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Rapid Report

Inhibition of lipoxygenase in lentil protoplasts by expression of antisense RNA

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Abstract

A number of plasmids were constructed containing chimeric genes consisting of fragments of antisense-oriented lentil lipoxygenase cDNA. The different constructs were tested for their ability to lower lipoxygenase activity in lentil protoplasts. Plasmids containing a full length lentil lipoxygenase cDNA proved to be the most effective, reducing the activity of the target enzyme to 70% of the control value. On the other hand, the full length lentil lipoxygenase cDNA in the sense orientation yielded a 20% increase of lipoxygenase activity.

Keywords: Lipoxygenase; Antisense RNA; Protoplast; Electroporation; Lentil (*Lens culinaris*)

Lipoxygenases (linoleate:oxygen oxidoreductase, EC 1.13.11.12, LOX) are non-heme iron containing enzymes that catalyse the reaction of oxygen with unsaturated fatty acids with one or more (1*Z*,4*Z*)-pentadiene moieties, yielding conjugated (*Z*,*E*)-hydroperoxydienoic acids. LOXs are found in most plant species, being particularly abundant in legumes [1]. Despite a long history of research, the physiological functions of these enzymes are not clear. LOXs have been proposed to be involved in growth and development [1], in the response to pathogenic attack [2,3], physical stress [4,5] and in the biosynthesis of regulatory molecules like jasmonic acid [6]. Recently, a possible role for LOX in the oxygenation of plant cell membranes has been suggested as well [7]. The development of mutant plants, with altered LOX activity, might give clues to the function of the enzyme, complementing the information obtained from natural mutants, when available, like in soybean [8–10]. Dent et al. [11] have recently introduced the soybean LOX2 cDNA into tobacco tissues under control of a CaMV-promoter. As a result, an increase of LOX activity was observed in undifferentiated calli, but there was no apparent difference in growth and development between the control and the transgenic plants. Therefore, reduction instead of enhancement of LOX activity might give better insight into the physiological function of the enzyme.

Antisense RNA-mediated suppression of a target gene is a valuable tool for obtaining mutant phenotypes (see [12] for a review). The ability of a fragment of antisense RNA in the inhibition of gene expression is difficult to predict, since the sequence homology between the antisense RNA and the target gene is not sufficient for effective inhibition. Different parts of the same gene may cause different inhibitions, even when they share 100% homology with the target [13]. Therefore, it is mandatory to test the effects of different constructs in a model system, before trying the modulation of gene expression in transgenic plants.

Previously, we reported the isolation of a full length cDNA clone coding for a vegetative lentil seedling LOX [14]. Moreover, we isolated lentil root protoplasts suitable for transient expression experiments [15,16]. Here, we evaluated the ability of the lentil LOX cDNA or fragments thereof, in the antisense orientation, to inhibit LOX activity in lentil root protoplasts.

All plasmid constructs (Fig. 1) were based on pAS6 [16], which is pEMBL18 with the GUS cassette, i.e.,

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CaMV promoter, GUS gene and NOS terminator [17], inserted between the *Hin*dIII and *Eco*RI sites. LOX cDNA fragments were inserted between the CaMV promoter and the GUS gene using the restriction sites of the GUS cassette (*Xba*I, *Bam*HI and *Sma*I), the internal *Bgl*II (890) and *Bam*HI (1860) sites of the lentil LOX cDNA, and restriction sites of the multiple cloning sites of plasmid pBS (Stratagene), in which the lentil LOX cDNA was cloned. A number of different pBS/LOX subclones were used as the starting material for construction of the pASL-plasmids. Orientation of the fragments in pASL constructs was verified by restriction analysis, in some cases supplemented with sequencing. The internal *Sac*I (656) site of LOX cDNA was used together with the external *Sac*I site of plasmid pBS to clone a fragment between the GUS gene and the NOS terminator. The internal *Sac*I (656) and *Bam*HI (1860) sites of LOX cDNA were used to replace the GUS gene in pAS6 with this LOX cDNA fragment. All antisense LOX fragments contained endogenous stop codons, preventing the formation of GUS fused to an erroneous peptide, which might be formed if protein synthesis starts in the antisense LOX sequence. Plasmids were purified with Qiagen 500 tips (Diagen, Hilden, Germany) according to the instructions of the manufacturer.

Lentil (*Lens culinaris*) root protoplasts were prepared as described [18]. Aliquots of the protoplast suspension, containing 10^6 protoplasts, were transferred into 0.4 cm electroporation cuvettes, each containing 15 μg of carrier DNA (sheared salmon sperm DNA) and 25 μg of plasmid in a final volume of 800 μl. Samples were electroporated with exponentially decaying pulses at a field strength of 400 V/cm and a capacitance of 960 μF (time constant: 28.8 $\pm$ 0.2 ms). These electroporation conditions were previously found to be optimal for the transfer of pAS6 and similarly sized plasmids into lentil root protoplasts, yielding the highest transfection efficiency [16]. Cell treatment after electroporation and preparation of cell extracts for assays were as described [16]. LOX activity was measured as reported before [16], using linoleic acid (150 μM) as substrate and recording the increase of UV absorbance at 234 nm. LOX activity was expressed as pmol hydroperoxyoctadecadienoic acid min^{-1} (mg protein)$^{-1}$. The amount of LOX protein was determined by ELISA, using the procedure reported elsewhere [16]. GUS (β-glucuronidase, EC 3.2.1.31) activity was assayed by the fluorimetric method of Jefferson [19], improved by adding 20% methanol to the assay buffer [20]. GUS activity was expressed as pmol 4-MU (4-methylumbelliferone) min^{-1} (mg protein)$^{-1}$. Protein content was determined according to [21], with bovine serum albumin as standard. All data represent the mean of three independent experiments (S.D. < 10%).

We constructed various plasmids to evaluate the ability of different fragments of the lentil LOX cDNA to inhibit LOX activity in lentil root protoplasts (Fig. 1). These fragments were placed between the CaMV promoter and the GUS gene of the GUS cassette [17]. This cloning strategy was chosen, because the presence of GUS activity

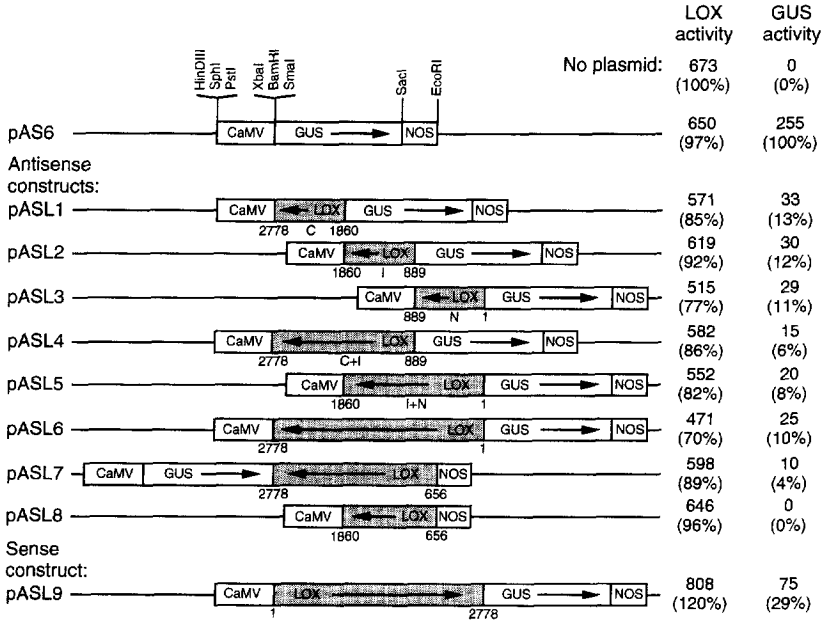

Fig. 1. (Left) Overview of the pAS6 constructs containing lentil LOX cDNA at various positions. CaMV: cauliflower mosaic virus promoter; GUS: β-glucuronidase gene; NOS: nopaline synthase terminator; LOX: lentil lipoxygenase cDNA. Numbers refer to restriction sites in the lentil LOX cDNA sequence [14]: *Sac*I(656), *Bgl*II(890) and *BamH*I(1860). N, I and C indicate the fragments of cDNA coding for the N-terminal (N), intermediate (I) and C-terminal (C) parts of the enzyme. Arrows indicate the direction of coding sequences. The LOX sequences are shown aligned with each other. (Right) Effect of the different plasmids on lipoxygenase (LOX) and β-glucuronidase (GUS) activities in lentil root protoplasts. Both LOX and GUS activities are expressed as pmol product min^{-1} (mg protein)$^{-1}$, values in brackets representing percentages of the controls, arbitrarily set to 100 (S.D. < 10%).

indicates the expression of antisense LOX mRNA. Using restriction sites conveniently located at about 1/3 and 2/3 of the lentil LOX cDNA sequence, LOX cDNA was dissected into fragments coding for the N-terminal (N), C-terminal (C) and intermediate (I) parts of the protein. The LOX inhibition by the three fragments, as well as by fragments representing 2/3 of the full length cDNA (N + I, I + C) and by full length cDNA itself, was tested. The possible influence of the GUS gene on the inhibitory effect of the LOX antisense mRNAs was also evaluated by testing constructs with antisense LOX fragments either replacing or being located downstream the GUS gene. Finally, the effect of a full length LOX mRNA in the sense orientation was evaluated.

We measured the GUS and LOX activities of lentil root protoplasts transfected with the various plasmids (Fig. 1). For the constructs with the strongest effects on LOX activity, i.e., pASL6 and pASL9, we also determined the amount of LOX protein, showing that the changes in activity could be entirely attributed to changes in the amount of protein expressed (70% and 132% of the control value, respectively). Although the presence of LOX cDNA fragments upstream the GUS gene severely reduced GUS expression in the plant cells (Fig. 1), GUS activity was still clearly detectable, thus indicating that the expected mRNA constructs were present in the cells. The full length LOX antisense mRNA appeared to be the most effective in reducing LOX activity, followed by fragment N. On the other hand, fragment I had the lowest impact on the target enzyme. These findings are in line with the results reported for petunia protoplasts [13], where a full length construct was the most effective in the inhibition of the *uidA*-gene, followed by the 5′ end of the gene, while hardly was observed an effect of the central part of the sequence. It is noteworthy that lentil root protoplasts, like a number of other plant cells, could contain more LOX isoforms [1]. The antisense constructs might be unable to bind all isotype sequences, which might reduce the extent of inhibition. Plasmids pASL4 and pASL7, carrying similar fragments of LOX cDNA (Fig. 1), but located upstream or downstream the GUS gene, showed similar GUS and LOX activities. Therefore, the effect of a construct is not influenced by the position of the LOX cDNA. Furthermore, the presence of the GUS gene did not interfere with fragment effectiveness, as shown by the results obtained with plasmids pASL8 and pASL2, which carried similar fragments, either replacing or being located upstream the GUS gene (Fig. 1). Finally, the results obtained with pASL9 showed that it is indeed possible to enhance LOX activity, which corroborates earlier results obtained by infecting tobacco leaves with *Agrobacterium tumefaciens* carrying sense-oriented soybean lipoxygenase [11].

In conclusion, the results presented in this paper show that inhibition of LOX in lentil tissue can be achieved with homologous antisense RNA expression, with the full length sequence being the most effective one. Work is in progress to use these constructs for the development of transgenic lentils with reduced LOX activity, which can be expected to give deeper insights into the function of LOX in plants. To our knowledge, this work is the first use of antisense RNA to reduce LOX activity in plant cells.

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