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Isolation and characterisation of a family of laccases in maize☆

David Caparrós-Ruiz, Silvia Fornalé, Laura Civardi, Pere Puigdomènech, Joan Rigau *

Laboratori de Genètica Molecular Vegetal, Consorci CSIC-IRTA, Jordi Girona 18-26, 08034 Barcelona, Spain

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Abstract

Plant laccases are enzymes that have been proposed to participate in the last step of lignin biosynthesis. The polymerisation event remains still much unknown, implicating other enzymes such as peroxidases. To gain more insight in how this polymerisation process takes place in maize, we isolated by differential screening of an elongation maize root cDNA library four cDNA clones encoding a family of laccases. Three of them (ZmLac2, ZmLac4, ZmLac5) were basic enzymes, while one of them (ZmLac3) was an acidic enzyme. Southern analysis indicates that laccases belong to a multigene family in maize. Phylogenetic analysis reveals that ZmLac2, ZmLac4, and ZmLac5 are closely related enzymes, whereas ZmLac3 is a slightly different enzyme. The pattern of mRNA accumulation of *ZmLac2*, *ZmLac3*, *ZmLac4*, and *ZmLac5* genes correlates with the maize regions undergoing lignification. Moreover, *ZmLac3* is induced by wounding, whereas *ZmLac2* and *ZmLac5* are repressed and *ZmLac4* gene expression is not affected.

Taken together, our results suggest that the acidic ZmLac3 enzyme could be involved in the polymerisation of phenolic compounds in maize. Instead, and in agreement with the idea that laccases are enzymes involved in a wide range of physiological processes, results obtained with ZmLac2, ZmLac4, and ZmLac5 lead us to exclude a direct role of these laccases in lignin polymerisation.

Keywords: *Zea mays*; Laccase; Lignification; Polymerisation; Cell wall formation; Secondary cell wall

1. Introduction

Laccases (EC 1.10.3.2) are copper-containing enzymes that have been studied in several organisms and have been proposed to participate in several physiological processes. At present, the most studied ones are the fungi laccases [1]. In that context, production of transgenic maize and tobacco plants over-expressing fungal laccases showed that these enzymes can be used as an alternative, and more efficient way in several industrial application, such as lignin degradation [2], soil detoxification [3] or polymerisation applications [4]. However, in contrast to the great knowledge acquired with fungi, much less is known on plants laccases. Indeed, as it happens with other enzymes involved in the last process of lignin polymerisation, such as peroxidases [5], laccases are also encoded by multigene families in plants. At present, the isolation and characterisation of laccases has been obtained mainly from dicot species, such as *Acer pseudoplatanus* [6–9], loblolly pine [10–12], tobacco [13,14], *Arabidopsis thaliana* [15,16,17], yellow-poplar [18], poplar [19], and cotton [20]. Although much less studied, a family of laccases has also been recently characterised in relation to the lignification process in a monocot species such as ryegrass (*Lolium perenne*) [21,22]. In rice, 17 genes encoding putative laccases are found in the rice databases (http://rgp.dna.affrc.go.jp/IRGSP/) and only one laccase, named *LAC1* (AY897208) was identified in a maize root cDNA library.

Although in many cases their precise role(s) remains to be clarified, it has been proposed that plant laccases could be implicated in the polymerisation step of the lignification

Abbreviations: pfu, plate forming units; 3′UTR, 3′end untranslated region; RT-PCR, reverse transcriptase PCR; ZmLac2–5, *Zea mays* laccase 2–5

☆ Sequence designated *ZmLac2*, *ZmLac3*, *ZmLac4*, and *ZmLac5* were submitted to the EMBL Nucleotide Sequence Database (Accession numbers: AM086214, AM086215, AM086216, and AM086217, respectively).

* Corresponding author. Tel.: +34 934006125; fax: +34 932045904.
E-mail address: rigau@ibmb.csic.es (J. Rigau).

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process [23–28]. Moreover, and in addition to lignification, a ferroxidase activity has recently been attributed to a poplar laccase enzyme when over-expressed in tobacco transgenic cells [29], indicating that this type of enzyme could be involved in a wide range of physiological processes. In that context, the protein responsible for the *A. thaliana transparent testa10* (*tt10*) mutant, that is affected in seed coat pigmentation, has been characterised as a laccase enzyme (AtLAC15) [16]. The authors demonstrated that AtLAC15 is involved in the oxidative polymerisation of flavonoids and provided new data that extend the function of laccases beyond their role in the oxidative polymerisation of lignin monolignols.

At present, few transgenic plants have been generated to study the implication of plant laccases in vivo [15,25,30]. In that context, poplar transgenic plants showed that even if the down-regulation of a laccase enzyme (PtLac3) does not result in altered or reduced lignin content, the decrease of this laccase activity affects the phenolic metabolism of the plant, as well as the structure of its cell walls [30], suggesting that this type of enzymes is somehow related to the last polymerisation step of lignification in poplar.

Remarkably, in an important crop such as maize, very few is known on the involvement of laccase activities in any physiological process and the work performed with maize plants has been addressed to study fungal laccase activities [2,4]. Therefore, to gain knowledge on the possible involvement of maize laccases in secondary cell wall formation, we have isolated four maize cDNAs (named *ZmLac2*, *ZmLac3*, *ZmLac4*, and *ZmLac5*) by differential screening of a cDNA library constructed from the elongation zone of the maize root. We determined that *ZmLac2*, *ZmLac3*, *ZmLac4*, and *ZmLac5*, although with different level of mRNA accumulation, show an expression pattern similar to other genes involved in lignification in maize, such as *COMT* [31,32], *CAD* [33], *CCR* [33,34], *CCoAOMT* [35] or peroxidases [5]. As it happens for other genes related to lignification, *ZmLac3* is up-regulated by wounding, whereas *ZmLac2* and *ZmLac5* are both down-regulated. Moreover, ZmLac3 is phylogenetically close to AtLAC15, a laccase involved in the polymerisation of flavonoids [16].

Based on these results, we propose *ZmLac3* as a new acidic laccase that could be involved in polymerisation of phenolic compounds such as flavonoids and lignin in maize. In contrast, and according to the proposed multifunctional roles of these enzymes, *ZmLac2*, *ZmLac4*, and *ZmLac5* may be involved in other physiological processes not related to lignification in maize.

2. Material and methods

2.1. Plant material and growth conditions

Dry seeds of *Zea mays* W64A inbred line were germinated in a growth chamber on wet Whatmann paper at 22 °C in dark conditions for 3 days and then with a 16 h light/8 h dark photoperiod during 4 days more. Plants used for Northern blot analyses were dissected at different growth stages, frozen immediately in liquid nitrogen at stored at −80 °C.

2.2. Differential cDNA library screening

We used the cDNA library obtained by Vignols et al. [36] to perform the differential cDNA screening to isolate genes expressed only in lignifying tissues of the maize root as already described [5]. Among all the isolated clones, one cDNA corresponded to a laccase that was named *ZmLac2*. This clone was then used to further isolate the *ZmLac3*, *ZmLac4*, and *ZmLac5* cDNA clones from the same cDNA library. *ZmLac4* cDNA was isolated as a partial clone and therefore, its 5′end was obtained using the 5′RACE amplification kit (Invitrogen) according to manufacturer's instructions.

The primers used for 5′RACE amplification were:

- Primer Lac4-race1: 5-ATTGAAGTCGTAGTGCCTCGTGATGCC-3.
- Primer Lac4-race2: 5-CAGCGAGATGTTGTGCTGGGCGTGGTTGGT-3.

2.3. Genomic DNA gel blot analysis

Genomic DNA was isolated from maize (*Z. mays* W64A) according to Sambrook et al. [37] and digested with appropriate restriction enzymes, fractionated on 0.7% agarose gel (20 μg per line) and transferred onto a nylon membrane. Finally, hybridisation was performed at 65 °C in a phosphate solution using the coding sequence of the ZmLac4 cDNA clone as unspecific probe. After hybridisation, the membrane was washed twice (15 min per wash) in 3× SSC, 0.1% SDS, and then twice in 0.5× SSC, 0.1% SDS at 65 °C and subsequently exposed to Kodak X-OMAT film at −80 °C for autoradiography.

2.4. RNA gel blot analysis

Total RNA (10 μg per line) was isolated from 9-day-old maize plants, separated on 1.5% formaldehyde-agarose gel and transferred to a nylon membrane. Membranes were hybridised with fragments corresponding to the 3′ untranslated region of each cDNA (see figures). After hybridisation, the membrane was washed twice (15 min per wash) in 3× SSC, 0.1% SDS, and then twice in 0.5× SSC, 0.1% SDS at 65 °C and subsequently exposed to Kodak X-OMAT film at −80 °C for autoradiography.

2.5. Wounding assays

Wounding assays were performed by transversal incisions with a scalpel blade in roots, leaf sheath, and leaf blade of 9-day-old plantlets. After 24 h, the different wounded parts of the plant were harvested and the effect of wounding on the expression of *ZmLac2*, *ZmLac3*, *ZmLac4*, and *ZmLac5* was then analysed by semi-quantitative RT-PCR. To ensure biological reproducibility, each sample was constituted by 10 plants.

2.6. Semi-quantitative RT-PCR

Total RNA was extracted with Trizol Reagent according to manufacturer's instructions (Invitrogen). Approximately 4 μg

of total RNA were reverse-transcribed using M-MLV Reverse Transcriptase (Invitrogen). First-strand cDNA was generated using an oligo(dT)$_{15}$ primer and 2 μl of the first-strand cDNA were used as a template in subsequent PCR reactions. Gene-specific primers were used to amplify a fragment of the 3′UTR regions of *ZmLac2*, *ZmLac3*, *ZmLac4*, and *ZmLac5* mRNAs. ''no-RT'' PCR assays were performed to verify the absence of genomic DNA contamination (data not shown). Different numbers of cycles were tested to ensure that the amplification was in the exponential range and each assay was repeated at least three times. The identity of the amplified bands was further confirmed by DNA sequencing.

The amplified fragment of the 3′UTR-ZmLac2 was 294 bp long. The amplified fragment was obtained at 33 cycles with a primer annealing temperature of 49 °C. The primers used were:

- Lac2-Forward: 5′-TCTGCAGTGGTTTTGATCCG-3′.
- Lac2-Reverse: 5′-CATTAGCTGGTCCAATCGAC-3′.

The amplified fragment of the 3′UTR-ZmLac3 was 205 bp long. The amplified fragment was obtained at 34 cycles with a primer annealing temperature of 52 °C. The primers used were:

- Lac3-Forward: 5′-CGCTCGATCAAACCAGCTAAT-3′.
- Lac3-Reverse: 5′-TGAACTAGCAGTAGACCGACACAA-A-3′.

The amplified fragment of the 3′UTR-ZmLac4 was 161 bp long. The amplified fragment was obtained at 33 cycles with a primer annealing temperature of 52 °C. The primers used were:

- Lac4-Forward: 5′-TACTTTCTTCATTGCCAATTGCA-3′.
- Lac4-Reverse: 5′-GCAAGTCACGCCATTCTTTATTTT-3′.

The amplified fragment of the 3′UTR-ZmLac5 was 169 bp long. The amplified fragment was obtained at 36 cycles with a primer annealing temperature of 51 °C. The primers used were:

- Lac5-Forward: 5′-TTTATCACCCGATCGAGGG-3′.
- Lac5-Reverse: 5′-GGATTCAGCGAGGATCGC-3′.

The amplified fragment of the 3′UTR-ZmUbiquitin (U29159) was 220 bp long. The amplified fragment was obtained at 29 cycles with a primer annealing temperature of 55 °C. The primers used were:

- Fw-ZmUbiq: 5′-TAAGCTGCCGATGTGCCTGCGTCG-3′.
- Rv-ZmUbiq: 5′-CTGAAAGACAGAACATAATGAGCA-CAGGC-3′.

2.7. Sequence alignment and phylogenetic tree

The alignment of the maize laccase sequences was done using the ClustalW program (http://www.ebi.ac.uk/clustalw/) [38]. Poorly aligned positions and divergent regions were eliminated by using Gblocks 0.91b following the given options for a less stringency [39]. The phylogenetic tree was performed using the PHYML (PHYlogenies by Maximum Likelihood) online execution program (http://atgc.lirmm.fr/phyml/) [40], using the JTT model of amino acids substitution with four substitution rate categories and the estimated proportion of invariable sites.

3. Results

3.1. Isolation of four maize laccases from an elongation root cDNA library

In maize, the pattern of lignification is a feature that could be used to identify genes involved in this biological process. Therefore, with the aim of isolate genes involved in lignification we generated a maize cDNA library constructed from the elongation root region, which corresponds to the region undergoing lignification in maize roots [36]. Thus, the screening of this cDNA library led to identify genes that were shown to be abundant in this region and absent in the root tip. These clones were considered as interesting cDNA clones, systematically sequenced and characterised [5,36]. Among others, tblastn analyses revealed that four isolated cDNA clones putatively encode four new laccases in maize, and were named *ZmLac2*, *ZmLac3*, *ZmLac4*, and *ZmLac5* (Fig. 1).

The presumably full-length *ZmLac2* cDNA sequence is 2176 bp long and the predicted protein has 582 amino acids, with a theoretical protein size of 63.5 kDa. *ZmLac3* cDNA is 2294 bp long, with a predicted protein of 584 amino acids and a theoretical size of 64.6 kDa. *ZmLac4* cDNA sequence is 2183 bp long, with a predicted protein of 587 amino acids, and a theoretical size of 63.8 kDa. Finally, *ZmLac5* cDNA is 2033 bp long, with a predicted protein of 586 amino acids and a theoretical size of 64.3 kDa. Despite their similarity in size, ZmLac2, ZmLac4, and ZmLac5 are basic enzymes, being their isoelectric point of 9.62, 8.74, and 8.87, respectively. Instead, ZmLac3 is the only acidic enzyme of the family, having an isoelectric point of 5.66. In addition to these four laccases, another laccase cDNA clone was found in maize databases (LAC1; AY897208). As ZmLac3, LAC1 is an acidic laccase having an isoelectric point of 6.15 and a theoretical size of 67.2 kDa.

As typically observed for this class of enzymes, the predicted ZmLac2, ZmLac3, ZmLac4, and ZmLac5 proteins have the typical features of laccases (Fig. 1). They have (i) a putative signal peptide predicted by the ScanProsite program, (ii) four highly conserved ligands for copper, and (iii) a multicopper oxidases signature (type 1 or blue) in the C terminal region, which is associated to oxido-reductase proteins acting on diphenols and related substances as donors. A particular feature of the maize LAC1 is the presence of a Proline-rich domain as an extension of its C terminal region. This domain has already been described for other plant laccases such as the ryegrass LpLAC5-4 enzyme [21]. In addition, and according to the fact that laccases are glycoproteins, ZmLac2, ZmLac4, and ZmLac5 have 14, 15 and 15 putative *N*-glycosylation sites (NX(S/T)X (X ≠ P)), respectively, whereas ZmLac3 and LAC1 have only 7 and 5 glycosylation sites, respectively.

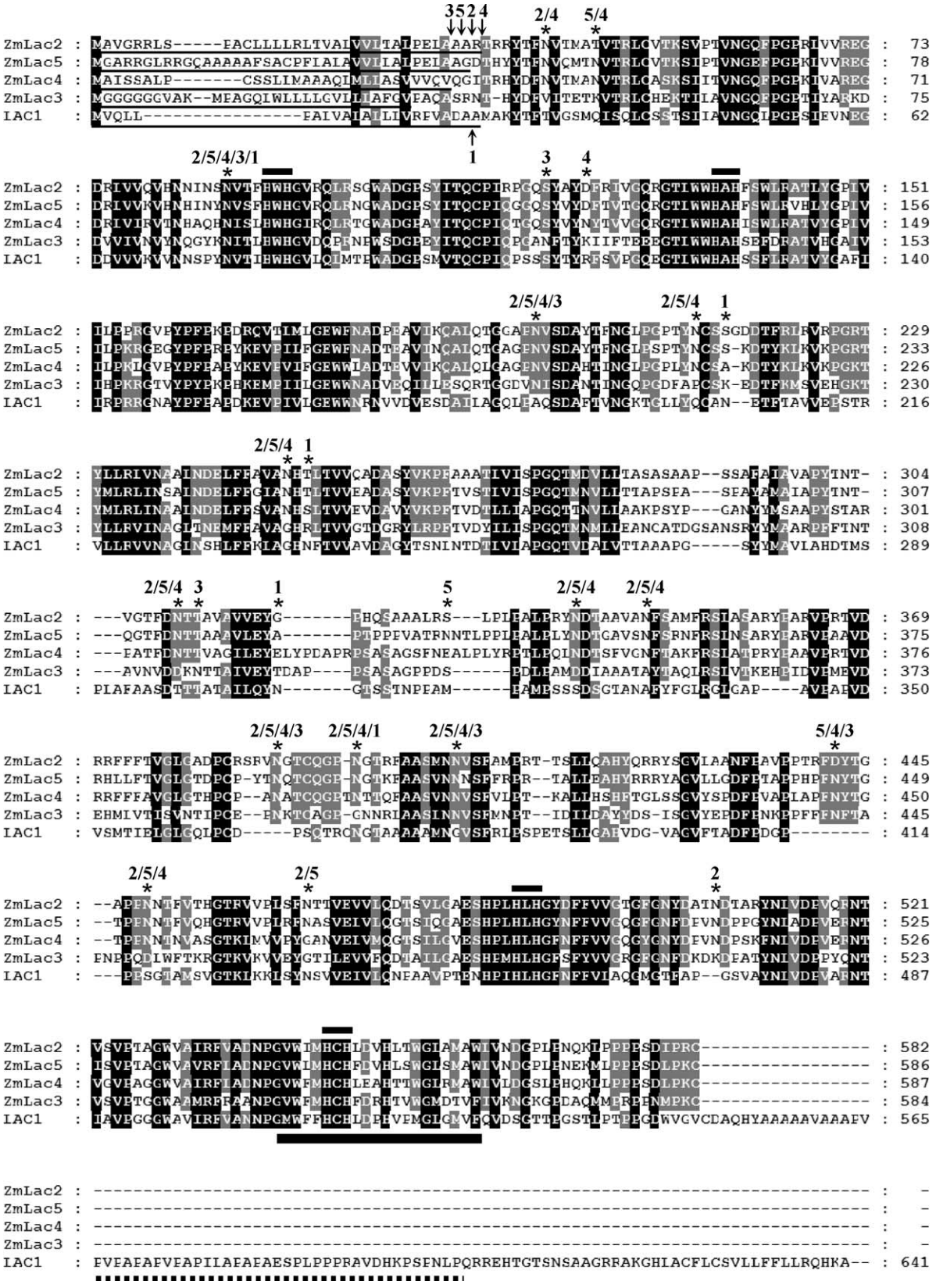

Fig. 1. Alignment of amino acid sequences of ZmLac2, ZmLac3, ZmLac4, ZmLac5, and the maize LAC1 (AY897208). Dark boxes refer to amino acid identity or similarity between the five sequences. Grey boxes refer to amino acid identity or similarity between four sequences. Arrows indicate the putative cleavage site and asterisks the putative *N*-glycosylation sites and 1, 2, 3, 4, and 5 at the top of the sequences refer to LAC1, ZmLac2, ZmLac3, ZmLac4, and ZmLac5, respectively. The N terminal peptide signal of the five proteins is underlined. Bars placed at the top of the sequences correspond to the four highly conserved ligands for copper whereas the bar placed on the bottom corresponds to the blue copper oxidase signature. Dashed line refers to the Proline-rich domain of LAC1. Finally, amino acid residue number is indicated on the right side of the figure for each protein.

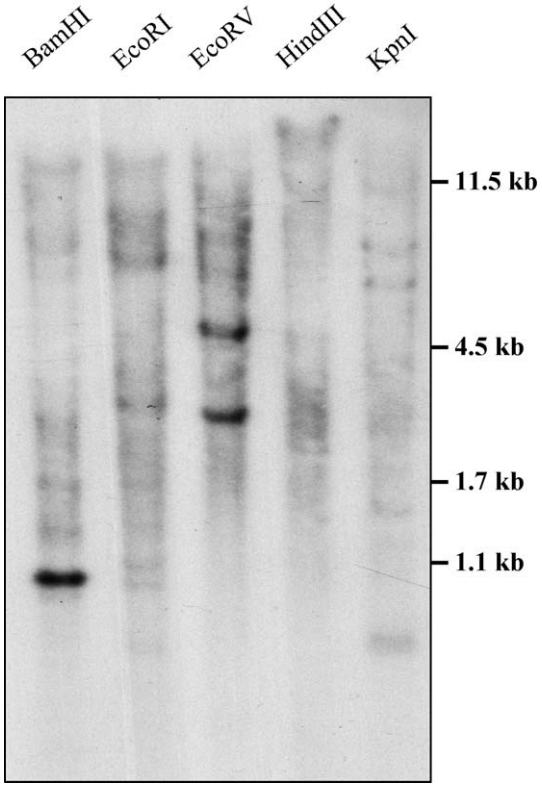

Fig. 2. Maize genomic DNA Southern blot analysis. Genomic DNA from maize inbred line W64A (20 μg per line) was digested by *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, and *Kpn*I, separated on a 0.7% agarose gel, blotted and probed with the coding sequence of the *ZmLac4* cDNA clone. Molecular weight markers are shown on the right side of the figure.

3.2. Maize laccases belong to a multigene family

As previously mentioned, laccases belong to multigene families in all species analysed so far [21]. Therefore, to obtain a rough estimation of the approximate number of genes belonging to the laccase family in maize, we performed Southern blot analyses using the coding sequence of *ZmLac4* gene as unspecific probe (Fig. 2). The appearance of multiple hybridised bands suggests that the four laccases isolated in this work plus LAC1 represents only a part of the maize laccase family, which could be constituted by more than 10 members. This is in agreement with the number of laccase genes identified in another cereal such as rice, in which this family is constituted by 17 members based on the International Rice Genome Sequencing Project (http://rgp.dna.affrc.go.jp/IRGSP/) and with *A. thaliana* whose laccase gene family is also constituted by 17 members [17].

3.3. Study of the mRNA accumulation pattern of ZmLac2, ZmLac3, ZmLac4, and ZmLac5 genes

In order to investigate whether these members of the laccase family could be involved in lignification in maize, we analysed their mRNA accumulation patterns in different regions of roots and leaves by Northern blot analysis using their 3′UTRs as a probe that showed to be specific for each of the four laccases (Fig. 3).

The mRNA accumulation pattern of *ZmLac2*, *ZmLac3*, *ZmLac4*, and *ZmLac5* was compared to the one corresponding to maize *Caffeic acid O-Methyl-Transferase* (*COMT*) gene, previously characterised as an enzyme involved in lignification [31]. In the root region, the pattern of mRNA accumulation of *ZmLac2*, *ZmLac4*, and *ZmLac5* mRNA is similar to the one observed for *ZmCOMT*. For *ZmLac3*, its mRNA accumulates at detectable levels only in roots of 9-day-old plantlets. Concerning the aerial part of maize plantlets, *ZmLac2*, *ZmLac4*, and *ZmLac5* mRNAs accumulate preferentially in the first stages of the leaves development, where lignification process is active.

3.4. Effect of wounding on the mRNA accumulation pattern of ZmLac2, ZmLac3, ZmLac4, and ZmLac5 genes

Expression of genes involved in lignification is typically induced by wounding, as they also participate actively in the

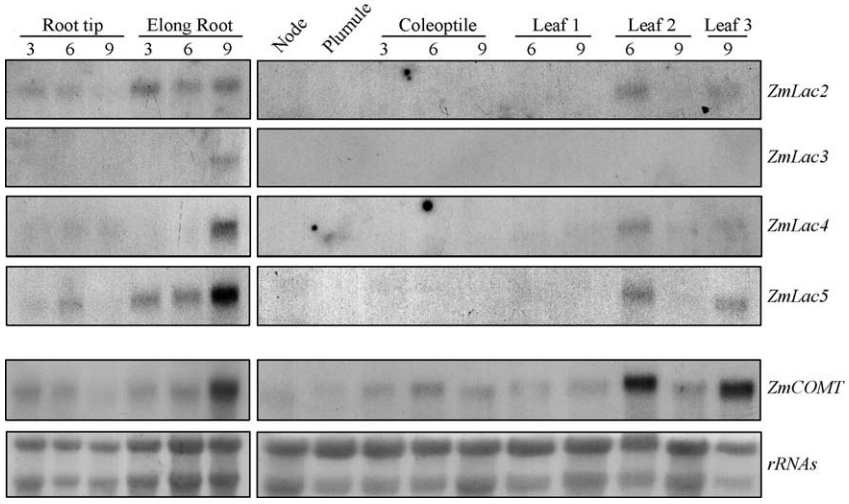

Fig. 3. RNA gel blot analysis of the *ZmLac2*, *ZmLac3*, *ZmLac4*, and *ZmLac5* genes in maize. RNA blot analysis was carried out with 10 μg of total RNA extracted from several regions of the maize plantlet at 3, 6 and 9 days after germination. The RNA extracted from these tissues was hybridized with the 3′UTR of *ZmLac2*, *ZmLac3*, *ZmLac4*, and *ZmLac5* cDNA. *COMT* gene was used as a control of a gene involved in lignification. rRNAs are shown as loading control.

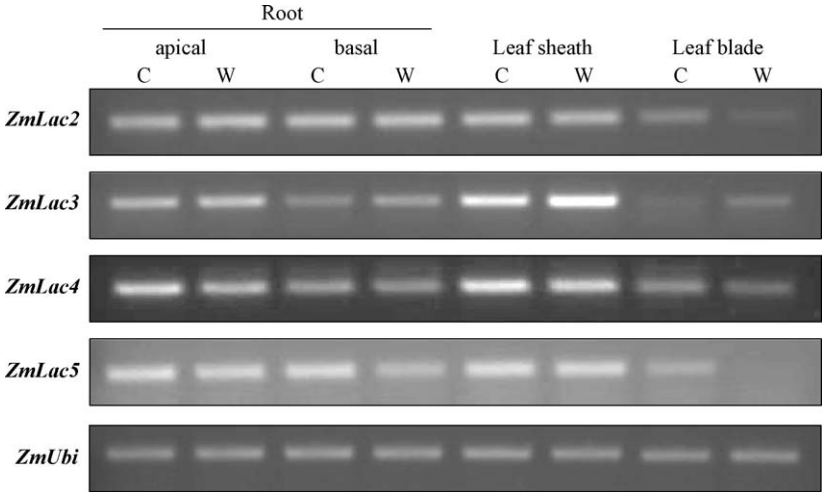

Fig. 4. Analysis of *ZmLac2*, *ZmLac3*, *ZmLac4*, and *ZmLac5* gene expression in response to wounding by semi-quantitative RT-PCR. (C) control and (W) wounded tissues. (ZmUbi) maize ubiquitin, used as a control of RT-PCR.

plant defence response [5,32]. In maize, the peroxidase ZmPox2 has been proposed as an enzyme involved in the last polymerisation step of lignification and its expression is strongly induced by wounding [5]. Therefore, to elucidate whether this family of laccases could be also involved in this polymerisation process, we analysed their mRNA accumulation patterns in different regions of maize plantlets previously submitted to wounding (Fig. 4). *ZmLac4* gene expression seems not to be regulated by wounding, contrarily to what expected for a gene implicated in lignification. On the other hand, the expression of *ZmLac2* and *ZmLac5* genes is severely reduced in leaves but not affected in roots. Instead, *ZmLac3* is the only gene whose expression level increases in wounded leaves, suggesting that it could be implicated in the polymerisation of lignin.

3.5. Phylogenetic analysis of maize laccases

Taken in consideration that ZmLac2 and ZmLac5 laccases are basic enzymes whose gene expression is repressed by wounding and that the acidic ZmLac3 laccase is induced by wounding, we performed a phylogenetic analysis to investigate whether these enzymes could be grouped based on their electrochemical properties. The family of laccases is structurally closely related to plant ascorbate oxidases [21]. The phylogenetic analysis performed in this work comprised initially the sequences of plant and fungal laccases as well as plant ascorbate oxidases. This tree grouped fungal laccases and ascorbate oxidases in two distinct clusters, evolutionary far from plant laccases (result not shown). As the maize ZmLac2, ZmLac3, ZmLac4, ZmLac5, and LAC1 were grouped within the plant laccases, we subsequently refined the phylogenetic analysis using only plant laccases (Fig. 5). Laccases are grouped within the six subgroups described in *Arabidopsis* by McCaig et al. [17]. Our results show that ZmLac3 and the maize LAC1 are clustered within the subgroups 4 and 5, respectively, which comprise the majority of acidic laccases. In addition, ZmLac3 is closely related to AtLAC15 [16,17], suggesting a possible role of this maize laccase in the polymerisation of flavonoids. In contrast, ZmLac2, ZmLac4, and ZmLac5 laccases cluster within the subgroup 1, suggesting that they could perform very closely related functions. Subgroups 1 and 2 are constituted by basic laccases, with one exception for each case and one-third of the laccases of the subgroup 3 are also acidic enzymes.

4. Discussion

This work reports the isolation and characterisation of a family of four laccases in maize obtained by differential screening of an elongation root cDNA library. Laccases has been deeply studied in fungi [1]. However, recent works undertaken with plant laccases suggest that this class of enzymes could participate in the secondary cell wall formation through the last polymerisation step of lignin and flavonoids biosynthesis [16,23–28]. The characterisation of ZmLac2, ZmLac3, ZmLac4, and ZmLac5 constitutes the first study performed with laccases in maize. These enzymes are *N*-glycoproteins of approximately 61–64 kDa in size containing the typical feature of laccases, such as a peptide signal necessary to putatively address the proteins to the cell wall, a copper-oxidase domain, and a C-terminal domain associated to oxido-reductase proteins acting on diphenols and related substances as donors.

At present, only peroxidases have been proposed to be involved in the last step of lignin polymerisation in maize [5]. This class of enzymes belongs to multigene family of more than one hundred members. In the case of maize laccases, our results also indicate that these enzymes belong to a multigene family, even if much smaller than peroxidases. Indeed, this finding is in agreement with results obtained with other plant species, such as *Arabidopsis* [15–17], poplar [19], ryegrasses [21], and rice (http://rgp.dna.affrc.go.jp/IRGSP/).

The study of *ZmLac2*, *ZmLac4*, and *ZmLac5* gene expression is in accordance with the expected mRNA accumulation pattern of genes encoding lignin-associated proteins. Their mRNA

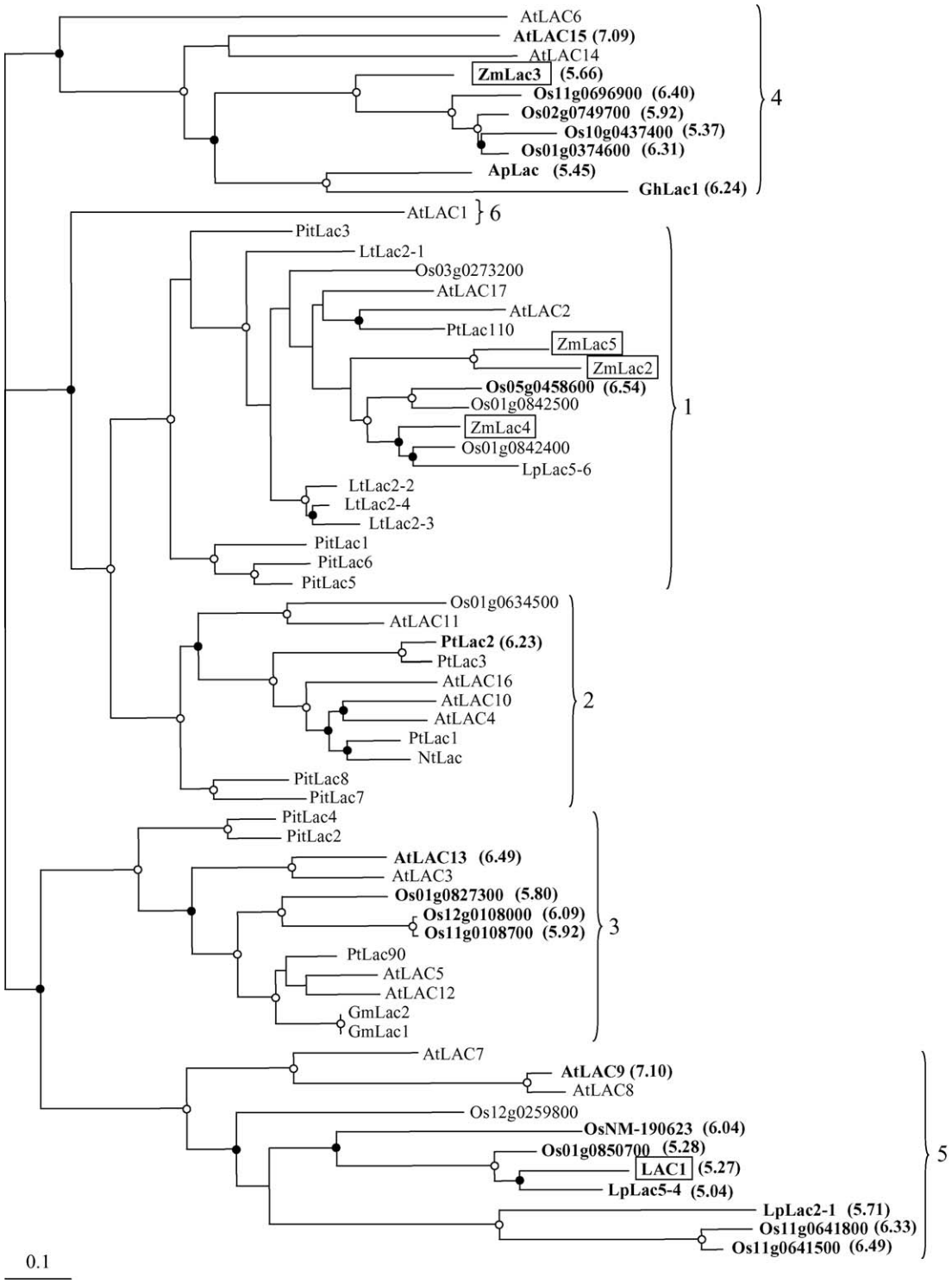

Fig. 5. Phylogenetic tree obtained by the protein sequence alignment of the four new laccases of maize and other plant laccases. White and dark dots refer to branches supported at a bootstrap proportion ≥90% and ≥50%, respectively. The scale bar represents 0.1 substitutions per position. The subgroup 4 was chosen as the outgroup as representative sequences of this subgroup were the earliest divergent sequences according to the tree containing plant ascorbate-oxidase, fungal laccases, and plant laccases. The maize laccases are boxed. The acidic laccases are shown in bold with their isoelectric point in brackets. Curly brackets refer to the six laccase subgroups based on McCaig et al. [17]. *Zea mays* LAC1 (AY897208). *Nicotiana tabacum* NtLac (U43542) [44]; *Populus trichocarpa* PtLac110, PtLac90, PtLac3, PtLac2, PtLac1 (Y13773, Y13772, Y13771, Y13770, Y13769, respectively) [19]; *Pinus taeda* PitLac1, PitLac2, PitLac3, PitLac4, PitLac5, PitLac6, PitLac7, PitLac8 (AF132119, AF132120, AF132121, AF132122, AF132123, AF132124, AF132125, AF132126, respectively) [11]; *Lolium perenne* LpLac2-1, LpLac5-4, and LpLac5-6 (AF465469, AF465470, AF465468, respectively) [21]; *Gossypium hirsutum* GhLac1 (AY423714) [20]; *Glycine max* GmLac1, GmLac2 (AF527604, AY113187, respectively); *Acer pseudoplatanus* ApLac (U12757); *Liriodendron tulipifera* LtLac2-1, LtLac2-2, LtLac2-3, LtLac2-4 (U73103, U73104, U73105, U73106, respectively); *Arabidopsis thaliana* laccases: AtLAC1-17 [16]; *Oryza sativa* laccases (Os01g0634500, Os01g0842500, Os03g0273200, Os01g0842400, Os05g0458600, Os02g0749700, Os11g0108700, Os12g0108000, Os01g0827300, Os11g0696900, Os10g0437400, Os01g0850700, Os12g0259800, Os01g0374600, Os11g0641800, Os11g0641500, OsNM_190623).

mainly accumulates in the elongation root region. This expression pattern was also observed for *ZmLac3* gene, even if its mRNA accumulation was much lower than the other three laccases.

In addition, ZmLac3 is the only acidic enzyme of the isolated family whereas the rest of the laccase family analysed in this work are basic enzymes. Interestingly, the evolutionary analysis of plant laccases revealed that the acidic ones are mainly clustered within two subgroups (4 and 5) of the phylogenetic tree opening the question of whether the functions of laccases could depend upon their electrochemical parameters.

It is well known that a variety of factors exert an effect upon the process of lignification [41–43]. This is the case of wounding that induces production of lignin in the damaged tissues by increasing the expression of several maize genes implicated in lignification, such *COMT* [32] or the *ZmPox2* peroxidase [5]. Our results suggest that the basic laccases ZmLac2, ZmLac4, and ZmLac5 are not implicated in lignification. In fact, even if their expression pattern is similar to the one expected for genes involved in lignification, these three basic enzymes were not induced by wounding (Fig. 4). Wounding did not affect *ZmLac4* gene expression and repressed *ZmLac2* and *ZmLac5* gene expression and this observation lead us to discard a role in lignification for these three maize basic laccases.

On the contrary, the acidic ZmLac3 is phylogenetically closely related to the AtLAC15 [16], suggesting that ZmLac3 could be implicated in the polymerisation of flavonoids. In addition, the expression pattern of *AtLAC15* [16,17] agrees with the one expected for a gene that could be also involved in the lignin polymerisation process. This result together with the fact that ZmLac3 is induced by wounding, leads us to suggest a possible implication of this laccase in the lignin polymerisation process, even if to a lesser extent than peroxidases. In fact, while maize peroxidases are strongly induced by wounding in the entire plant [5], the induction of *ZmLac3* was observed only in leaves, suggesting a more localised involvement in lignin polymerisation. In addition, both the basal expression level of *ZmLac3* in the plant and the level of induction by wounding of *ZmLac3* gene suggest that the involvement of this laccase in this process may be more subtle than the one observed for peroxidases.

In summary, this work reports the first characterisation of a family of laccases in maize. Our data lead us to propose the acidic ZmLac3 as a candidate enzyme involved in the polymerisation of phenolic compounds such as lignin and flavonoids. Therefore, further studies will be addressed to confirm the role of ZmLac3 in these processes in maize. On the other hand, our results indicate that the basic ZmLac2, ZmLac4, and ZmLac5 enzymes may be involved in other physiological processes, according to the wide range of functions attributed to this class of enzymes.

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