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Characterisation of maize *peroxidases* having differential patterns of mRNA accumulation in relation to lignifying tissues☆

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Abstract

Among other enzymes, peroxidases have been proposed to participate in the latest steps of lignin biosynthesis. In order to identify new proteins involved in such mechanism of lignification in maize, we have isolated three cDNAs coding for three different peroxidases, named *ZmPox1*, *ZmPox2*, and *ZmPox3*, respectively. Computational analyses of these three proteins correlate with features typically attributed to heme-containing plant peroxidases of approximately 300 amino acid residues.

Although with different expression levels, *ZmPox2* and *ZmPox3* mRNAs are accumulated in the elongating region of young roots but not in the root tips. In addition, the *ZmPox2* mRNA levels are up-regulated by wounding and ethylene treatments. However, *ZmPox1* is also expressed in the root tip meristems, where lignification does not occur.

Finally, in situ hybridisations indicate that *ZmPox2* mRNA localises in vascular tissues and epidermis. Although *ZmPox1* mRNA localises in the same regions as *ZmPox2* mRNA in root tips, its mRNA is only detected in the epidermis but not in the vascular tissues of young roots, suggesting that the function of ZmPox1 is not correlated to lignification. In addition, although *ZmPox3* mRNA is also detected in the regions where lignification occurs, the involvement of this peroxidase in such a mechanism remains to be further investigated due to its very low expression level.

Therefore, based on its amino acid sequence and mRNA accumulation and localisation patterns, the involvement of ZmPox2 in the latest steps of lignification is discussed.

Keywords: Differential screening; In situ hybridisation; Lignin biosynthesis; Maize; Peroxidase

1. Introduction

Plant peroxidases (EC. 1.11.1.7) are donor: hydrogen peroxide oxidoreductases and form a class of heme-containing enzymes that act on a large variety of substrates. They can receive two oxidation equivalents from different hydroperoxides with which a wide range of aromatic hydrogen donors are oxidised and subsequently released as oxidised radicals (Teichmann et al., 1997). It has been suggested that these enzymes play an important role in several physiological processes including indole-3-acetic acid metabolism (Grambow, 1986), pathogen resistance (Mohan et al., 1993), response to stress (Mohan et al., 1993), suberisation (Espelie et al., 1986; Roberts et al., 1988; Quiroga et al., 2000), and lignin biosynthesis (Whetten et al., 1998; Christensen et al., 2001; Quiroga et al., 2000; Hatfield and Vermerris, 2001; Onnerud et al., 2002).

Although peroxidases are ubiquitous in vascular plants and are involved in many different functions such as plant growth and response to environmental stimuli, many of the proposed functions for peroxidases are based on in vitro

Abbreviations: bp, base pair(s); BSA, bovine serum albumin; CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl CoA 3-OMT; CCR, cinnamoyl CoA reductase; cDNA, complementary to RNA; COMT or OMT, caffeic *O*-methyltransferase; Kb, kilobase(s) or 1000 bp; mRNA, messenger RNA; NADH, nicotinamide-adenine dinucleotide reduced form; pfu, plaque-forming unit(s); pI, isoelectric point; UTR, untranslated region(s).

☆ The nucleotide sequences reported appear in the EMBL Nucleotide Sequence Database under the accession numbers AC: AJ401274 for *ZmPox1*, AC: AJ401275 for *ZmPox2*, and AC: AJ401276 for *ZmPox3*.

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data. The many potential substrates and multitude of isozymes have made very difficult to determine the in vivo role for the majority of peroxidases using standard biochemical techniques.

In that way, a great deal of research has been directed towards the identification of peroxidases specifically involved in the process of lignification. Increased levels of wall-associated anionic peroxidases have been correlated with the lignification of specific tissues (Imberly et al., 1985). Other groups have found increased levels, and even novel cationic isozymes, of peroxidases associated with lignifying tissues (Church and Galston, 1988). It has been postulated that cell wall anionic peroxidases are involved in the formation of phenoxy radicals needed for lignification in situ while cationic ones are involved in the formation of hydrogen peroxide used by anionic isozymes in the formation of these phenoxy radicals. Therefore, mounting results arising using a biochemical approach suggest that peroxidases are key enzymes involved in the latest steps of lignification (Lagrimini, 1991; Pomar et al., 2002).

Lignin biosynthesis in the cell walls of land plants proceeds initially through the dehydrogenation of substituted *p*-hydroxycinnamyl alcohol and coupling of phenol-radicals to give phenol polymers. For a long time peroxidases have been considered the unique enzymes responsible for this reaction, although further investigations suggest a role for laccases in lignin biosynthesis (Bao et al., 1993). It seems that cell wall associated peroxidases have two roles in the lignification of plant cells. First, they oxidise cinnamyl alcohols in a reaction that requires hydrogen peroxide to form mesomeric phenoxy radicals that polymerise to form lignin. Second, they can oxidise NADH produced in muro to form H_2O_2 (McDougall, 1992). Recently, the existence of a redox shuttle/peroxidase system involved in a new vision of how polymerisation of monolignols occurs in plants has also been proposed (Onnerud et al., 2002).

In addition, recent studies have shown a more precise understanding of the role of specific isozymes in plants by applying a molecular analysis approach and the production of transgenic plants (for review, Anterola and Lewis, 2002). In that way, peroxidases associated to lignification have already been described in several plants, such as wheat (Baga et al., 1995), tomato (Quiroga et al., 2000), poplar (Christensen et al., 2001), and *Pinus* (Charvet-Candela et al., 2002). In addition, a tomato peroxidase has been proposed as an enzyme involved in the latest steps of both lignin and suberin production (Quiroga et al., 2000), as the oxidation of cinnamyl alcohols prior to their polymerisation is a common process for both lignin and suberin (Whetten et al., 1998). Finally, very little is known concerning maize peroxidases. Indeed, only one peroxidase, ZmAP1, has been well characterised and related to suberisation process (Teichmann et al., 1997). However, the precise role(s) of peroxidases is difficult to establish as these enzymes belong to a very large multigene family. Although several tobacco transgenic plants in which the expression of specific peroxidases have been diminished (McIntyre et al., 1996; Lagrimini et al., 1997) or tomato transgenic plants in which a specific peroxidase is over-expressed (Sherf et al., 1993), no effect on lignin content of the cell could be detected. The authors attributed that several peroxidases may be involved in the synthesis of lignin and/or suberin. However, a recent transgenic tomato plant over-expressing an specific peroxidase has been able to significantly increase the lignin content of the cell (Quiroga et al., 2000), strongly supporting the role of peroxidases in the ligno-suberisation process.

Although the molecular mechanisms involved in the regulation of the peroxidase genes is very poorly understood, the very large variety of peroxidases agrees with fact that many hormones have been implicated in the regulation of different types of peroxidases. That is the case for the abscisic acid and ethylene (Kim et al., 2000), auxin (Charvet-Candela et al., 2002), or methyl-jasmonate (Curtis et al., 1997).

In this work, we report the identification of two presumably full-length *ZmPox1* and *ZmPox2* cDNAs and a truncated *ZmPox3* cDNA clones isolated by differential screening from a maize cDNA library. We have determined their tissue-specific expression by Northern blot and in situ hybridisation analyses and the up-regulation of *ZmPox2* by wounding and ethylene. From these results, we propose that ZmPox2 is a new peroxidase involved in the latest steps of lignin biosynthesis in maize. In contrast, our results also indicate that ZmPox1 is probably not involved in the process of lignification.

2. Materials 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 under at 22°C in dark conditions for 3 days and then with a 16 h light/8 h dark photoperiod 4 days more. Plants used for Northern blot analysis were dissected at different growth stages, frozen immediately in liquid nitrogen at stored at −80°C.

2.2. Differential cDNA library screening

The construction of the cDNA library used for this work has been already published (Vignols et al., 1999). Briefly, total RNA was isolated from 9-day old tip-less roots and Poly(A)$^+$ RNA was isolated from total RNA using the PolyATrack kit (Promega), and 5 μg of Poly(A)$^+$ RNA were used for the synthesis of double-stranded cDNA using the λZAPII-cDNA synthesis kit (Stratagene). Finally, ligation and packaging of cDNA (Gigapack, Stratagene) were performed according to manufacturer's conditions.

Then, the tip-less root cDNA library was plated on *Escherichia coli* MRF$^-$ XL1-Blue at low density (5000 pfu/plate). To perform the differential screening, duplicated filters from the library were hybridised with two probes. The positive probes corresponding to cDNAs obtained from mRNAs isolated from 9-day old tip-less roots, while the negative probes contains the cDNAs obtained from mRNAs isolated from the remaining root tips. Both probes were labelled with ^{32}P random priming for hybridisation to a series of duplicated filters. Thus, the plaques giving a signal only with the positive probe were further screened. The clones of interest were excised in vivo to generate pBluescript phagemids containing the cDNA inserts. Finally, positive clones were sequenced using an Automated Laser Fluorescence sequencer (Pharmacia).

2.3. Genomic DNA gel blot analysis

Genomic DNA was isolated from maize (*Z. mays* W64A) 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 specific probes for each clone (see figures).

2.4. RNA gel blot analysis

Total RNA (10 μg per line) was isolated from 9-days-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). Hybridisation conditions were the same as described by Vignols et al. (1999).

2.5. In situ hybridisation

These assays were carried out with 7-days-old maize plantlets grown in wet filter paper. Tissue sections (2–3 mm) were fixed in ethanol/acetic acid 3:1 for 30 min at room temperature. The fixative was removed and the samples were stored in 70% ethanol at 4°C until their hybridisation.

Non-radioactive riboprobes were labelled with digoxygenin-dUTP according to manufacturer's instructions (Amersham). Digoxygenin hybrids were detected by immunoreaction with alkaline phosphatase conjugated to anti-digoxygenin antibody. The antibody was diluted 1/1000 in TBS and bovine serum albumin fraction V (100 mM Tris–HCl pH 7.5, 400 mM NaCl, 0.5% BSA). The incubation with the antibody was performed for 1–4 h. Tissues were then washed for 3 × 10 min in TBS and BSA. Detection was performed according to the protocol of the manufacturer (Amersham) following incubation overnight. Tissue preparations were photographed using bright-field microscopy on Ektachrome 160 ASA film (Kodak).

2.6. Wounding assays

Wounding effect was performed by longitudinal incisions in roots of 9-day-old plantlets with a scalpel blade and dialysis pincers was used in leaves. The various parts of the plant were harvested for analysis at 24 h after treatment, and the effect of wounding on the expression of *ZmPox1*, *ZmPox2*, and *ZmPox3* was then analysed by Northern blot as described above.

2.7. Peroxidases activation in response to hormones

2.7.1. Treatment with methyl-jasmonate

Plants were grown as previously for 6 days and then transferred to a precipitate glass containing 10 μM of methyl-jasmonic acid (>90% pure; Apex Organics, Leicester, UK) in sterile water. The various parts of the plant were harvested 24 h after.

2.7.2. Treatment with ethylene

Plants were grown as previously for 6 days and then transferred to closed bells where they were infiltrated with ethylene to a final concentration of 100–200 ppm over 24 h. Ethylene concentration was measured each 2 h by gas chromatography.

2.8. Sequence alignment and phylogenetic tree

The alignment of the peroxidase sequences has been done using the ClustalW program (Higgins et al., 1994). The bootstrap consensus tree (phylogenetic tree) has been done by the Phylip package programs (Felsenstein, 1993).

2.9. Equivalences of names of peroxidase proteins with their accession number (see Fig. 1)

The name of each sequence corresponds to the deduced protein of the follow DNA accession numbers: *A. thaliana*-08: X70220; *A. hypogaea*-2: M37637; *A. rusticana*-1: A00740; *A. rusticana*-2: D90115; *A. rusticana*-3: D90116; *A. rusticana*-4: M37156; *A. rusticana*-5: M37157; *A. rusticana*-6: M60729; *A. thaliana*-01: AF030132; *A. thaliana*-02: AJ000469; *A. thaliana*-03: AJ000470; *A. thaliana*-04: D14442; *A. thaliana*-05: M58380; *A. thaliana*-06: M58381; *A. thaliana*-07: X59600; *A. hypogaea*-1: M37636; *A. thaliana*-09: X71794; *A. thaliana*-10: X80036; *A. thaliana*-11: X89866; *A. thaliana*-12: X98189; *A. thaliana*-13: X98190; *A. thaliana*-14: X98275; *A. thaliana*-15: X98276; *A. thaliana*-16: X98313; *A. thaliana*-17: X98314; *A. thaliana*-18: X98315; *A. thaliana*-19: X98316; *A. thaliana*-20: X98317; *A. thaliana*-21: X98318; *A. thaliana*-22: X98319; *A. thaliana*-23: X98320; *A. thaliana*-24: X98321; *A. thaliana*-25: X98322; *A. thaliana*-26: X98323; *C. pepo*: Y17192; *N. sylvestris*-3: P30708; *Cucurbita* sp: D83656; *G. hirsutum*: L08199; *G. max*: L10292; *H. vulgare*-1: AJ003141; *H. vulgare*-2: AJ006358;

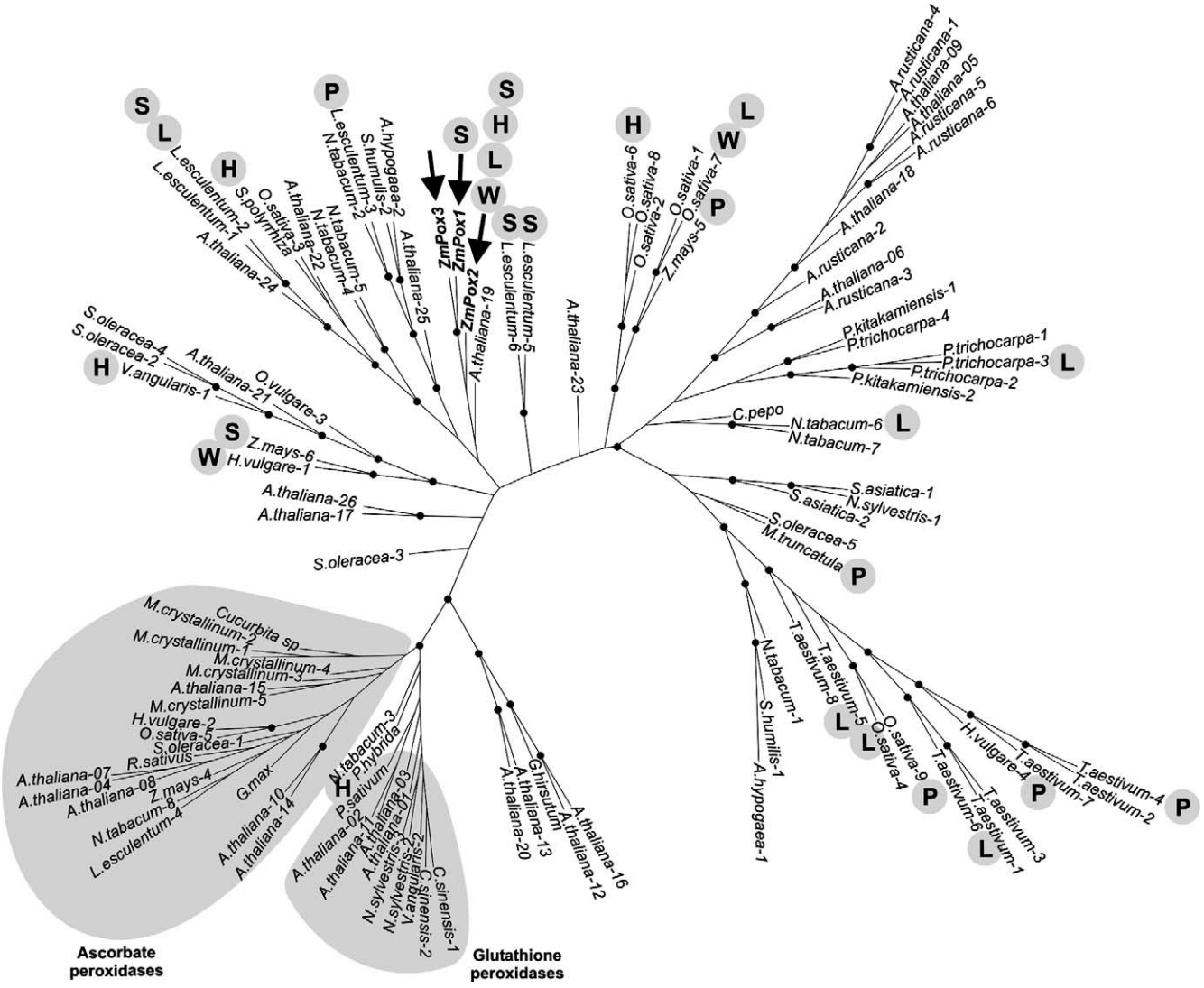

Fig. 1. Phylogenetic tree obtained by the protein sequence alignment of the three new peroxidases of maize and the 108 peroxidase sequences found in the databanks. Dots refer to branches supported at a bootstrap proportion of >90%. ZmPox1 (AC: AJ401274), ZmPox2 (AC: AJ401275), and ZmPox3 (AC: AJ401276). Equivalences with names and accession numbers of the rest of peroxidases are described in Section 2. Ascorbate and glutathione peroxidases are grouped in grey bubbles. **S** refers to peroxidases related to suberisation. *Z. mays*-6 refers to reported ZmAP1 protein (Teichmann et al., 1997). **W** refers to peroxidases induced by wounding. **H** refers to peroxidases induced by hormones. **P** refers to peroxidases induced by pathogens or fungus. **L** refers to peroxidases related to lignification: *L. escutelum*-2 refers to the reported TPX1 protein (Quiroga et al., 2000). *P. trichocarpa*-3 refers to the reported PXP-3-4 protein (Christensen et al., 2001), and *T. aestivum*-5, -6, and -8 correspond to the reported POX1, POX2, and POX4, respectively (Baga et al., 1995). *N. tabacum*-6 refers to the reported protein from tobacco (Lagrimini et al., 1987). Finally, *O. sativa*-6 refers to an unpublished peroxidase (AC: D8440).

H. vulgare-3: M73234; *H. vulgare*-4: X58396; *L. esculentum*-1: L13653; *L. esculentum*-2: L13654; *L. esculentum*-3: X94943; *L. esculentum*-4: Y16773; *L. esculentum*-5: X15853; *L. esculentum*-6: X15854; *M. crystallinum*-1: AF069315; *M. crystallinum*-2: AF069316; *M. crystallinum*-3: AF079512; *M. crystallinum*-4: AF079513; *M. crystallinum*-5: AF139190; *M. truncatula*: U16727; *N. sylvestris*-1: M74103; *N. sylvestris*-2: X60219; *N. tabacum*-1: AB027752; *N. tabacum*-2: AB027753; *N. tabacum*-3: D29976; *N. tabacum*-4: D42064; *N. tabacum*-5: D42065; *N. tabacum*-6: J02979; *N. tabacum*-7: L02124; *N. tabacum*-8: U15933; *O. sativa*-1: D14481; *O. sativa*-2: D14482; *O. sativa*-3: D14997; *O. sativa*-4: D16442; *O. sativa*-5: D45423; *O. sativa*-6: D49551; *O. sativa*-7: D84400; *O. sativa*-8: E04422; *O. sativa*-9: X66125; *P. hybrida*: U31094; *P. kitakamiensis*-1: D38050; *P. kitakamiensis*-2: D38051; *P. sativum*: AJ000508; *R. sativus*: X78452; *S. asiatica*-1: AF043234; *S. asiatica*-2: AF043235; *S. humilis*-1: L37790; *S. humilis*-2: L77080; *S. oleracea*-1: L20864; *S. oleracea*-2: Y10462; *S. oleracea*-3: Y10463; *S. oleracea*-4: Y10464; *S. oleracea*-5: Y10465; *S. polyrrhiza*: Z22920; *T. aestivum*-1: S54871; *T. aestivum*-2: S55687; *T. aestivum*-3: X53675; *T. aestivum*-4: X56011; *T. aestivum*-5: X85227; *T. aestivum*-6: X85228; *T. aestivum*-7: X85229; *T. aestivum*-8:

X85230; *V. angularis*-1: D11337; *V. angularis*-2: E11682; *Z. mays*-4: Z34934; *Z. mays*-5: AF037033; *Z. mays*-6: Y13905; *C. sinensis*-1: X66377; *C. sinensis*-2: Q06652.

3. Results

3.1. cDNA cloning of three closely related maize peroxidases

Genes involved in the lignin biosynthesis pathway have a characteristic pattern of mRNA accumulation in maize. This is the case for *OMT* and *CCoAOMT* (Collazo et al., 1992; Civardi et al., 1999). Based on that, it is therefore possible to identify specific regions of the plant where genes involved in the formation of the secondary cell wall are highly expressed. One of these regions is the growing root behind the root tip. In order to detect new genes that could be involved in the formation of the secondary cell wall in maize, a cDNA library was constructed from this region (Vignols et al., 1999). The cDNAs that were shown to be abundant in this region were systematically sequenced and analysed for expression in the maturing region of the root and not in the root tip. A previously published example of these cDNAs is the one coding for a highly repetitive proline-rich protein (Vignols et al., 1999). These results indicate that the differential screening is a successful approach to identify genes involved in lignification. Therefore, three cDNA sequences with similarity to reported plant peroxidases were detected in the course of this screening, and named *ZmPox1*, *ZmPox2*, and *ZmPox3*; for *Z. mays* peroxidase 1–3, respectively.

As many peroxidases have been described in plants we want to know whether or not these three new maize peroxidases are evolutionary related to known lignin-associated peroxidases. The phylogenetic tree obtained by the alignment of ZmPox1, ZmPox2 and ZmPox3 with the 108 peroxidase proteins (or protein deduced sequences) present in databases is shown in Fig. 1. The three new maize peroxidases are closely grouped, and diverge from the other, described as ascorbate and glutathione peroxidase proteins, which in turn are evolutionary grouped in two superfamilies. Instead, lignin- and suberin-related peroxidases, or hormone, pathogen and wounding induced peroxidase are distributed widespread on the dendogram.

3.2. Structure and comparison of ZmPox1, ZmPox2, and ZmPox3 proteins

The differential screening has revealed three different peroxidase cDNAs. *ZmPox1* and *ZmPox2* cDNAs have 1398 and 1498 bp, in which the start codon of the deduced proteins are at position 122 and 64, respectively. The deduced ZmPox1 and ZmPox2 proteins have 344 and 335 amino acid residues, which is in agreement with the size of classical plant peroxidases (Welinder, 1992). *ZmPox3* cDNA is a truncated clone lacking the start codon. However, sequence alignment of ZmPox3 with ZmPox1 and ZmPox2, together with the size of the *ZmPox3* mRNA (see below) suggests that only few amino acid residues lack in the full ZmPox3 protein. Therefore, it is reasonable to suggest that the size of the full-length ZmPox3 protein is similar to ZmPox1 and ZmPox2 (Fig. 2).

In addition, the majority of these types of peroxidases are *N*-glycosylated (Welinder, 1992). In that way, ZmPox1, ZmPox2, and ZmPox3 have several potential sites for *N*-glycosylation (NX(S/T)X (X ≠ P). In addition, SMART prediction program indicates a N terminal peptide signal for both ZmPox2 and ZmPox3 proteins, but not for ZmPox1. Furthermore, the same prediction program identifies a C terminal peptide signal, Asn-His-Tyr in ZmPox2 that suggests a possible localisation of this protein in the cell wall. When these proteins are compared (Fig. 2), a C terminal extension region is detected for ZmPox1 and ZmPox3 with respect ZmPox2. Similar amino acid residue extensions have been identified as vacuolar targeting signals in other described peroxidases (Bednarek et al., 1990). Finally, ZmPox1, ZmPox2, and ZmPox3 have eight conserved Cys residues for putative disulfide bridges, a typical feature of plant secretory peroxidases. However, PSORT program predicts that although ZmPox2 and ZmPox3 are localised outside the cell, ZmPox1 seems to be a cytoplasmic protein. Therefore, taken together, it seems clear that ZmPox2 and ZmPox3 are extracellular proteins, whereas the precise localisation of ZmPox1 remains to be further analysed.

Finally, plant peroxidases have several motifs that are essential for their enzymatic activity. The deduced ZmPox1, ZmPox2, and ZmPox3 proteins are suggested to be acidic peroxidases as their calculated pI is 6.11, 5.39, and 6.49, respectively, and contain the essential motifs required for the enzymatic activity of plant heme peroxidases. Thus, ScanProsite program predicts the active site signature of ZmPox1, ZmPox2, and ZmPox3 from amino acid residue 43 to 54, from 66 to 77, and from 55 to 66, respectively (for detail, see Fig. 2). Equally, the proximal heme-ligand signature of ZmPox1, ZmPox2, and ZmPox3 are predicted from amino acid residue 171 to 181, from 197 to 207, and from 183 to 193, respectively (for detail, see Fig. 2).

3.3. ZmPox1, ZmPox2, and ZmPox3 genes could be analysed independently

As previously shown, peroxidases belong to a very large multigene family. Bioinformatic data agrees with the fact that when the maize genome is analysed by Southern blot with a common region of *ZmPox1*, *ZmPox2*, and *ZmPox3*, a smear of hybridisation is detected (result not shown).

The fact that ZmPox1, ZmPox2, and ZmPox3 are closely related (see Fig. 1), together with the fact that there are more than one hundred peroxidases identified on databases, raised the question of whether the study of the expression pattern

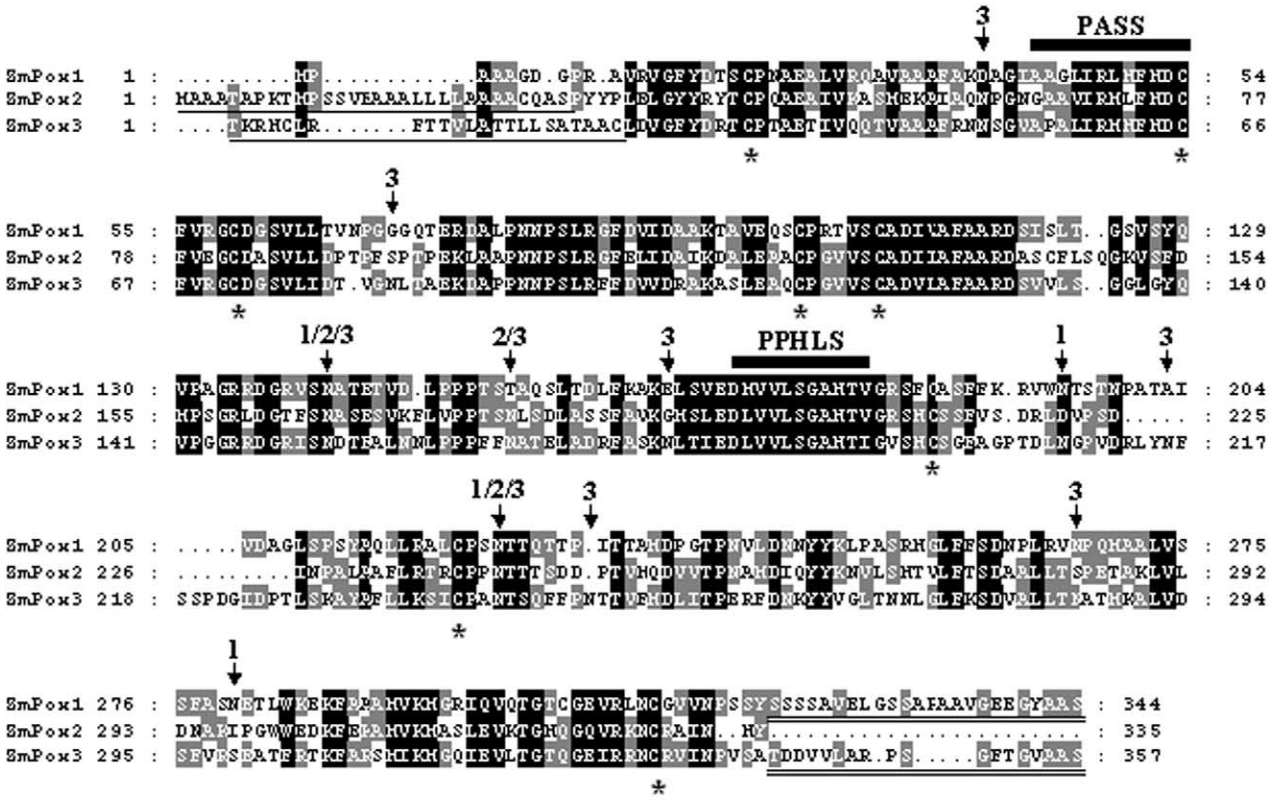

Fig. 2. Alignment of amino acid sequences of ZmPox1, ZmPox2, and ZmPox3. Dark and grey boxes refer to amino acid identity or homology between three or two sequences, respectively. Thick bars refer to peroxidases active site signature (PASS) and peroxidases proximal heme-ligand signature (PPHLS). Asterisks refer to eight conserved Cys residues. N terminal peptide signals of ZmPox2 and ZmPox3 are underlined. C terminal amino acid extensions of ZmPox1 and ZmPox3 are double-underlined. Arrows indicate putative *N*-Glycosylation sites. The number(s) of each arrow refer to the protein(s) in which the *N*-Glycosylation site is found. Finally, amino acid residue number is indicated on both sides of the figure for each protein.

of these three peroxidases could be studied independently. Therefore, as this report is consecrated to the study of the specific expression of these three peroxidases, it was crucial to determine a specific probe of each cDNA in order to unambiguously differentiate the expression pattern of *ZmPox1*, *ZmPox2*, and *ZmPox3*.

In that way, results showed in Figs. 3A–C demonstrate that using the 3′UTR of each cDNA as a probe, only its own peroxidase gene (but neither the other two closely sequence-related peroxidases nor other peroxidases present in the maize genome) could be detected by Southern blot analysis.

3.4. mRNA accumulation of ZmPox1, ZmPox2, and ZmPox3 in different tissues of the maize plant

Using the probes shown to be specific for each of the three cDNAs, it was therefore possible to analyse the specific expression pattern of each peroxidase in different parts of the maize plant. Therefore, the mRNA accumulation pattern of *ZmPox1*, *ZmPox2*, and *ZmPox3* has been studied in depth in different parts of the root and leaves (Fig. 4).

ZmPox1 mRNA is detected in all different parts of the roots, including the root tip, the elongation root region, and mesocotyl but not in leaves (Fig. 4A). In contrast, both *ZmPox2* and *ZmPox3* mRNA are detected predominantly in young (elongation zone) and mature roots, but not in the root tips (Figs. 4B,C). These two genes show a mRNA accumulation pattern very similar to those involved in the biosynthesis of lignin, such as maize *OMT* gene (Collazo et al., 1992), supporting a role of these two peroxidases in the process of lignification in maize. However, it is important to note that the *ZmPox3* gene is expressed at a very low level with respect *ZmPox2*.

3.5. ZmPox2 is up-regulated by wounding and ethylene treatments

Another feature of some peroxidases is that they are induced by wounding stimuli to actively participate in plant defence reactions. Therefore, in order to elucidate whether some of these three maize peroxidases could be involved in such mechanism, we have analysed the mRNA accumulation pattern of their genes in different regions of the maize root, previously submitted to wounding.

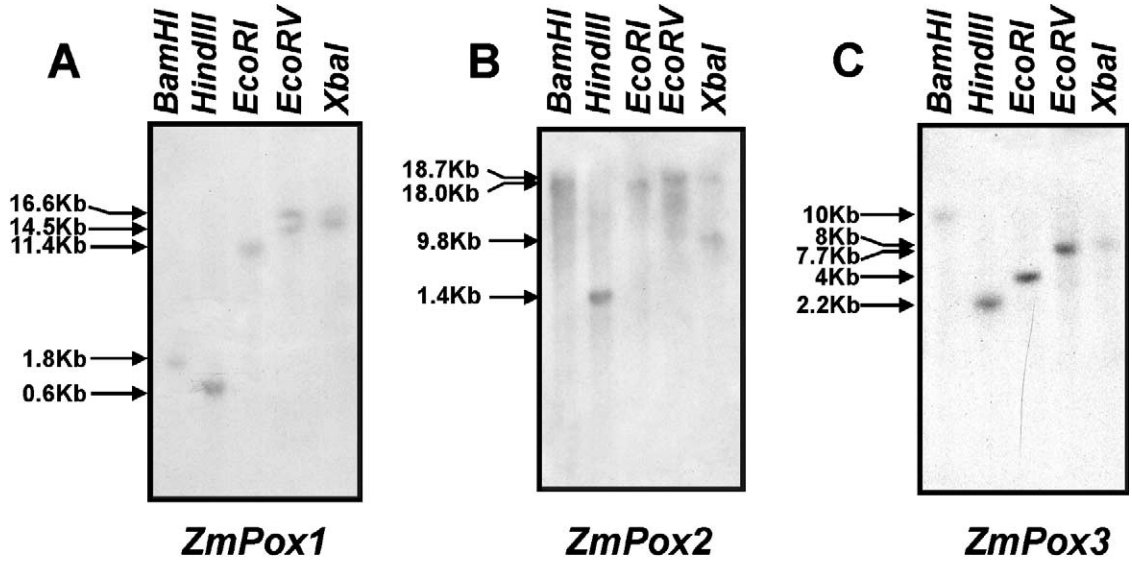

Fig. 3. Maize genomic DNA Southern blot analysis. Genomic DNA from maize line W64A (20 μg per line) was digested by *Bam* HI, *Hin*dIII, *Eco* RI, *Eco* RV, and *Xba* I, separated on a 0.7% agarose gel, blotted and probed with the 3′UTR of each peroxidase cDNA clone. **A**. *ZmPox1*, **B**. *ZmPox2*, and **C**. *ZmPox3*. The size of each genomic band is indicated on the left of each picture in Kb.

Results shown in Fig. 5B demonstrate that *ZmPox2* is severely up-regulated by wounding. Indeed, densitometry analysis reveals that accumulation of *ZmPox2* mRNA in elongation root, maturation root, and meristems of wounded plantlets increases approximately 150 times with respect the control plants. In addition, a similar mRNA accumulation of *ZmPox2* is detected in leaves of wounded plants, although no significant levels of its mRNA could be detected in leaves of control plants. In contrast, neither *ZmPox1* nor *ZmPox3* are induced by wounding (Figs. 5A,C).

In addition, as *ZmPox2* gene is induced by wounding we also have tested the effect of the hormones, such ethylene and jasmonic acid in its mRNA accumulation. Results shown in Fig. 5D reveal that *ZmPox2* mRNA is slightly more accumulated in plants treated with ethylene than the non-treated plants (approximately ten times), suggesting that this hormone could be up-regulating *ZmPox2* gene.

In contrast, when plants are treated with methyl-jasmonate, no effect on the *ZmPox2* RNA accumulation is detected when compared with control plants (Fig. 5E), suggesting that in this case, methyl-jasmonate is not involved in the regulation of *ZmPox2* gene.

3.6. mRNA localisation of ZmPox1 and ZmPox2 by in situ hybridisation

In order to study which cell types accumulate *ZmPox1* and *ZmPox2* mRNA, in situ hybridisation studies were performed. Therefore, maize root tips and mature roots were

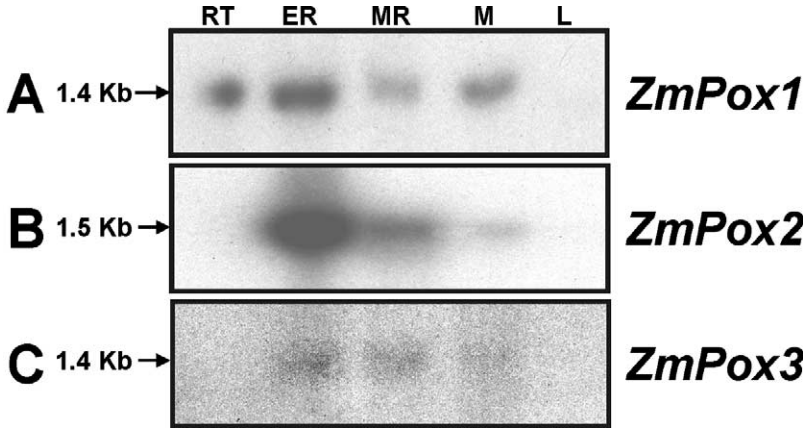

Fig. 4. RNA gel blot analysis of the *ZmPox1*, *ZmPox2*, and *ZmPox3* genes in maize. RNA blot analysis was carried out with 10 μg of total RNA extracted from root tips (RT), elongation zone of the root (ER); maturation zone of the root (MR); mesocotyl (M); and leaf (L). The RNA extracted from these tissues were treated and hybridised with the 3′UTR of *ZmPox1, ZmPox2, or ZmPox3* cDNA (panels A–C). The size of each hybridised transcript is indicated on the left of each picture in Kb.

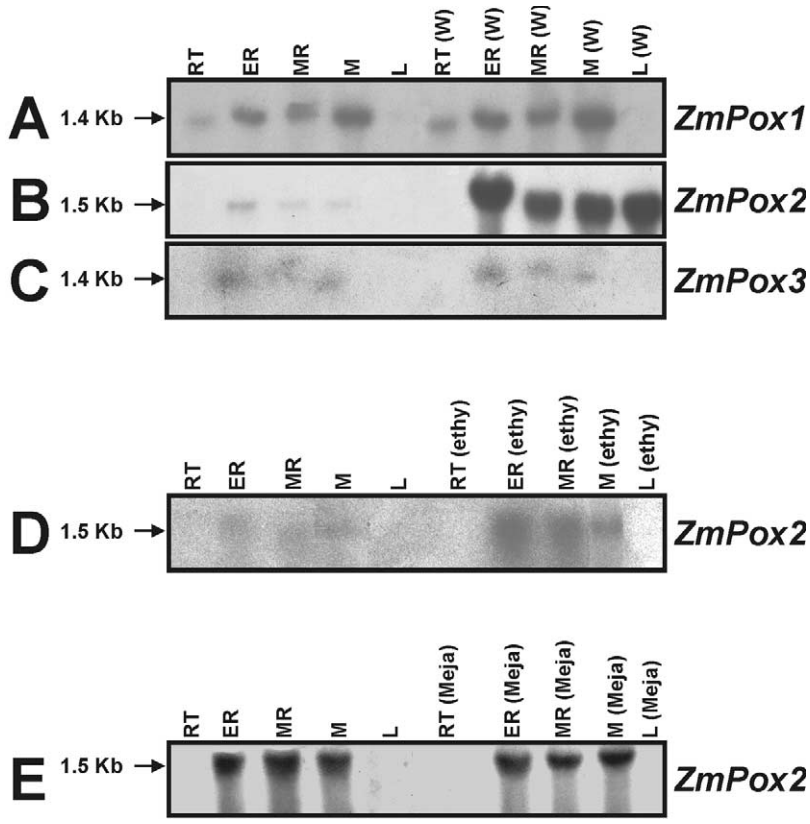

Fig. 5. RNA gel blot analysis of the *ZmPox1, ZmPox2*, and *ZmPox3* genes in maize. The same conditions as in Fig. 4 were used. RT, ER, MR, M, and L indicate the same as Fig. 4. Wounded tissue (W), tissue treated with ethylene (ethy), and tissue treated with methyl-jasmonic acid (Meja). Panels A, B, and C refer to RNA extracted from wounded plants and hybridised with the *ZmPox1*, *ZmPox2*, and *ZmPox3* probes, respectively. Panels D and E refer to RNA extracted from plants treated with ethylene or methyl-jasmonic acid and hybridised with the *ZmPox2* probe, respectively.

the chosen regions of the plant, as they correspond to the region with highest *ZmPox1* mRNA accumulation (Fig. 4A). For the same reason, the region of the plant used for *ZmPox2* corresponds to young roots (Fig. 4B). Finally, *ZmPox3* was not analysed by this technique due to its low expression level (Fig. 4C).

As expected by Northern blot analysis, *ZmPox1* was detected in the longitudinal and cross-section root tips (Figs. 6A,B). The cross-section of the root tip hybridised with the antisense *ZmPox1* probe shows that its mRNA accumulates preferentially in the vascular tissue and epidermis. In addition, and according to the Northern blot analysis, *ZmPox1* mRNA is also detected in the young root (Fig. 6C). However, its mRNA localises near completely in the epidermis region and no signal is detected in lignified vascular tissues. This result indicates that ZmPox1 may not be implicated in lignification processes but rather suberisation, as suberin accumulates in the epidermis zone of roots (Wallace and Fry, 1994; Teichmann et al., 1997; Quiroga et al., 2000). Finally, no hybridisation signal is observed when sections in the mature regions of the root were hybridised with the sense *ZmPox1* probe as a negative control (Fig. 6C).

When the *ZmPox2* mRNA is analysed, its localisation pattern agrees with that expected for genes involved in lignification process. Thus, *ZmPox2* is mainly detected in vascular tissues and epidermis (Figs. 6E,F). As expected, no hybridisation signal is observed when sections in the mature regions of the root were hybridised with the sense *ZmPox2* probe as a negative control (Fig. 6G).

4. Discussion

Formation of the secondary cell wall is a complex process that includes the biosynthesis of lignin. The structure and expression of genes coding for enzymes involved in the pathway leading to the synthesis of monomer alcohols is being unravelled, and probes corresponding to most of them have already been published in different species. Another stage necessary for the synthesis of lignin and suberin is the polymerisation of the final product, a process in which peroxidases are suggested to play a key role (Teichmann et al., 1997; Quiroga et al., 2000).

In this work, three peroxidase cDNAs, *ZmPox1*, *ZmPox2,* and *ZmPox3*, have been found by differential screening as genes preferentially expressed in lignin-associated regions of the maize root.

In the plant species analysed so far, peroxidases belong

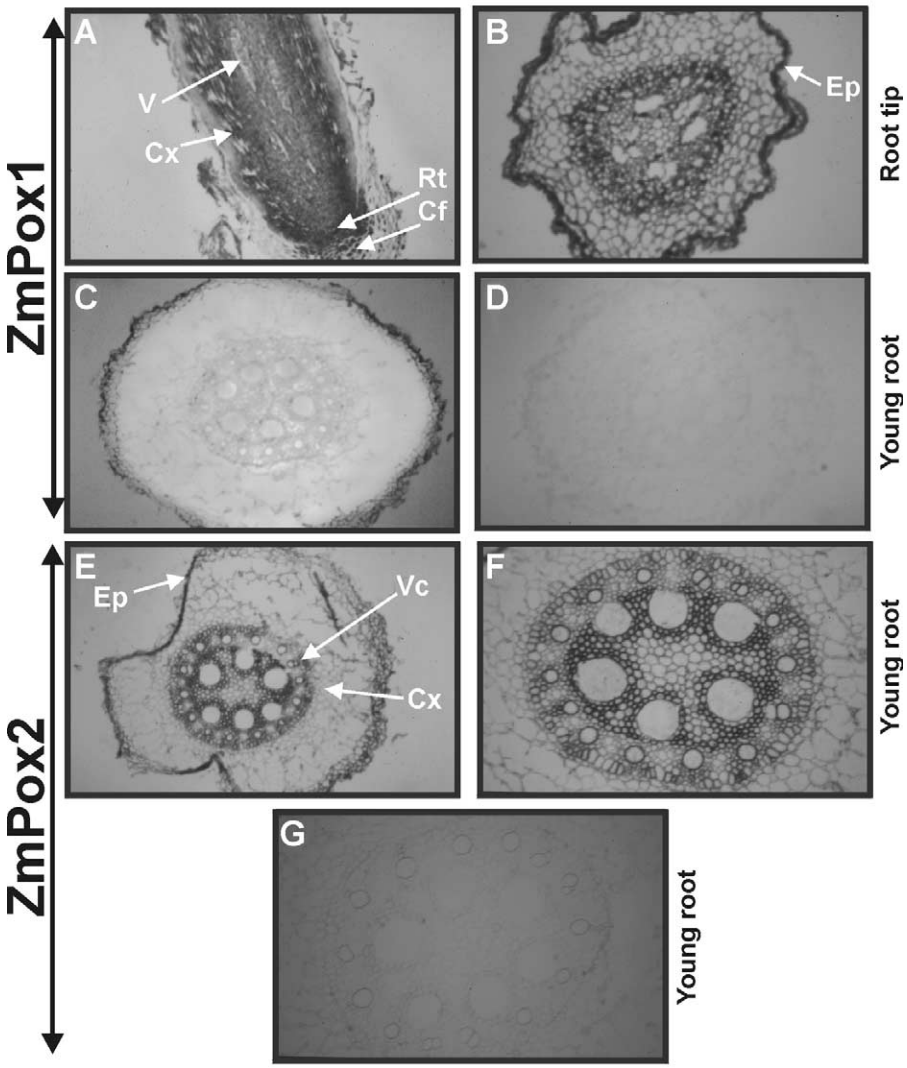

Fig. 6. Cell-specific localisation of *ZmPox1* and *ZmPox2* in young roots and root tips in maize. In situ hybridisations were performed using digoxygenin-labelled *ZmPox1* antisense sequence (A–C). *ZmPox2* antisense (E and F), *ZmPox1* sense (D) and *ZmPox2* sense (G) probes. Except for Panel A, which corresponds to a longitudinal section of the root tip, the rest of hybridisations have been performed in cross sections of the root tip or young root. Panel F is a closed-up of panel E. V refers to vascular tissues. Cx refers to cortex. Rt refers to root tips. Cf refers to cofia. Ep refers to epidermis, and Vc refers to vascular cylinder.

to a large multigene family (e.g. 26 in *Arabidopsis*). The phylogenetic tree reported in this work shows that both ascorbate and glutathione peroxidases could be evolutionary grouped in two families, whereas peroxidases involved in other functions, such lignin- and/or suberin production, are distributed widespread the phylogenetic tree. This is the case for *ZmPox1*, *ZmPox2* and *ZmPox3*.

In maize, Southern blot analysis also indicates that when using a non-specific probe the number of hybridised bands is too large for distinguishing specific genes. This result agrees with the fact that at present more than 100 putative peroxidases could be identified when searching on maize data bases. However, Southern blot analyses performed with specific regions of each of our three maize peroxidases unambiguously demonstrate that the *ZmPox1*, *ZmPox2*, and *ZmPox3* gene expression could be studied without interference of other peroxidase genes present in the maize genome.

The three maize peroxidases reported in this work contain all the features described for plant peroxidases involved in lignin and/or suberin biosynthesis. Thus, these three proteins have a similar size of approx. 300 amino acid residues and the active and proximal heme-ligand sites, which are the typical signatures of plant heme-peroxidases. In addition, other important features, such eight putative cysteine disulfide bridges and *N*-glycosylation sites are also found in the structure of the three maize peroxidases.

Finally, one of the major features of these three maize peroxidases is their putative cell localisation. Computational analysis suggests that ZmPox1 is a cytoplasmic

protein, whereas ZmPox2 and ZmPox3 are localised outside the cell, where the lignification takes place.

The study of mRNA accumulation of *ZmPox2* and *ZmPox3* agrees with the expected mRNA accumulation pattern of genes encoding lignin-associated proteins. Thus, *ZmPox2* and *ZmPox3* mRNAs are detected in lignifying tissues such elongation and maturation root regions. There is no accumulation in the non-lignifying tissues such the root tip. Although these two genes have a very similar mRNA accumulation pattern, the amounts of *ZmPox3* mRNA are lower than *ZmPox2* mRNA. This makes unpractical further studies of *ZmPox3* mRNA localisation by in situ hybridisation.

mRNA localisation of *ZmPox2* further support the involvement of this enzyme in lignification. In situ hybridisations show that *ZmPox2* mRNA localises in the vascular vessels and epidermis.

Instead of *ZmPox2* and *ZmPox3*, the mRNA accumulation pattern of *ZmPox1* does not correlate with the pattern of genes linked to lignification process. Although its mRNA is detected in lignifying tissues, it is also detected in the root tips, where lignification does not occurs. This suggests that ZmPox1 might not be involved in lignification. This putative function is reinforced by its mRNA localisation. Its mRNA localises preferentially in the epidermis region of the young roots, excluding the vascular vessels. This localisation profile is more similar to peroxidases involved in suberisation rather than lignification (Teichmann et al. 1997; Quiroga et al., 2000).

A variety of factors, both environmental and endogenous, have been found to exert and effect upon the process of lignification (Lewis and Yamamoto, 1990). In addition, another features of genes involved in the phenylpropanoid pathway, such maize and tobacco *COMT* (Capellades et al., 1996), is the fact that they are induced by wounding in order to produce lignification and/or suberisation in the damaged tissues. Our results indicate that *ZmPox2* gene, but neither *ZmPox1* nor *ZmPox3* genes, is up-regulated by wounding.

Although the regulatory mechanisms by which the wound-induced expression of peroxidases is still poorly understood, different wounding-induced compounds have been reported to be involved in the regulation of plant peroxidase expression (Kim et al., 2000). Our results suggest that ethylene might be involved in the wounding-induction of *ZmPox2* gene. Instead, methyl-jasmonate compound seems to do not have any effect on the wounding-induction signal of *ZmPox2* gene.

Therefore, due to *ZmPox1* mRNA accumulation and tissue-localisation patterns, the question of whether ZmPox1 enzyme could be involved in suberisation rather than lignification is opened. Instead, we propose ZmPox2 as a new peroxidase involved in the production of lignin and/or suberin in maize, as all the features studied in this work for this enzyme accomplish the characteristics expected for an enzyme involved in the synthesis of lignin and/or suberin.

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