticifer formation in opium poppy. In mutation breeding program at CSIR-CIMAP, Lucknow (India), a latex-less and low alkaloid cultivar ('Sujata') of *P. somniferum* was developed from the latex and alkaloid-rich opium poppy parent cultivar ('Sampada') (Sharma et al. 1999a, Sharma et al. 2004). No latex or near zero latex oozes out when the plant 'Sujata' is lanced. This plant is considered to be having the potential for commercial cultivation, specifically for seed with less worry for lancing and opium extraction. But the peduncle/stem of the plant 'Sujata' is weak compared to its parent 'Sampada' and prone to lodging during high wind. Hence, this generated our interest to investigate the mechanism underlying the relationship between less latex character and succulent nature of the stem. Beside this, the two genotypes were from same lineage (the genesis of the variety has been described in Fig. 1, Sharma et al. 1999a), with similar external appearance but contrasting characters. In this article, we report differential anatomy of the peduncle/stem for less latex and the functional role of *laccase* causing weak stem in 'Sujata' compared to 'Sampada'.

Materials and methods

Plant material

The genotypes 'Sampada' and 'Sujata' seeds were obtained from the National Gene Bank for Medicinal and Aromatic Plants at CSIR-CIMAP, Lucknow and grown and maintained under greenhouse conditions (60–75% relative humidity and $25 \pm 2^{\circ}C$ temperature). Thin transverse sections of peduncles and stems from 90 days old plants of both the genotypes were observed under microscope for the latex vessels. Peduncle tissue was also taken up for quantitative RT-PCR analysis. Because it was difficult to infiltrate the whole stem along with the plant, infiltration assay was standardized in the leaf tissue of 1-month-old seedlings. Two lower leaves of each plant were infiltrated for either suppression or overexpression. After incubation the leaves from each plant were sampled separately constituting one replicate. Likewise at least three plants constituted one treatment.

Microarray data resource used

The comparative microarray analysis of early stage (75 days) capsules of 'Sujata' and 'Sampada' has been reported in our earlier study (Chaturvedi et al. personal communication). The candidate taken up for functional characterization in this study has been derived from the earlier experiment described under series accession number GSE37983 [associated sample data GSM931298 (Sujata) and GSM931299 (Sampada)] conducted on Platform GPL14625 [GEO (NCBI)].

Quantitative RT-PCR

Peduncle RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA). The expression levels of *laccase* at various conditions were measured by real-time PCR with SYBR green I chemistry (Applied Biosystems, Foster City, CA) following the protocol described by Misra et al (2012). The primer sequences are provided in Supporting Information, Table S1. Threshold cycle (Ct) values obtained after Real-Time PCR were used for calculation of ΔCt value (target-endogenous control). The relative quantification was carried out by calculation of $\Delta\Delta$Ct to determine the fold difference in gene expression (ΔCt target $-$ ΔCt calibrator). Relative quantity was determined by $2^{-\Delta\Delta CT}$ (RQ).

Isolation, characterization and heterologous expression of *laccase*

The contig sequence [CL177Contig1@3 (Psom225 of the microarray)] assembled from ESTs (FE966243.1, JG634065.1, FE968600.1, FE967277.1, FE964188.1, JG630456.1, JG630757.1, FE965835.1, FE967384.1, JG631477.1, JG630926.1) was having the full coding sequence of the *P. somniferum laccase*. It was used with primers (5′ATGATCAGCATGGAACGTTTCG 3′ and 5′ TTAACACTTGGGGAGATCAGAAGGTG 3′) for full-length gene cloning in pGEM®-T Easy vector (Promega, Madison, WI) and the identity was confirmed by sequence homology match. The *laccase* sequences from 'Sampada' (JX861092) and 'Sujata' (JX861093)

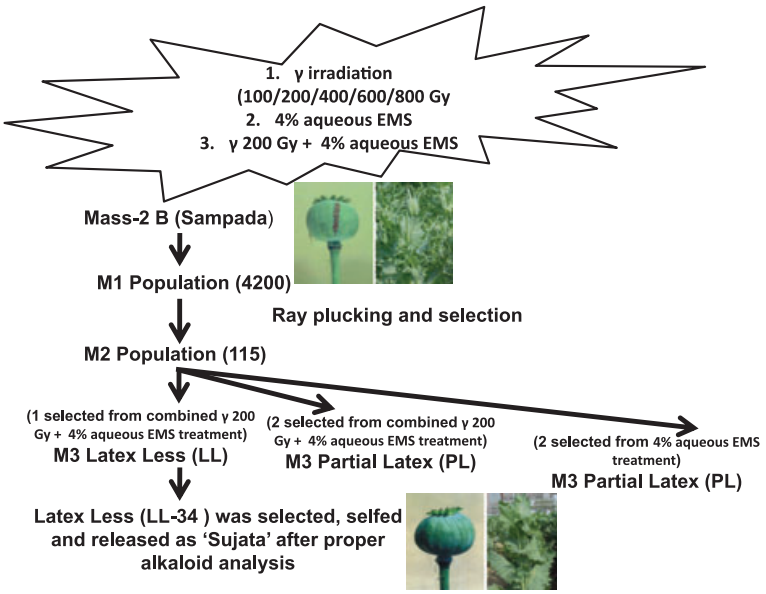

Fig. 1. Breeding history of 'Sujata' from 'Sampada' (Sharma et al. 1999a, 1999b). Sujata genotype originated from combined gamma irradiation and EMS treatment of Sampada seeds as depicted. The morphology of both the plants is similar but 'Sujata' lodges during high wind. Also shown, latex oozes out upon lancing the capsule of 'Sampada', whereas no latex oozes out in 'Sujata'.

were submitted to GenBank (NCBI) database. Cluster analysis of laccases was carried out according to Misra et al. (2012).

Analysis of laccase enzyme

The open reading frame of *laccase* was cloned in pET 28a(+) vector (Novagen, Darmstadf, Germany) in-frame with C-terminus His_6-tag and expressed in *Escherichia coli* Rosetta–Tuner (DE3) as described by Koeduka et al. (2006). Protein was extracted from exponentially growing bacteria after 2 h induction at 37°C with 1 m*M* IPTG ($A_{600} = 0.7$). Pelleted cell sample was suspended in potassium phosphate buffer (pH 6.8) and sonicated for 5 min. Then crude protein was estimated using Bradford reagent (Sigma, St. Louis, MO). Monolignol oxidation was analyzed spectrophotometrically by mixing crude protein extract (0.1 mg) with coniferyl alcohol (200 µ*M*) in 20 m*M* potassium phosphate buffer (pH 6.8) in 1 ml spectrophotometric cell at 25°C temperature. The change in absorbance of the reaction mixture was noted every 1 min at 400 nm. The enzyme activity was estimated by using an extinction coefficient of 13 090 M^{-1} cm^{-1} (Sterjiades et al. 1992).

Overexpression and transient suppression for laccase

The ORF of *laccase* from 'Sampada' was cloned under CaMV 35S promoter in pGreenII binary vector between *Bam*HI and *Sac*I restriction sites for overexpression (pGreenII-*laccase*). Similarly, 400 bp fragment from ATG codon of *laccase* was amplified in both sense and antisense orientations, and cloned sequentially on either side of the intron of pHANNIBAL (CSIRO, Australia) at *Eco*RI/*Kpn*I and *Xba*I/*Hin*dIII restriction sites, respectively. This sequence was exactly identical to the nucleotide sequence of 'Sujata' *laccase* except a single base change at position 224 (C to T). The hairpin cassette was then cloned into pART27 binary vector under CaMV 35S promoter using the *Not*I restriction sites (pART27-*ilaccase*) and transformed into GV3103 strain of *Agrobacterium tumefaciens*. Transient overexpression and RNAi suppression *in planta* were carried out by 'Syringe Infiltration'. Fresh culture of *Agrobacterium* was inoculated in 10 ml YEP broth with antibiotics (Gentamycin 40 µg ml^{-1} and kanamycin 30 µg ml^{-1}) and was grown overnight at 28°C for 18 h. The bacterial pellet obtained after centrifugation at 956 *g* (4°C, 15 min) was washed with 10 ml of infiltration buffer (50 m*M* MES pH 5.7, 0.5% (w v^{-1}) glucose, 2 m*M* Na_3PO_4, 100 µ*M* acetosyringone) and resuspended in same solution to get an OD_{600nm} of 0.3. Needleless syringe (1 ml) was loaded with the *Agrobacterium* mixture. Using the corner of a razor blade the underside of the leaves of the plant was gently nicked. The mouth of the syringe was placed on the nick. A seal was created on the other side of the leaf by placing a finger from the other gloved hand just beneath the nick. Using minimal

pressure, the *Agrobacterium* culture was injected into the leaf. This step was repeated until the whole leaf had been infiltrated and the plants were grown in 4% sucrose solution. The samples for expression analysis were collected 7 and 14 days post-infiltration.

Lignin estimation

Lignin content was estimated using the protocol as described by Mann et al. (2009). This method involves a two-step acid hydrolysis for fractionating carbohydrates and lignin into forms that are easily quantifiable.

Results

Anatomy: vascular bundles and laticifers

The networks of laticifers are described to be embedded in the cortex and phloem of vascular bundles. Interestingly, the transverse section of peduncle/stem revealed the major anatomical difference between the genotypes 'Sampada' and 'Sujata' (Fig. 2). Thick layers of laticifers were observed in the cortex and the phloem of the genotype 'Sampada'. Although the numbers of vascular bundles do not vary in the cross section of peduncles/stem of both the genotypes, the numbers of laticifers are much less in the genotype 'Sujata' (Table 1).

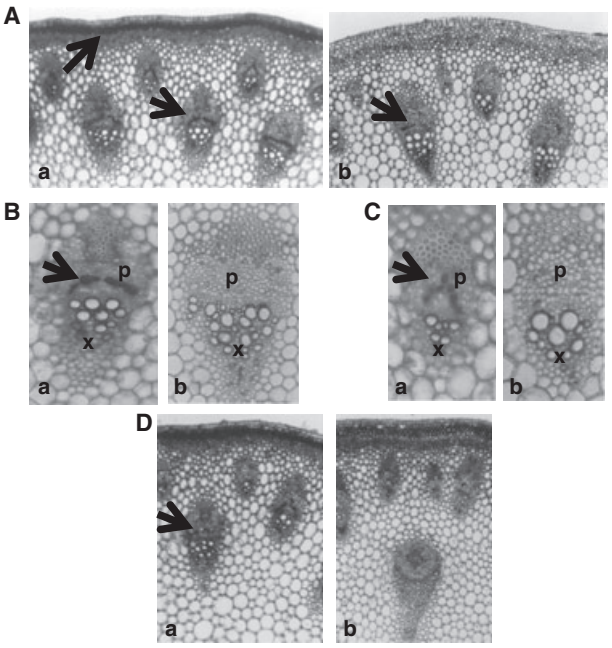

Fig. 2. Transverse section (TS) of 90-days old *Papaver somniferum* peduncle/stem in contrasting genotypes (a) 'Sampada' and (b) 'Sujata'. (A) TS of peduncles, arrows indicates laticifers in the cortex and phloem. (B) Large vascular bundle. (C) Small vascular bundle. P, Phloem; X, Xylem. (D) TS of stems showing similar differences in the anatomy.

Table 1. Anatomical differences in vascular bundles and laticifers.

	Large vascular bundles (number of laticifers per vascular bundle)	Small vascular bundles (number of laticifers per vascular bundle)
Sampada	17 ± 3 (14 ± 3)	35 ± 3 (7 ± 2)
Sujata	17 ± 3 (7 ± 2)	31 ± 6 (3 ±2)

qRT-PCR for differentially expressing *laccase* gene

Since, the anatomical differences between the genotypes showed reduced number of laticifers in 'Sujata', which is also prone to lodging, the most interesting candidate (*laccase*) from the earlier microarray analysis, belonging to the cell wall-related category with significantly less expression in 'Sujata' was analyzed further by qRT-PCR (Fig. 3).

Characterization of laccase gene

The gene '*laccase*' was taken up further for functional characterization in *P. somniferum*. The sequences isolated from both the genotypes were found to be 1728 bp long, encoding polypeptides of 575 amino acid residues each. Sequence information on laccases of 'Sampada' and 'Sujata' can be found in NCBI database under accession numbers JX861092 and JX861093, respectively. Amino acid sequences of laccases from both the genotypes were identical except eight substitutions at positions 75, 124, 159, 435, 455, 457, 459 and 501 with P-L, E-K, P-Q, T-I, L-V, F-L, A-G, E-K, respectively. The changes in amino acids do not fall in the conserved domain described earlier (Kumar et al. 2003, Pourcel et al. 2005) (Fig. 4, Appendix S1). The amino acid sequence proximity of these laccases was with BT013137.1LELAC (approximately 68%), NM_001246887.1SLLAC (approximately

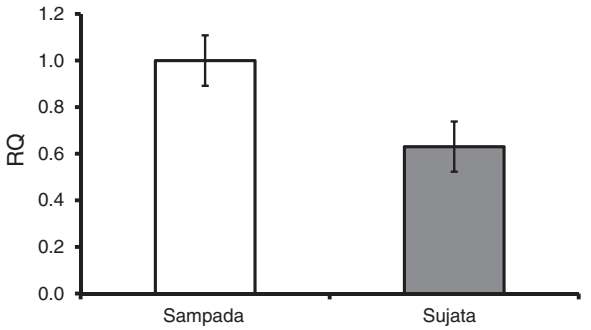

Fig. 3. Comparison of quantitative expression levels for *laccase* (Psom225) in 90 days old 'Sampada' and 'Sujata' peduncles. Data are means ± SD ($n = 3$ biological replicates) and *Y*-axis represents relative quantity (RQ).

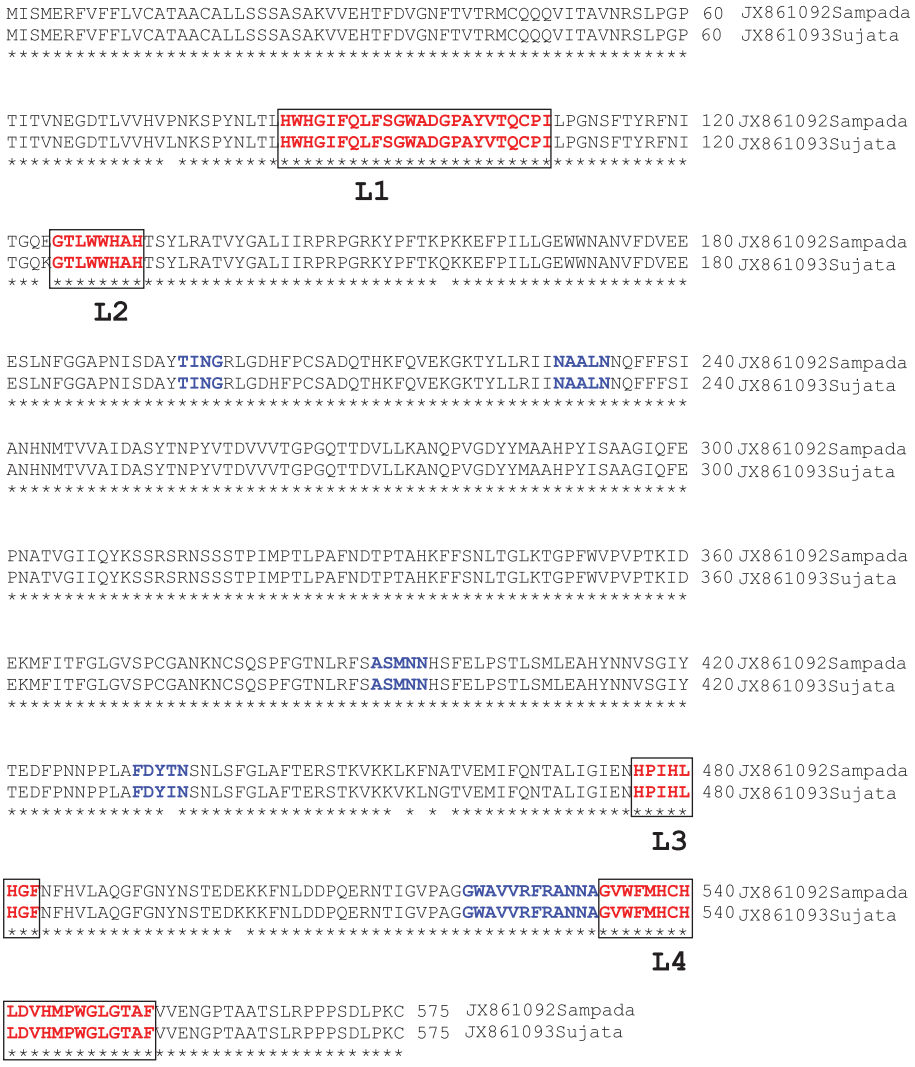

Fig. 4. Alignment of amino acids of laccases from 'Sampada' and 'Sujata'. L1, L2, L2 and L4 are conserved cupper binding domain sequences for laccases represented in boxes. Other plant specific conserved sequences are represented by blue color bold letters (as per Kumar et al. 2003, Pourcel et al. 2005). Identical amino acids are indicated with asterisks.

68%), XM_002276379.1VVLAC7 (approximately 67%), XM_002308160.1PTLAC110a (approximately 67%), and XM_002527842.1RCLAC (approximately 63%) (Fig. 5).

Enzyme assay

The protein fraction isolated from cells transformed with empty vector (control) showed similar activity due to the presence of native host protein. But a 3.1 folds higher activity was observed (Table 2) in the proteins isolated from cells expressing laccases from 'Sampada' and 'Sujata' compared to the protein of cells transformed with empty vector.

Over expression and RNA interference analysis

The overexpression construct of the gene (pGreenII-*laccase*) was transiently infiltrated and expressed in greenhouse-grown 'Sujata' plants having less expression of *laccase*, while RNAi construct (pART27-*ilaccase*) of the same was infiltrated to transiently suppress *laccase* in 'Sampada' having higher expression. After 7 days, infiltrated 'Sujata' plant showed about five folds higher *laccase* transcript due to overexpression of *laccase* compared to control (Fig. 6A(a)). Similarly, substantial reduction of transcript was observed when 'Sampada' was suppressed transiently with the RNAi construct of the laccase (Fig. 6A(b)). After 14 days of infiltration, the trend of increase and decrease in the transcript levels

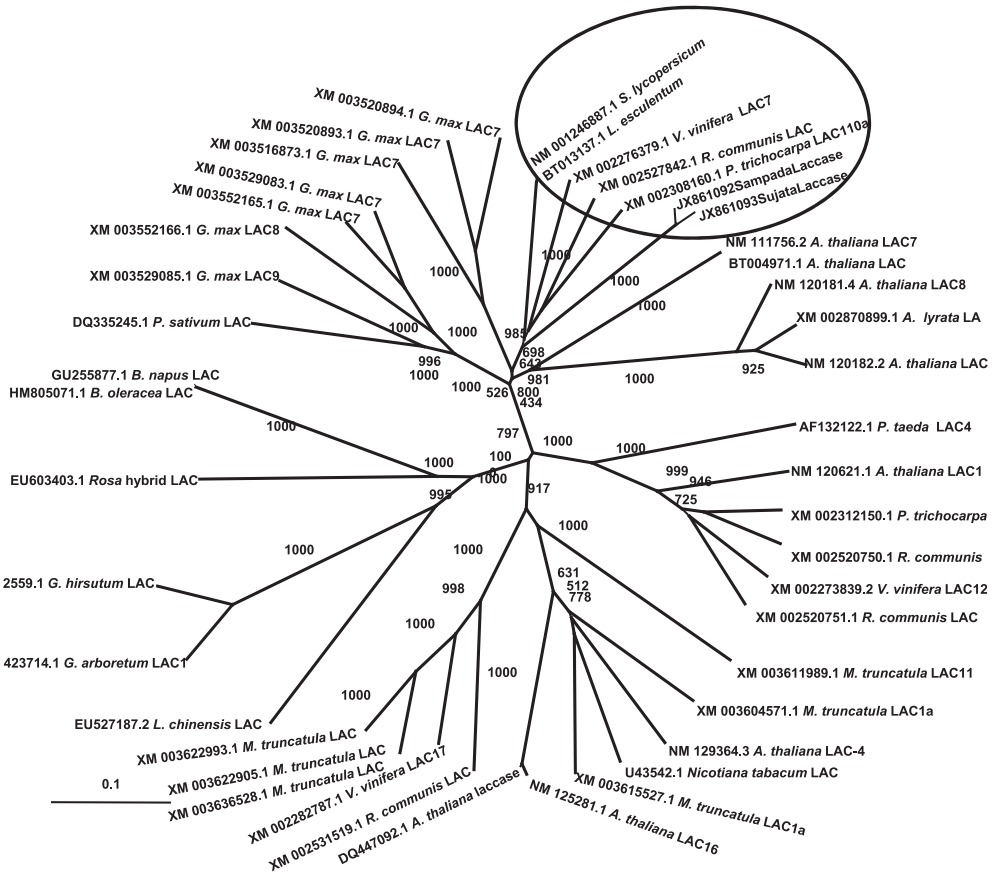

Fig. 5. Unrooted neighbor-joining tree comparing the predicted amino acid sequence of *Papaver somniferum laccase* compared to the *laccase*s of other species. GenBank accession numbers for the corresponding amino acid sequences are: BT004971.1 *A. thaliana* LAC (*Arabidopsis thaliana*), NM_111756.2 *A. thaliana* LAC7 (*A. thaliana*), NM_120182.2 *A. thaliana* LAC9 (*A. thaliana*), NM_120181.4 *A. thaliana* LAC8 (*A. thaliana*), NM_125281.1 *A. thaliana* LAC16 (*A. thaliana*), NM_129364.3 *A. thaliana* LAC-4 (*A. thaliana*), DQ447092.1 *A. thaliana* laccase/diphenol oxidase (*A. thaliana*), NM_120621.1 *A. thaliana* LAC12 (*A. thaliana*), XM_002870899.1 *A. lyrata* LAC (*A. lyrata*), GU255877.1 *B. napus* LAC (*Brassica napus*), HM805071.1 *B. oleracea* LAC (*Brassica oleracea*), XM_003552165.1 *G. max* LAC7 (*Glycine max*), XM_003529083.1 *G. max* LAC7 (*G. max*), XM_003516873.1 *G. max* LAC7 (*G. max*), XM_003520893.1 *G. max* LAC7 (*G. max*), XM_003529085.1 *G. max* LAC9 (*G. max*), XM_003552166.1 *G. max* LAC8 (*G. max*), XM_003520894.1 *G. max* LAC7 (*G. max*), AY423714.1 *G. arboretum* LAC1 (*Gossypium arboretum*), EU642559.1 *G. hirsutum* LAC (*Gossypium hirsutum*), EU527187.2 *L. chinensis* LAC (*Litchi chinensis*), BT013137.1 *L. esculentum* LAC (*Lycopersicon esculentum*), XM_003604571.1 *M. truncatula* LAC1a (*Medicago truncatula*), XM_003611989.1 *M. truncatula* LAC11 (*M. truncatula*), XM_003615527.1 *M. truncatula* LAC1a (*M. truncatula*), XM_003622905.1 *M. truncatula* LAC (*M. truncatula*), XM_003622993.1 *M. truncatula* LAC (*M. truncatula*), XM_003636528.1 *M. truncatula* LAC (*M. truncatula*), U43542.1 *N. tabacum* LAC (*Nicotiana tabacum*), Sampada Laccase JX861092 (*Papaver somniferum*), Sujata Laccase JX861093 (*P. somniferum*), AF132122.1 *P. taeda* LAC4 (*Pinus taeda*), DQ335245.1 *P. sativum* LAC (*Pisum sativum*), XM_002312150.1 *P. trichocarpa* LAC90a (*Populus trichocarpa*), XM_002308160.1 *P. trichocarpa* LAC110a (*P. trichocarpa*), XM_002520750.1 *R. communis* LAC (*Ricinus communis*), XM_002520751.1 *R. communis* LAC (*R. communis*), XM_002527842.1 *R. communis* LAC (*R. communis*), XM_002531519.1 *R. communis* LAC (*R. communis*), EU603403.1 *Rosa* hybrid LAC (*Rosa* hybrid), NM_001246887.1 *S. lycopersicum* LAC (*Solanum lycopersicum*), XM_002276379.1 *V. vinifera* LAC7 (*Vitis vinifera*), XM_002273839.2 *V. vinifera* LAC12 (*V. vinifera*), XM_002282787.1 *V. vinifera* LAC17 (*V. vinifera*).

were similar to the observation recorded after 7 days (Fig. 6B).

Lignin analysis

Seven days after infiltration with pGreenII-*laccase,* the total lignin content in 'Sujata' was enhanced, compared to vector-infiltrated plant. Same pattern was observed for acid insoluble lignin content but no significant difference was detected for acid soluble lignin (Fig. 7A). Similarly, 'Sampada' plants infiltrated with vector pART27-*ilaccase,* showed decreased total lignin as well as acid insoluble lignin but not acid soluble lignin content. Similar trend was detected 14 days after infiltration (Fig. 7B).

Table 2. Specific activity of *Escherichia coli*-expressed laccase recombinant protein. *Specific activities were mean ± SD ($n = 3$ independent assays).

Sample	Specific activity,* nmol min^{-1} mg^{-1} protein	
pET 28a(+)	pET28a(+)-*laccase* Sampada	pET 28a(+)-*laccase* Sujata
1.20 ± 0.08	3.72 ± 0.21	3.74 ± 0.18

Discussion

Weak stem of latex-less genotype – function of *laccase* and the mechanism involved

As the two genotypes 'Sampada' and 'Sujata' are of same lineage and contrasting in latex and stem strength characters, the anatomy of the peduncle was initially analyzed for vascular bundles. The availability of contrasting genotypes from the same lineage made the task simpler for functional characterization. The comparison of the anatomy of 'Sujata' and 'Sampada' peduncle and stem, implicated lower laticifer number in the former genotype (Fig. 2, Table 1). Besides, a dense layer of laticifers was observed in the cortex of 'Sampada'.

Since *laccase* expression was significantly lower in 'Sujata' having less laticifers, and its probable role was perceived to be related to the differences in the stem characters of the two contrasting genotypes, it was taken up for further characterization. Thus its selection for functional characterization was based on a combination of factors including cues from literature and the nature of genotypes being handled. Evidences on functions for various *laccase*s are available in the form of expression analysis and biochemical characterizations of members of *laccase* multigene family. Unfortunately, the sequence enzyme link has been established only for a few *laccase*s so far. Fungal laccases are by far the most extensively studied group and have been shown to be associated with a large number of physiological processes including morphogenesis, pathogenicity and lignin degradation (Thurston 1994, Hoegger et al. 2006). In plants, the role of laccases has never been demonstrated clearly, although there are several indications suggesting their involvement in lignin biosynthesis (O'Malley et al. 1993, Ranocha et al. 2002, Dwivedi et al. 2011). This is based on their capacity to oxidize lignin precursors (*p*-hydroxycinnamyl alcohols) and their localization in lignifying xylem cell walls (Davin et al. 1992, Driouich et al. 1992, Sterjiades et al. 1992, Bao et al. 1993, McDougall and Morrison 1996). Owing to contrasting functions of *laccase*s, including lignin biosynthesis (in plants) and lignin degradation (in fungi and bacteria), this group of enzymes are interesting to study from the point of view of their structure, function and application (Dwivedi et al. 2011).

In opium poppy, we could find 11 ESTs representing one contig showing similarity to *A. thaliana laccase* 7. Furthermore, sequence alignment and cluster analysis

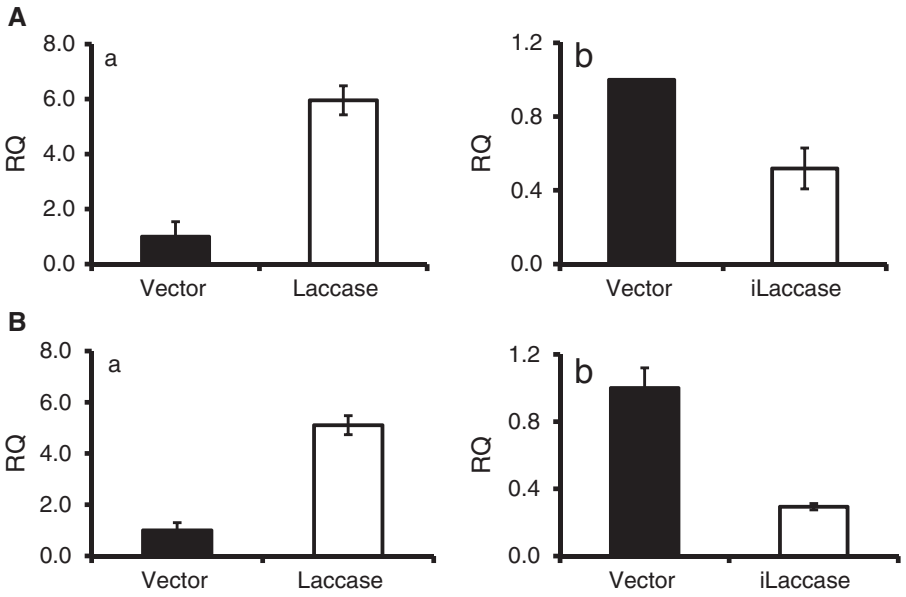

Fig. 6. Expression pattern of *laccase* transcript in the leaf, 7 (A) and 14 (B) days post-infiltration. (a) 'Sujata' infiltrated with pGreenII-*laccase* (overexpression construct); (b) 'Sampada' infiltrated with pART27-*ilaccase* (RNAi) construct compared to the vector infiltrated plants. Data are means ± SD ($n = 3$ biological replicates) and Y-axis represents relative quantity (RQ).

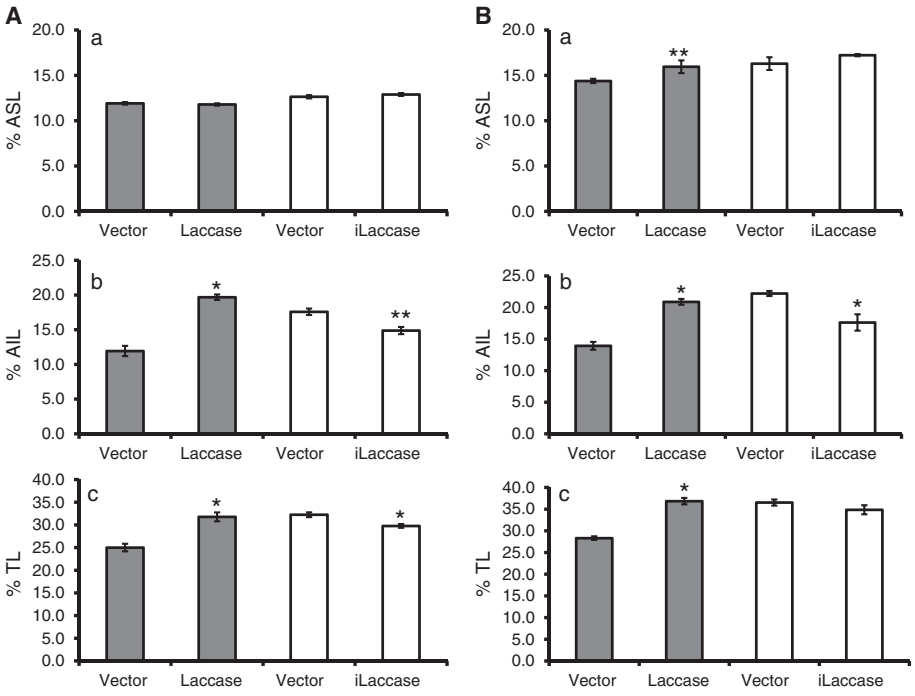

Fig. 7. Lignin content of transiently overexpressed / suppressed plants, 7 (A) and 14 (B) days post-infiltration. (a) Acid soluble lignin (ASL). (b) Acid insoluble lignin (AIL) and (c) Total lignin (TL). Shaded bars represent 'Sujata' infiltrated with pGreenII-*laccase* and vector, whereas unshaded bars represent 'Sampada' infiltrated with pART27-*ilaccase*. *Y*-axis represents lignin percentage (w/w). Data are means ± SE ($n = 4$ biological replicates for 7 days experiment and 3 biological replicates for 14 days experiment). $*P < 0.01$ and $**P < 0.05$ by Student's *t*-test of infiltrated samples to control.

revealed similarity to *laccase*s of *Lycopersicon esculentum*, *Vitis vinifera*, *Ricinus communis* and *Populus trichocarpa* (Fig. 5). This gene showed higher expression in the peduncle of 'Sampada' (32–36% lignin) compared to 'Sujata' (25–28% lignin). In an attempt to study the function, the effects of overexpression and suppression in 'Sujata' and 'Sampada', respectively were analyzed with the assumption that overexpression of *laccase* in 'Sujata' and suppression of the same in 'Sampada' may have some effects on total lignin content. Overexpression in 'Sujata' was chosen to complement low native laccase expression. With the same logic, suppression in 'Sampada' was undertaken to observe a reduction in expression. In both cases, comparisons were made with vector infiltrated samples.

Lignin is predominantly synthesized and deposited in the secondary cell walls of certain specialized cells such as xylem vessels, tracheary elements, phloem fibers and periderm (Lewis et al. 1999, Rogers and Campbell 2004). To gain more insight into the function of *laccases* in plants, especially their role in lignification, Wang et al (2008) produced transgenic poplar plants overexpressing a cotton *laccase* cDNA (GaLAC1) under the control of the cauliflower mosaic virus 35S promoter and observed a 2.1–13.2 fold increased laccase activity with a 2.1–19.6% increase in total lignin content, though plant growth rate and morphological characters remained similar to control plants. The genetic transformation of opium poppy has been reported, but remains a relatively difficult and inefficient procedure primarily because of recalcitrant regeneration (Desgagne-Penix et al. 2010). To overcome this, transient methods like infiltration have facilitated the study of gene function through overexpression and suppression. Since, injuring the stem kills one month old plants, which need to survive for more than

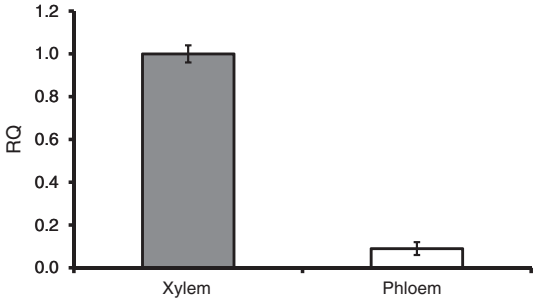

Fig. 8. Expression of *laccase* in phloem enriched tissue compared to xylem enriched tissue. Data are means ± SD ($n = 3$ biological replicates) and *Y*-axis represents relative quantity (RQ).

7 days for subsequent analysis, the growing leaf was preferred in the live seedlings for infiltration. Quantitative expression analysis of infiltrated plants confirmed higher transcript abundance in 'Sujata' (Fig. 6A(a)) while *laccase* expression in 'Sampada' decreased (Fig. 6A(b)) 7/14 days post infiltration. No change in acid soluble lignin in both transiently overexpressed and suppressed plants indicates synthesis of normal level of phenolics compared to the vector infiltrated samples. Interestingly, in 'Sujata' percentage of acid insoluble lignin content along with total lignin content were found to be increased significantly with overexpression construct, while there was a comparable decrease in the content of both when 'Sampada' plants were infiltrated with the RNAi construct. This result indicates facilitation of monomer incorporation for lignin biosynthesis and was confirmed by the utilization of coniferyl alcohol in vitro by the enzyme, implicating the involvement of laccase in lignin polymerization. Since, this is the only *laccase* so far detected from the EST database showing differential expression in the two contrasting genotypes under study in the array-based profiling, the full length sequences isolated from both 'Sujata' and 'Sampada' were compared. A total of eight substitutions were observed at the positions 75, 124, 159, 435, 455, 457, 459 and 501 with P-L, E-K, P-Q, T-I, L-V, F-L, A-G, E-K amino acid replacement in 'Sujata', respectively. These differences do not fall in the conserved region (Fig. 4) and might be due to the result of exposure to heavy dose of mutagen, during the development of variety. Hence, both the *laccase* sequences were assumed to be similar as the enzyme activities of both the laccases are comparable. This indicates that the decrease and increase in lignin in 'Sampada' and 'Sujata' may be due to the decrease or increase in the level of expression of *laccase* mRNA in transiently suppressed or overexpressed leaves with the possible control by the regulatory factors.

Are laticifer development and lignification -dependent stem strength linked?

Initially, laccase was considered to be involved in influencing the cellular anatomy of laticifers because of lower expression in 'Sujata' having less number of laticifers. However, the quantitative expression analysis using xylem tissue rich RNA and RNA isolated from phloem rich region of plant of genotype 'Sampada' indicated negligible expression in phloem compared to the xylem-rich part (Fig. 8). Xylem is generally considered as the tissue of strength for the plant and any decrease in lignin in xylem will weaken the stem. There seems to be no direct evidence for involvement of laccase in laticifer development, which also indicates that the latex-less character due to stunted laticifer is independent of the strength of stem/peduncle. Thus the functional characterization of *P. somniferum laccase* explains the weak peduncle/stem character and the phenomena of lodging in the genotype 'Sujata'.

Acknowledgements – The work was carried out under the Council of Scientific and Industrial Research (CSIR)-funded EFYP Network Project (NWP08) and TFYP (BSC0203). NC was supported by CSIR (SRF). The authors express their sincere gratitude to the National Gene Bank for Medicinal and Aromatic Plants, CSIR-CIMAP for providing the plant material and the Director, CSIR-CIMAP for keen interest and for providing facilities for the experiments.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers used in this investigation.

Appendix S1. Alignment of aminoacids from laccases.

Edited by E. Scarpella