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MINI-REVIEW

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Molecular biology and application of plant peroxidase genes

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Abstract Peroxidases are a family of isozymes found in all plants; they are heme-containing monomeric glycoproteins that utilize either H_2O_2 or O_2 to oxidize a wide variety of molecules. These important enzymes are used in enzyme immunoassays, diagnostic assays and industrial enzymatic reactions. Peroxidase genes and their promoters can be used for molecular breeding of useful plants. Transgenic techniques have also been used to investigate the physiological and molecular functions of peroxidase genes in plants. Here, we review transgenic studies of peroxidase genes, including the functional analyses of the enzymes and their promoters. Regarding application of peroxidase genes, it has been reported that overexpression of the tomato *TPX2* gene or the sweet potato *swpa1* gene conferred increased salt-tolerance or oxidative-stress tolerance, respectively. The growth stimulation effect in transgenic tobacco and hybrid aspen upon overexpression of horseradish peroxidase gene is also discussed.

Plant peroxidase genes

Peroxidase (EC 1.11.1.7) oxidizes a vast array of compounds (hydrogen donors) in the presence of H_2O_2. Plant peroxidases are heme-containing glycoproteins and are usually classified as acidic, neutral, or basic, according to their isoelectric points. Most higher plants possess a large number of peroxidase isoenzymes, which are encoded by multigene families. Several physiological functions for peroxidases in plants have been reported, such as removal of H_2O_2, oxidation of toxic reductants, biosynthesis and degradation of lignin in cell walls (Grisebach 1981; Mäder and Füssl 1982; Lagrimini 1991), auxin catabolism (Hinnman and Lang 1965; Gazaryan and Lagrimini 1996), defensive responses to wounding (Espelie et al. 1986; Dowd and Lagrimini 1997), and defense against pathogen or insect attack (Ye et al. 1990; Dowd and Lagrimini 1997). Hoyle (1977) found 42 isoenzymes and/or isoforms in commercial preparations of horseradish (*Armoracia rusticana*) peroxidase (HRP). Four genomic DNAs (*prxC1a*, *prxC1b*, *prxC2* and *prxC3*) encoding HRP (Fujiyama et al. 1990), and three cDNAs (*prxC1a*, *prxC1b* and *prxC1c*) have been isolated (Fujiyama et al. 1988). The amino acid sequence deduced from the *prxC1a* gene is the same as that determined for the purified isoenzyme C of HRP by Welinder (1974). More than 100 expressed sequence tags encoding different peroxidase isoenzymes are found in *Arabidopsis*. The cloning, expression and analysis of a number of cDNAs and genomic DNAs encoding peroxidases in higher plants has been performed (Table 1).

The biochemistry and enzymology of the plant peroxidase superfamily has been reviewed extensively (Welinder 1992; Hiraga et al. 2001). Here, we summarize reports of peroxidase-expressing transgenic plants, especially those relating to the regulation of peroxidase gene expression. We also describe in detail growth-rate stimulation by overexpression of the neutral HRP gene *prxC1a* in tobacco and hybrid aspen as examples of useful applications of peroxidase genes.

Mode of peroxidase gene expression

Expression of plant peroxidase genes is complicated since they are regulated at different times and places by various kinds of biotic and abiotic stressors. A large number of studies, however, have been conducted describing the temporal and spatial control of the expression of specific peroxidase genes: physical wounding induces the expres-

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Table 1 Principal plant peroxidase genes

Plant	Gene	Expression and/or functional analysis	Reference
Picea abies	*SPI2*	Pathogen inducible	Fossdal et al. 2001
Gossypium hirsutum (cotton)	n.d.[a]	Bacterial inducible	Assigbetse et al. 1999
Cucumis sativas (cucumber)	*Cuper2*	Ethylene inducible	Morgens et al. 1990
Cucurbita pepo (zucchini)	*APRX*	Cell-wall-bound	Carpin et al. 2001
Populus	prxA3a, prxA4a	Stem	Osakabe et al. 1995
	PXP1, *PXP11*, *PXP22*, *PXP3–4*	Xylem of stem and root	Christensen et al. 2001
Arabidopsis thaliana	*prxCa*, *prxEa*, *prxCb*	Promoter	Intapruk et al. 1994
	Nine *prxr* genes	All organs	Capelli et al. 1996
	ATP1a, *ATP2a*	All organs	Kjaersgard et al. 1997
	ATP15a, *ATP24a*	Wound inducible (*ATP2a* also)	Cheong et al. 2002
	ATPA2	Promoter	Ostergaard et al. 2000
Armoracia rusticana (horseradish)	*prxC1*	Promoter, transgenic analysis	Fujiyama et al. 1988
	prxC2	Wound inducible, promoter	Fujiyama et al. 1990
Glycine max (soy bean)	*SPOD4.1*, *Prx2*, *Ep*	Seed coat	Huangpu et al. 1996
Stylosanthes humilis	*Shpx6a*, *Shpx6b*	Pathogen and JA inducible, promoter	Curtis et al. 1997
Arachis hypogaea (peanut)	*prxPNC1*, *prxPNC2*	Wound inducible	Breda et al. 1993
Medicago sativa (alfalfa)	*Msprx1A*, *Msprx1b*, *Msprx1C*	Pathogene inducible	el-Turk et al. 1996
Nicotiana tabacum (tobacco)	*tpoxC1*	TMV inducible	Hiraga et al. 1999
	tpoxN1	TMV, wound, and ethylene inducible	Hiraga et al. 2000
	NtpoxAN	Promoter, transgenic analysis	Klotz et al. 1998
Lycopersicon esculentum (tomato)	*TPX1*	Salt and wound inducible	Botella et al. 1993
	TPX2	Transgenic analysis	Amaya et al. 1999
	TAP1	Promoter (wound), antisense analysis	Mohan et al. 1993
	TAP2	Wound inducible, antisense analysis	Sherf et al. 1993
Solanum tuberosum (potato)	*Stprx2*	Wound inducible	Collinge and Boller 2001
Ipomoea batatas (sweet potato)	*Swpa2*, *Swpa3*	Promoter, transgenic analysis	Huh et al. 1997
Oryza sativa (rice)	*POX22.3*, *POX8.1*, *POX5.1*	pathogen inducible	Chittoor et al. 1997
	prxRPA, *prxRPN*	wound and ethephone inducible	Ito et al. 1994
		promoter (*prxRPN*)	Ito et al. 2000
Triticum aestivum (wheat)	*pox1*, *pox2*, *pox3*, *pox4*	powdery mildew induced (*pox2*)	Baga et al. 1995

[a] Not determined

sion of rice *poxA* and *poxN* (Ito et al. 2000), tobacco *tpoxN1* (Hiraga et al. 2000), sweet potato *swpa1*, *swpa2*, *swpa3*, and *swpn1* (Huh et al. 1997; Kim et al. 1999), horseradish *prxC2* (Kawaoka et al. 1994b), and tomato *tap1*/*tap2* (Mohan et al. 1993); phytohormone ABA induces the expression of pine *PpPrx75* (Charvet-Candela et al. 2002), duckweed basic peroxidase (Chaloupkova and Smart 1994), and tomato and potato anionic peroxidase (Roberts and Kolattukudy 1989); and cooling at 4°C induces the expression of sweet potato *swpa1*, *swpa2*, *and swpa3*, but reduces the expression of sweet potato *swpn1* (Huh et al. 1997; Kim et al. 1999). Information about the timing and tissue specificity of peroxidase gene expression is important, as it may reveal the real function of a specific peroxidase isozyme encoded by that gene. For example, the detailed expression profile of a wound-inducible tobacco *tpoxN1* implied that the gene encodes a peroxidase isozyme that functions in the wound-healing process (Sasaki et al. 2002). Profiles of tissue-specific expression for a number of plant peroxidase genes were also compared to those of lignified tissues, since some specific plant peroxidases are thought to be important in the biosynthesis of lignin, a compound with great commercial value (Klotz et al. 1998). Some of these studies supported the role of plant peroxidases in lignin biosynthesis (Ostergaard et al. 2000), while others suggested a role for plant peroxidase in defense, growth, and development (Klotz et al. 1998).

There is, however, only limited information regarding the mechanisms by which plants regulate specific expression of their peroxidase genes. Promoter analysis in a heterologous host confirmed wound- and fungus-responsive activity of the legume *Shpx6b* promoter, which contains a motif similar to the methyl jasmonate (MeJA) responsive element (Curtis et al. 1997). Analysis of the rice *poxA* promoter in transgenic tobacco plants has revealed UV and wound-responsive *cis* elements within 144 bp of the *poxA* translation start site (Ito et al. 2000). In the expression of the HRP (*prxC2*) gene, two cis elements and two cognate trans factors have been reported (Fig. 1) (Kawaoka et al. 1994c, 2000; Kaothien et al. 2000). These two sets of cis elements and trans factors are important in both basal and wound-inducible activity of the *prxC2* promoter in transgenic tobacco plants (Kaothien et al. 2002).

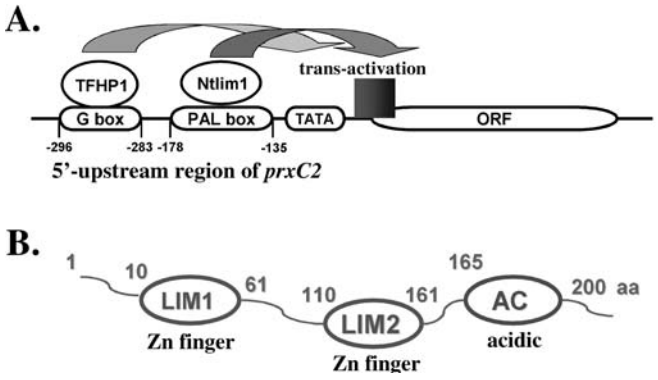

Fig. 1A,B Molecular mechanism of the regulation of *prxC2* promoter activity. **A** Cis- and trans-acting factors of the wound-inducible *prxC2* promoter. The first cis element contains a *G-box* consensus, a common element found in promoter regions of several stress-responsive genes; the second cis element was found to contain a sequence similar to the *PAL-box* element, an elicitor-responsive cis element in the promoter of the parsley phenylalanine ammonia-lyase gene (Lois et al. 1989). A trans factor (*TFHP-1*) containing bZip and helix-loop-helix motifs that binds to the G-box region, and a trans factor (*Ntlim1*) containing two LIM-domains that binds to the PAL-box region have been isolated. **B** Schematic drawing showing the domain structure of the Ntlim1 protein

Generation of transgenic plants harboring peroxidase genes

Recombinant DNA technology has been used to investigate the precise physiological functions of plant genes. Some plant peroxidase genes have been expressed transgenically and the phenotypes of these transgenic plants yield information about their functions. The *NtpoxAN* gene, which encodes an anionic peroxidase in tobacco (*Nicotiana tabacum*), was placed under the control of the CaMV 35S promoter and introduced back into tobacco (Lagrimini et al. 1990, 1997). The transgenic tobacco plants exhibited peroxidase activity 2- to 10-fold higher than that of the wild-type plants and displayed chronic severe wilting through loss of turgor in leaves, initiated at the time of flowering. Although alteration of indoleacetic acid (IAA) metabolism by overexpression of anionic peroxidase was examined, no significant difference in IAA levels was observed. In transgenic plants expressing higher peroxidase activity, however, root elongation was insensitive to exogeneously applied IAA. Lagrimini et al. (1997) proposed that the overexpression of the tobacco anionic peroxidase results in diminished root mass from fewer root branches, which contributes to wilting.

TPX2 cDNA, which encodes a cell-wall-associated peroxidase involved in modifying of cell wall architecture in tomato (*Lycopersicon esculentum*), was placed under the control of the CaMV 35S promoter and introduced into tobacco plants (Amaya et al. 1999). By investigating ten independent transgenic plants, Amaya et al. (1999) found that overexpression of the *TPX2* gene had no effect on wild-type development under normal growth conditions; however, the germination rate of the transgenic plants was increased greatly under conditions of high salt (250 mM NaCl) or osmotic stress (470 mM mannitol). Thermoporometry calculations indicated a lower mean pore size in the walls of transgenic seeds. Therefore, they speculated that the salt-tolerant phenotype seen in the transgenic tobacco is due to a higher water-retaining capacity in transgenic seeds.

The genes *tap1* and *tap2* encode anionic peroxidases from tomato and potato that are inducible by wounding, fungus, elicitors and ABA (Sherf 1993). Transgenic tomato plants producing a chimeric antisense transcript corresponding to the 5' region of both *tap1* and *tap2* were generated. The resulting defect in TAP1 or TAP2 activity did not appear to significantly inhibit the suberization of the wound periderm of the tomato fruit.

Anionic (swpa1) and neutral (swpn1) peroxidases from sweet potato (*Ipomoea batatas*) were overproduced in transgenic tobacco plants under the control of the CaMV 35S promoter. Leaves of transgenic plants with either the *swpa1* or *swpn1* gene showed higher peroxidase activity than those of nontransgenic control plants. When tobacco leaf discs were treated with 10 μM methyl viologen (MV; paraquat), *swpa1*-transgenic plants showed a reduction in membrane damage of about 25% relative to *swpn1*-transgenic or untransformed control plants. Leaves of the *swpn1*-transgenic and the control plants were also bleached more than those of the *swpa1*-transgenic by 1 μM MV treatment. These results indicate that the increased H_2O_2-scavenging capacity provided by overproduction of swpa1 peroxidase (a guaiacol-type peroxidase) confers increased oxidative-stress tolerance on the transgenic plants (Yun et al. 2000).

Growth stimulation by overexpression of the HRP gene

The 1.1 kb *prxC1a* cDNA was ligated to either the CaMV 35S or the HRP *prxC2* promoters, and the chimeric genes were separately introduced into tobacco plants (*N. tabacum* L. cv. W-38). Crude extracts of leaves from ten independent transgenic plants showed 2- to 10-fold higher peroxidase activity than that of the wild-type plants, and the T-1 transformed plants clearly exhibited increased growth rate as compared to untransformed control plants (Kawaoka et al. 1994a). In a related study, the self-pollinated seeds of these transformed tobacco plants were harvested and the phenotypes of about 50 seedlings were subjected to statistical analysis. After being grown for 3 months in a greenhouse, T-2 plants with the CaMV 35S promoter-*prxC1a* construct were 20% taller than control plants (Fig. 2A) and northern blot analysis showed that the fast-growing plants also expressed *prxC1a* at a high level. Furthermore, in order to clarify whether the size of the transgenic plants is a result of an increase in growth rate or an increase in stem elongation, timing of flower formation in 40 transformed and 40 wild type plants, each grown for 4 months in a greenhouse, was analyzed. Approximately 30 flower-forming *prxC1a*-overexpressing plants were observed, as

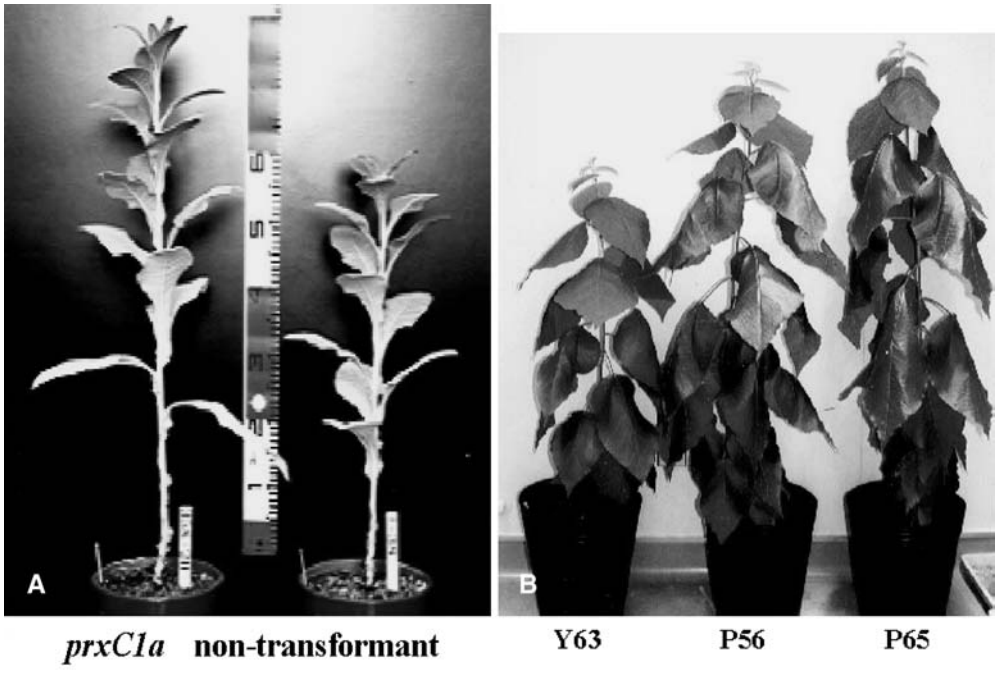

Fig. 2A,B Growth stimulation of transgenic plants by overexpression of the *prxC1a* gene. **A** The average stem length of transgenic tobacco plants expressing the *prxC1a* gene is approximately 20% greater than that of the wild-type plant. **B** The average stem length of transformed hybrid aspen plants (P56 and P65) was 25% greater than that of the wild-type plant (Y63)

oppose to 10 flower-forming wild-type control plants. The final stem length after flowering, however, was almost identical in the transformed and wild-type plants, indicating that the product of the *prxC1a* gene had an effect not on stem elongation, but rather on plant growth promotion.

The CaMV 35S promoter-*prxC1a* construct was also introduced into hybrid aspen (*Populus siebollidi* × *P. grandidentata*), one of the most important species for providing pulping material (Kawaoka et al. unpublished data). The growth rates of 32 independent plants transformed with the CaMV 35S promoter-*prxC1a* construct were compared with those of 8 plants transformed with pBI121 [CaMV 35S promoter-*β-glucuronidase* (*GUS*)] and 10 non-transformed plants as controls by measuring stem lengths for 60 days after transfer to culture tubes. After this period, the average stem lengths of CaMV 35S promoter-*prxC1a*, CaMV 35S promoter-*GUS* and wild-type plants were 42.0, 33.9 and 34.4 mm, respectively. Furthermore, the growth rate of transformed hybrid aspen plants was increased substantially under greenhouse conditions; the average stem length was 25% greater than that of wild-type plants (Fig. 2B). Fast-growing transformed hybrid aspen showed high *prxC1a* transcript levels and had elevated peroxidase activities. These results suggest that overexpression of HRP in plants could be an effective strategy for producing biomass. However, it is very important that the growth stimulation effect of HRP observed in the greenhouse is verified under field conditions.

Future prospects in plant peroxidase research

Recently, microarray technology has been used in plant peroxidase gene expression studies. Three *Arabidopsis* peroxidase genes (*ATP2a*, *ATP15a* and *ATP24a*) were found to be upregulated by wounding in a microarray focused on changes in transcriptional profiling of *Arabidopsis* genes in response to wound stress (Cheong et al. 2002). Therefore, we can expect to learn more about the expression profiles of other plant peroxidase genes from different microarrays. This strategy could be helpful for understanding the role of each peroxidase. Peroxidase has also become a model enzyme to study the molecular mechanisms of vesicular transport accompanied with glycosylation. In our study, we found that the C-terminal 15 amino acid residues in a precursor of HRP C1a possibly function as signal for localization of the enzyme to the vacuole (T. Matsui et al. unpublished data). Although the whole picture of how peroxidase genes regulate various aspects of plant metabolism is still unclear, the information accumulated so far is a promising starting point for further studies to identify their functions and to apply them to the molecular breeding of useful plants.

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