Developmentally and wound-regulated expression of the gene encoding a cell wall copper amine oxidase in chickpea seedlings

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Abstract   A chickpea cDNA encoding a cell wall copper amine oxidase (CuAO) was cloned and characterised. The 2010 bp open reading frame encodes a protein of 76.5 kDa which shares significant primary structure homology with other known CuAOs. Southern blot analysis indicates that in chickpea CuAO is encoded by a single gene or a small gene family. This cDNA was essential for studying the role of CuAO during seedling development and wound healing in chickpea seedlings. CuAO transcript level and activity were modulated during seedling development in parallel with cell maturation. Moreover, mechanical wounding induced a rapid increase of CuAO mRNA accumulation and enzyme activity which remained high during the wound-healing process. Aminoguanidine, a specific CuAO inhibitor, decreased the deposition of lignin-suberin barrier along the lesion. CuAO may be a limiting factor in H_2O_2 production in the cell wall of chickpea seedlings and its expression seems to integrate with the remodelling of plant cell wall occurring during ontogenesis and wound healing.

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Key words: Copper amine oxidase; Chickpea;
Hydrogen peroxide; Peroxidase; Polyamine; Wound healing

1. Introduction

The cell wall of higher plants forms a unique extracellular matrix that controls growth and developmental processes during ontogenesis. These physiological events are intimately linked with the synthesis and deposition of wall polymers during cell division, elongation and differentiation [1,2]. An essential role in the modification of cell walls is carried out by peroxidases (PODs) which are involved in lignin and suberin biosynthesis and intramolecular isodityrosine generation in hydroxyproline-rich glycoproteins [2,3]. Each of these wall-associated events occurs not only during wall differentiation, but also following wounding and in resistance responses during pathogen attack [4]. Hydrogen peroxide is an essential co-substrate in the oxidative reactions catalysed by wall-bound PODs [2]. Furthermore, H_2O_2 has been implicated in direct killing of pathogen and host cells as a part of the hypersensi-

tive response and as an intracellular signal regulating gene expression [5]. Several classes of enzymes have been proposed to produce H_2O_2 in the cell walls: NADPH oxidase [6], oxalate oxidase [7], amine oxidase [8] and POD itself [2]. Amine oxidases can be divided into two groups: copper-containing amine oxidases (CuAOs) and flavin polyamine oxidases [8]. CuAOs are glycoproteins which catalyse the oxidation of polyamines at the primary amino group giving the corresponding amino-aldehydes, with concomitant production of hydrogen peroxide and ammonia. They are homodimers of 70–90 kDa, each containing a copper ion and a 2,4,5-trihydroxyphenylalanine quinone cofactor (TPQ) generated by a post-translational autocatalytic modification of a tyrosine residue in the active site [9]. CuAOs occur at high levels in dicots and are the most abundant soluble protein of cell wall from pea, chickpea, lentil and soybean etiolated seedlings [8]. CuAO activity, which is under phytochrome control [10], has been proposed to be functionally correlated with PODs during development of legume seedlings [11], in response to wounding [12] and during pathogen infection [13]. In this study, we report the characterisation of a CuAO gene from *Cicer arietinum* and we present evidence for a positive regulation of CuAO expression during development and wound healing.

2. Materials and methods

2.1. Plant material
Chickpea (*Cicer arietinum* L.) seeds were soaked overnight in aerated water and germinated in a greenhouse under natural light condition for 15 days. Various organs were harvested, frozen in liquid N_2, and stored at $-70°C$ until use.

2.2. Molecular cloning
A cDNA library was constructed from chickpea total RNA using a RiboClone cDNA Synthesis and Cloning Kit (Promega) and screened by a previously characterised partial-length lentil CuAO cDNA as a probe [14]. Positive plaques were analysed for cDNA insert size by PCR with vector-specific primers and the identity of the amplified products was confirmed by Southern blot analysis. The largest cDNA inserts were sub-cloned and sequenced. Rapid amplification of the 5′ and 3′ ends (5′, 3′ RACE) was done as reported by Frohman et al. [15]. For 5′ RACE, an antisense-specific primer PD1 was used in a reverse transcription reaction with 2 μg of chickpea total RNA. An oligo(dC) anchor sequence was added to the 3′ end of RT reaction products and the obtained cDNA was amplified by PCR using the nested antisense-specific primer PD2 and the anchor primer provided in the RACE kit (Gibco BRL). For the 3′ RACE the first strand cDNA was obtained using the oligo(dT) primer AP1 and amplified using the 5′ sense-specific primer PD4 and the antisense primer AP2. Full-length chickpea cDNA (*csao*) was obtained from the first strand cDNA pool using two specific primers, PD9 and PD4. PCR reactions were performed with AmpliTaq DNA polymerase under standard reaction conditions. The PCR products were purified with the Qiaex

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Abbreviations: CuAO, copper amine oxidase; *Csao*, chickpea seedling amine oxidase cDNA; ORF, open reading frame; POD, peroxidase; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction

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ccccuuuuuuuuuucuuuuuuuuuucuuuycuccaycccyacccc                    2226
atggcttccaccaccatcaaactttcatttttctttgctattacacttatcttcttacaa      85
M  A  S  T  T  I  K  L  S  F  F  F  A  I  T  L  I  F  L  Q       -2
gccgttacaccattgaatcttcaacatccacttgaccctttaactaaagaagagttttcc     145

A  V  T  P ┌L  N  L  Q  H  P  L  D  P  L  T  K  E  E  F┐ S        19
attgtccaaaccgtagttctacacaaatatcccacctcaaaaaatagtgttgccttccac     205
I  V  Q  T  V  V  L  H  K  Y  P  T  S  K  N  S  V  A  F  H        39
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Y  I  G  L  D  D  P  D  K  D  D  V  L  K  Y  E  T  Q  P  T        59
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L  I  T  I  P  R  K  S  F  V  V  A  I  I  N  S  Q  N  H  E        79
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ttccctatcttatctgctgatgaacaaggtgttgctataaaactacctttgaaatatcct     445
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P  F  I  A  S  V  N  K  R  G  L  N  L  S  E  I  V  C  S  T       139
ttctcaatgggttggtttggagaagaaaaaaaatagtagaacagtgagagttgattgtttc     565
F  S  M  G  W  F  G  E  E  K  N  S  R  T  V  R  V  D  C  F       159
atgaaagaaaacactgttaatatatatgtgagaccaatatctggaataacaattgttgtt     625
M  K  E  N  T  V  N  I  Y  V  R  P  I  S  G  I  T  I  V  V       179
gatcttcaacttatgaaaattgttgagtataatgatagaggtattgaagctgtgcctact     685
D  L  Q  L  M  K  I  V  E  Y  N  D  R  G  I  E  A  V  P  T       199
gctgagaaaactgactatagattttcacatcagaacccaccatttggaccaaaactaaat     745
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S  L  V  S  H  Q  P  Q  G  S  G  A  H  I  N  G  Y  S  V  S       239
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R  D  V  N  W  P  G  C  S  N  *                                  649
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tgaggacaaaaatgtattggctccagttatttttatgtgaatgagaggtttatttggaag    2185
cttaaaaaaaaacaaaaaaaaacaaaagcactagtcgaatat                      2226
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Fig. 1. Nucleotide and predicted amino acid sequences of chickpea copper amine oxidase. Initiation ATG codon and the putative polyadenylation site are underlined. The translated sequence is shown as single letter amino acid code (in bold) and numbered +1 from the start of the mature coding region. The arrow indicates the predicted cleavage site for the secretion signal peptide. The boxed region corresponds to the N-terminal amino acid sequence determined by automated Edman degradation of the intact protein. Termination TAA codon is indicated by an asterisk.

gel extraction kit (Qiagen) and cloned in the pGEM-T Easy vector (Promega). Both strands of three independent clones were completely sequenced by the Sanger method using the Amplicycle sequencing kit (Perkin Elmer). Oligonucleotides used were: PD1, 5′-TAC CAG CTC

GCG CAT CAA TAG CC-3′; PD2, 5′-GCA GTA GGC ACA GCT TCA ATA C-3′; AP1, 5′-CGT CTA GAG TCG ACT AGT GCT T$_{(19)}$-3′; AP2, 5′-CGT CTA GAG TCG ACT AGT GC-3′; PD9, 5′-AAC TCG CTA GGA GCA AAG CTC ACT-3′; PD4, 5′-TTA ATT GGA GCA ACC TGG CC-3′.

2.3. Northern and Southern blot analyses

Total RNA was isolated using the Trizol Reagent (Gibco BRL) and following the manufacturer's instructions. Chromosomal DNA was extracted according to D'Ovidio et al. [16]. Blotting and hybridisation procedures were performed as reported by Sambrook et al. [17]. Membranes were hybridised with the digoxigenin-labelled *csao* using high stringency conditions.

2.4. Enzyme assay

Enzymatic assay were performed as previously reported [18]. The estimated S.D. was < 5% for each value. Proteins were quantified according to Bradford [19] using bovine serum albumin as a standard reference.

2.5. Wounding experiments

Ten-day-old light-grown seedlings were detached from the cotyledon and transferred in a beaker containing 0.1% MS medium [20] in the presence or absence of 1 mM aminoguanidine. After 12 h, the 4th internode of these epicotyls was longitudinally injured with a blade. To determine CuAO activity and mRNA level, wounded and unwounded (control) internodes were collected at different times. For histochemical analysis, 3 days after wounding the 4th internode was cut into 4–5-mm segments, oriented in 4% agar and cut to a thickness of ~60 μm with a vibratome. CuAO activity was visualised using an indirect method based on tissue staining due to the POD-mediated oxidation of 3,3′-diaminobenzidine (DAB) upon putrescine addition [11]. Lignin-suberin depositions were detected by the phloroglucinol/HCl method according to [21].

3. Results and discussion

3.1. Isolation and analysis of CuAO cDNA from chickpea seedlings

The isolation of *csao* was accomplished by screening a cDNA library from chickpea and by the 5′ and 3′ RACE procedures. A partial-length CuAO clone from lentil [16] was used to screen the cDNA library. This analysis yielded six independent clones related to the CuAO as demonstrated by Southern blot hybridisation. The largest clone, pCuAO 3.1, was sequenced on both strands, had an insert size of 912 bp which showed a strong similarity with the C-terminal region of CuAO cDNA from pea and lentil [23,14], and contained a single open reading frame (ORF) encoding a 304-amino acid peptide with high sequence homology compared to the pea and lentil CuAO. Northern blot analysis of total RNA extracted from chickpea seedlings using pCuAO 3.1 as a probe showed that this cDNA hybridised to a single mRNA species ~2.4 kb long (data not shown). The complete *csao* sequence was obtained by RT-PCR and 5′ and 3′ RACE approaches using specific primers designed on the basis of the sequence of the pCuAO 3.1 clone (Fig. 1). The PCR product obtained was directly sequenced and then cloned. *Csao* is 2226 nucleotides long. This size corresponds to the size of the 2.4-kb band estimated by Northern blot analysis. The complete CuAO cDNA contains an ORF of 2010 bp that extends from the ATG at position 26 to the termination codon at position 2036 (Fig. 1). The sequence context of the first ATG matches well the consensus start site (TAAACAATGGCT) for plant genes [22]. The 5′- and 3′-untranslated region are 25 and 192 bp long, respectively. The 3′-untranslated region includes a putative polyadenylation signal AATAAA located 75 bp upstream of the poly(A) tail. *Csao* encodes a mature protein of 649
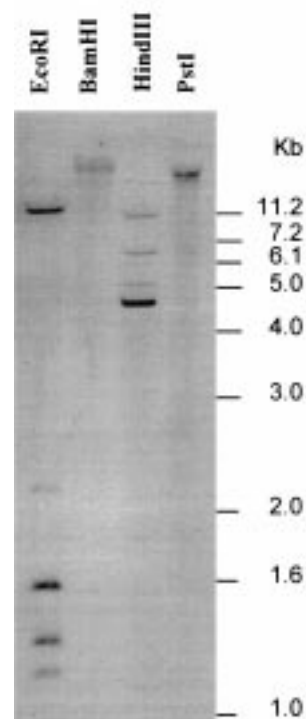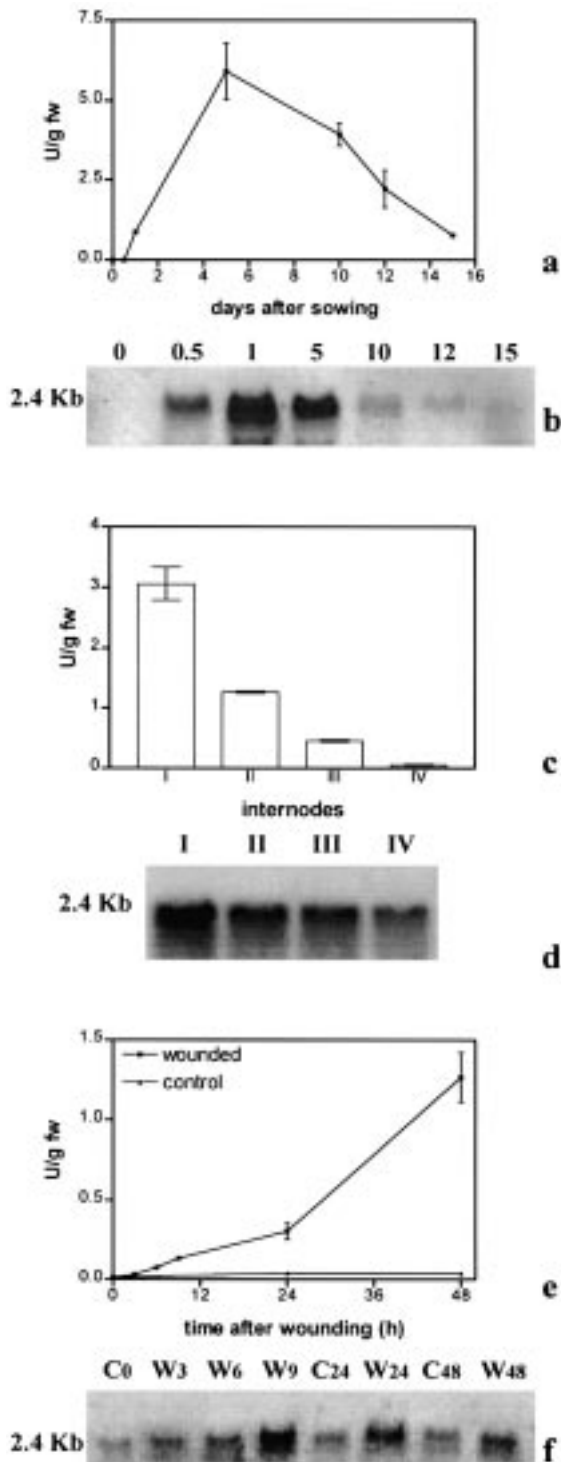

Fig. 2. Southern blot analysis of chickpea genomic DNA. Chromosomal DNA (10 μg) was digested with the indicated restriction enzymes, electrophoresed on 1% agarose gel, transferred on nylon membrane and probed with the full-length digoxigenin-labeled CuAO cDNA.

amino acids (Fig. 1) with a predicted molecular mass of 76.5 kDa. Chickpea CuAO identity was confirmed by comparison to previously reported CuAO primary structures in the SwissProt protein database. In addition, a perfect match of the N-terminal region of the pure protein (boxed in Fig. 1), determined by Edman degradation, was found within the deduced amino acid sequence. Comparative analysis performed on 19 amine oxidase primary structures from bacteria, yeast, plants and mammals, revealed a high sequence homology in the central region and the C-terminal end. Remarkably, these regions contain the 33 conserved amino acids previously observed among CuAOs [23] and, among these, we can highlight the Tyr[387] proposed to form the TPQ cofactor, the three histidines (His[442], His[444] and His[357]) implicated in co-ordination with the copper atom and the Asp[383] probably involved in proton abstraction during the catalysis. The chickpea CuAO presents three putative *N*-glycosylation sites at positions N[131], N[458] and N[558] and of these, only the N[131] and N[558] are conserved in pea and lentil CuAO [23,14]. The amino acid residues from −21 to −1 of the predicted CuAO primary structure show the characteristics expected for a secretion signal peptide. This finding is in agreement with biochemical and histochemical evidence indicating the occurrence of CuAO in the cell wall [11].

3.2. Genomic organisation of chickpea clone

To determine the organisation of the CuAO gene in the chickpea genome, chromosomal DNA was digested with the *Eco*RI, *Bam*HI, *Hin*dIII and *Pst*I restriction enzymes. As shown in Fig. 2, *Eco*RI and *Hin*dIII digestions contain three and two major bands, respectively, as expected, since there are

specialisation in its structure and composition depending on the tissue, developmental stage and environmental growth conditions [1,2]. To check the possible involvement of apoplastic CuAO in cell wall modifications occurring during germination, the steady-state level of CuAO was analysed in chickpea seedlings (Fig. 3). Total RNA and proteins were extracted from embryo and the first internode of epicotyls at different times after sowing and examined by Northern blot analysis and enzyme activity determination. CuAO mRNA is detectable 12 h after sowing, its accumulation reaches a maximum at 1 day, persists at high level in the four successive days, and then gradually decreases until the second week (Fig. 3b). CuAO activity is detectable 1 day after sowing, peaks on day 5, and then slowly decreases until day 15 (Fig. 3a). Interestingly, the accumulation of CuAO mRNA precedes enzyme activity by approximately 12 h. The lag between mRNA accumulation and enzyme activity increase seems to be consistent with the required time for de novo synthesis, high stability, and slow turnover of CuAO, but other controlling mechanisms may be involved.

The spatial expression of CuAO in chickpea seedlings was also studied. Northern analysis was performed in all the four internodes of 10-day-old plants. As shown in Fig. 3d, CuAO mRNA distribution is not uniform, being higher in the basal segment and decreasing sharply toward the seedling apex. The same distribution pattern along the epicotyl was found when CuAO activity was examined (Fig. 3c). This analysis evidences a close correlation between CuAO transcript level, activity and developmental state. These results may be explained hypothesising an active role of CuAO in cell wall remodelling during development. It is evident that cell maturation and growth involve many physical changes in wall structure that participate in the cessation of wall expansion. Among these, the biosynthesis of lignin and suberin and the wall stiffening process which entails the cross-linking of phenolic groups in the wall, such as those of the structural proteins, hemicellulose and lignin [1]. A plethora of evidence has shown a parallelism between CuAO and POD localisation and activity: this correlation has led to the hypothesis that CuAO activity may be a limiting factor in H_2O_2 biosynthesis essential for POD during catalysis of the above mentioned phenomena [11]. This hypothesis is consistent with the data presented, since the cell walls of mature tissues in the basal region of chickpea seedlings contain a higher amount of POD as compared to the cell walls of young expanding tissues in the apical zone [11].

two EcoRI and one HindIII restriction sites in the cDNA clone. Furthermore, in the same digestions other faint bands occur; as a whole, these results provide evidence that in chickpea genome CuAO may either exist as a single gene or belong to a small gene family. However, we cannot exclude that these faint bands correspond to any related genes.

3.3. CuAO activity and mRNA level during development of chickpea seedlings
The cell wall of higher plants displays a high degree of

3.4. CuAO activity and mRNA levels upon wounding
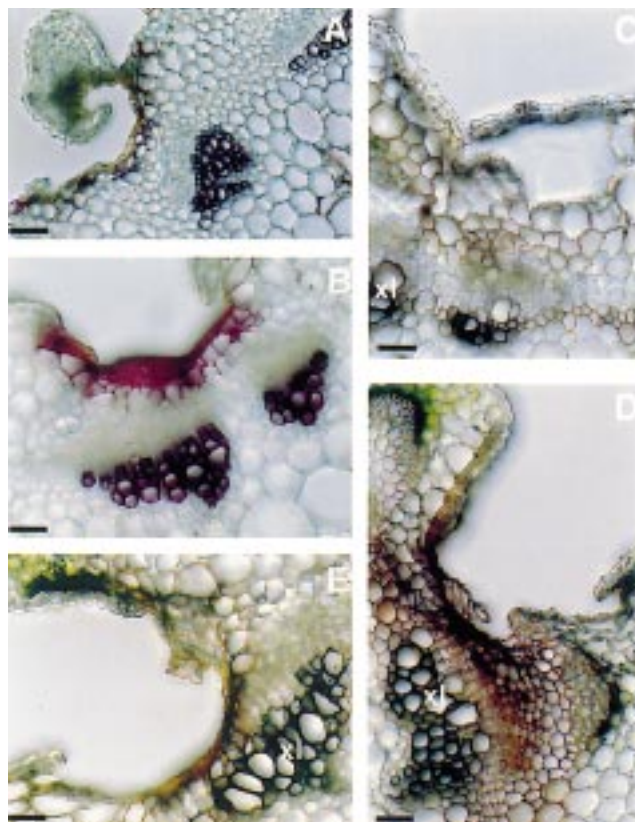Plant cell walls undergo dynamic changes in response to

Fig. 4. Effect of aminoguanidine on CuAO activity and lignin-suberin deposition during wound healing of chickpea seedlings. Ten-day-old chickpea epicotyls were detached, pre-treated with 1 mM aminoguanidine for 12 h, wounded and left in the presence or absence of the inhibitor as described in Section 2. Lignin-suberin deposition (A,B) and CuAO activity (C,D,E) were visualised 3 days after wounding as described in Section 2. Transverse sections from wounded (A) aminoguanidine-treated and (B) untreated chickpea epicotyls, incubated in phloroglucinol/HCl. Histochemical visualisation of CuAO activity in transverse sections from wounded, aminoguanidine-treated (C) and untreated (D) chickpea epicotyls. Control section (E) incubated in absence of putrescine. Magnification: (A) 40×, bar = 137 μm; (B,C,D,E) 50×, bar = 110 μm.

pathogen attack and mechanical wounding that increase wall strengthening, hydrophobicity and redox state. It has been shown that mechanical wounding and pathogen attack induce an increase of CuAO activity in chickpea seedlings [12,13]. This increase is related to the deposition of a lignin-suberin barrier along the wound that should protect the tissues underneath from dehydration and attack by pathogens [4]. To determine whether wounding also modulates CuAO expression, the accumulation of CuAO transcript was analysed in the 4th internode of 10-day-old detached epicotyls injured with a blade. Detachment did not significantly alter the wound-healing process since experiments using the whole seedling showed similar results (data not shown). These experiments were carried out on that internode because, as shown before, it has a low constitutive level of CuAO activity. Fig. 3f shows that an increase in the accumulation level of CuAO mRNA is already detectable at 3 h after wounding. This accumulation reaches a maximum after 9 h and then declines to reach levels similar to those of the corresponding control plants. It is important to underline that during this period the CuAO mRNA levels in the control unwounded plants increased, remaining always

lower than those in the corresponding wounded plants. Fig. 3e shows that CuAO activity has a similar trend during the first phases after wounding and it continued to increase up to 48 h; this result suggests that the newly synthesised enzyme has a very slow turnover. The close correspondence between CuAO activity and mRNA accumulation indicates that transcriptional modulation may be the major event governing the phase of rapid increase in enzyme activity. Post-transcriptional control mechanisms may become important in the later stage of the response, when the level of CuAO transcript decreases. The rapidity of CuAO mRNA induction and the close correlation with stimulation of enzyme synthesis implies that activation of CuAO genes by wounding is an early event. The kinetics of CuAO mRNA accumulation matches very closely that observed for elicitors, pathogens and wound-induced mRNAs encoding the phytoalexin biosynthesis enzymes [24], many enzymes of the phenylpropanoid pathway [25], the hydroxyproline-rich glycoproteins [26], as well as the polygalacturonase-inhibiting proteins [27]. These results, together with the previously reported data on CuAO activity induction in chickpea seedlings upon pathogen attack [13], suggest that this enzyme may have a protective role in defence mechanisms.

3.5. Effect of aminoguanidine on wound-induced CuAO activity and lignin-suberin deposition

To check a possible involvement of CuAO activity in the synthesis of the lignin-suberin protective barrier during wound healing, detached plants were incubated in a solution of aminoguanidine, an irreversible CuAO inhibitor [8] and then wounded. As shown in Fig. 4A, aminoguanidine treatment strongly reduced the deposition of lignin-suberin along the wound as compared to plants not pretreated with the inhibitor (Fig. 4B). Histochemical determination of CuAO showed a parallel effect of aminoguanidine on enzyme activity. CuAO activity is very high along the lesion, as well as in xylem elements of control-wounded plants (Fig. 4D), while it disappears along the wound of plants treated with the inhibitor (Fig. 4C). Sections incubated in the medium for histochemical CuAO detection in the absence of putrescine showed a certain amount of enzyme activity along the wound and in the xylem (Fig. 4E) that could be due, as previously reported [11], to oxidation of the endogenous polyamines released during sectioning. Aminoguanidine did not affect POD activity as deduced by determination of enzyme activity in inhibitor-treated tissues and histochemical POD assay (data not shown). These findings strongly support a role of CuAO as a H_2O_2-delivering system in the POD-mediated synthesis of the lignin-suberin barrier during the wound-healing process.

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