

## Article Addendum

# Wound healing in plants

## Cooperation of copper amine oxidase and flavin-containing polyamine oxidase

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Copper amine oxidases (CuAO) and flavin-containing amine oxidases (PAO) are hydrogen peroxide ( $H_2O_2$ )-producing enzymes responsible for the oxidative de-amination of polyamines. Currently, a key role has been ascribed to apoplastic amine oxidases in plants, i.e., to behave as  $H_2O_2$ -delivering systems in the cell wall during cell growth and differentiation as well as in the context of host-pathogen interactions. Indeed,  $H_2O_2$  is the co-substrate for the peroxidase-driven reactions during cell-wall maturation and a key signalling molecule in defence mechanisms. We recently demonstrated the involvement of an apoplastic PAO in the wound-healing process of the *Zea mays* mesocotyl. Experimental evidence indicated a similar role for an apoplastic PAO in *Nicotiana tabacum*. In this addendum we suggest that a CuAO activity is also involved in this healing event.

Amine oxidases (AO) are an heterogeneous group of enzymes including CuAO and PAO, which oxidize polyamines at the primary and secondary amino group, respectively. Identity of reaction products varies depending on substrate type and mode of oxidation. However,  $H_2O_2$  is a shared product in all the AO-mediated oxidations.<sup>1</sup>

In plants, several biological functions ascribed to AO have been linked to the production of  $H_2O_2$ . Indeed, it has been suggested that important developmental and defence responses depend on the apoplastic production of  $H_2O_2$  carried out by cell wall-localized CuAO and PAO through polyamine oxidation.<sup>1</sup> Polyamine-derived  $H_2O_2$  is involved in cell wall differentiation by driving peroxidase-mediated oxidative cross-linking of cell wall components<sup>2,3</sup> or behaves as second messenger in signalling programmed cell death (PCD) such as, for instance, developmental PCD during vascular differentiation in *Arabidopsis* (*Arabidopsis thaliana*)<sup>4</sup> or hypersensitive response (HR) cell death in tobacco (*Nicotiana tabacum*)

plants infected with tobacco mosaic virus.<sup>5</sup> However, CuAO mediated- $H_2O_2$  production has also been linked to cell expansion in fast-growing tissues of soybean seedlings, inasmuch as the simultaneous apoplastic production of superoxide anion ( $O_2^{\cdot-}$ ) can lead to peroxidase-mediated  $H_2O_2$  reduction to form the wall-loosening agent hydroxyl radical ( $\cdot OH$ ).<sup>6</sup>

CuAO and PAO, due to their abundance in the apoplast of *Leguminosae* and *Gramineae* respectively, have been long considered distinctive enzymes of these two plant families,<sup>7</sup> leaving open the question concerning polyamine catabolism in other plant families and/or cellular compartments. More recently, genetic, biochemical and molecular approaches supported by data-base analysis revealed the presence of a number of putative CuAO and PAO in several plant species,<sup>1</sup> belonging to different plant families. Moreover, prediction of subcellular localization based on the analysis of primary structures suggested the occurrence of AO also in intracellular compartments. In *Arabidopsis* five cDNA encoding for putative PAO<sup>8</sup> and ten cDNA encoding for putative CuAO (Cona unpublished) can be retrieved by database search, only a few of which showing apoplastic localization. In barley (*Hordeum vulgare*) two paralogous PAO genes code for two protein isoforms, namely the likely apoplastic HvPAO1<sup>9</sup> and the vacuolar HvPAO2.<sup>10</sup> Moreover, a CuAO activity has been involved in the supply of  $H_2O_2$  to peroxidase-catalysed reactions during grain filling in the same plant.<sup>11</sup> In maize (*Zea mays*), three PAO gene copies encoding for identical apoplastic proteins have been characterized.<sup>12</sup> This growing information about physiological, biochemical and molecular features of AO in the plant kingdom, made evident the complexity of polyamine catabolism. The apparent redundancy of AO in plants could reflect the need for compartment-specific  $H_2O_2$  synthesis in different phases of development and differentiation as well as in the course of defence mechanisms.

By means of both pharmacological and genetic approaches, we have recently demonstrated that PAO, similarly to what previously demonstrated for CuAO in chickpea (*Cicer arietinum*) plants,<sup>2</sup> plays a role in wound-healing by delivering the  $H_2O_2$  required for suberin polyphenolic domain and lignin synthesis catalyzed by peroxidase.<sup>13</sup> The involvement of *Zea mays* PAO (ZmPAO)-mediated  $H_2O_2$  production in wound-healing events was analyzed both in wounded maize mesocotyl by exploiting the in vivo use of *N*-prenylagmatine (G3), a specific and selective ZmPAO inhibitor, as well as in transgenic tobacco plants constitutively expressing high level of ZmPAO

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in the cell wall. These experiments indicated that G<sub>3</sub> strongly inhibited lignin and suberin polyphenolic domain deposition along the wound periderm in maize mesocotyl and that ZmPAO over-expression in tobacco cell wall greatly accelerates this phenomenon in wounded tobacco stem.<sup>13</sup> As expected, treatment of wounded tobacco plants with the ZmPAO substrate spermidine, enhanced lignosuberized deposition in transgenic-tobacco plants.<sup>13</sup> However, spermidine had a similar effect also in wild type-tobacco plants,<sup>13</sup> suggesting that an endogenous amine oxidase could be involved in wound-healing processes in tobacco plants.

Being spermidine one of the preferential ZmPAO substrate, the identity of the endogenous AO conceivably appeared to be a PAO, likely the cell-wall localized *Nicotiana tabacum* PAO (NtPAO) previously demonstrated to be involved in defence response against pathogens.<sup>5</sup>

However, we unavoidable must consider that spermidine can be oxidized also by CuAO, although with a lower catalytic constant, and that a cell-wall localized CuAO activity has been reported to be involved in defence response against pathogens in tobacco plants as well.<sup>14</sup> Consequently, we explored the possibility that CuAO participates to wound-healing in tobacco, by treating wounded tobacco plants with putrescine (Put), an exclusive CuAO substrate, and two CuAO inhibitors, aminoguanidine (Fig.1) and 2-bromoethylamine (not shown). 8-Week-old tobacco (*Nicotiana tabacum* cv Petit Havana SR1) plants were blade-wounded on the second internode (numbered from shoot apex). After injury, some plants were treated with  $1 \times 10^{-3}$  M Put in 0.01 M sodium phosphate buffer, pH 6.5, by brushing 50  $\mu$ L of the polyamine solution along the wound every 24 h after wounding. Others plants were treated with  $1 \times 10^{-3}$  M aminoguanidine or  $5 \times 10^{-3}$  M 2-bromoethylamine in the presence or absence of Put. After 72 h from injury, cross sections of wounded internodes from treated and control plants, were examined for UV-light induced yellow autofluorescence, as indicative of lignosubertization level along wound periderm cell walls, accordingly to Sherf et al.<sup>15</sup> The presence of a strong yellow autofluorescence in the innermost part of the wound periderm of Put-treated plants (Fig. 1B), not yet detectable in untreated plants (Fig. 1A), indicates that this polyamine accelerates deposition of lignin and suberin polyphenolic domain along the wound, presumably through CuAO-mediated H<sub>2</sub>O<sub>2</sub> delivering in the cell wall. The absence of yellow autofluorescence in cross section of plants simultaneously treated with Put and aminoguanidine (Fig. 1C) or 2-bromoethylamine (not shown), revealed that CuAO inhibition blocks Put-induced lignosubertization along wound periderm, suggesting the involvement of an apoplastic CuAO activity in the cicatrization process.

Presently, a number of evidence suggest the occurrence of both CuAO and PAO activity in the same cellular compartment of tobacco plants. Moreover, our experiments seem to suggest that these enzymes could be active at the same time in the same tissues. In our opinion, the likely simultaneous presence of CuAO and PAO activity in tobacco cell wall indicate either the need of a composite control on polyamine catabolism-dependent H<sub>2</sub>O<sub>2</sub> production, or, analogously to what previously suggested in tobacco vascular tissues undergoing lignification,<sup>16</sup> the occurrence of a cooperation of these two enzymes in the AO-mediated oxidative burst during plant defence responses following mechanical damage or pathogen attack.

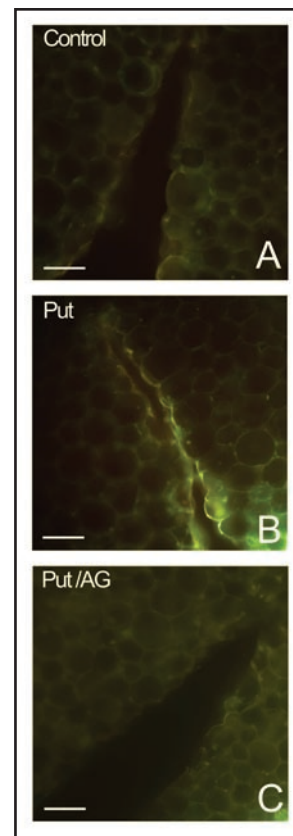


Figure 1. UV-induced autofluorescence microscopic analysis in wound-healing tobacco plants. Histochemical analysis was performed in hand-cut cross sections (~100  $\mu$ m thick) obtained from the wounded zone of the second internode (numbering from the shoot apex) at 72 h after wounding. Sections were directly mounted on slides and observed for autofluorescence under UV light. (A) Put-untreated plants (Control); (B) Put-treated plants; (C) plants simultaneously treated with Put and aminoguanidine (AG). Bar = 100  $\mu$ m.

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