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Full Length Research Paper

Change in antioxidant and lignifying enzyme activities in rubbing tomato (*Solanum lycopersicum*) internodes

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In tomato plant, rubbing applied to a young internode inhibits elongation of the rubbed internode and its neighbouring one. These morphological changes were correlated with an increase in lignification enzyme activities, phenylalanine ammonia-lyase (PAL) and peroxidases (POD), 24 h after rubbing of the forth internode. Furthermore, a decrease in indole-3-acetic acid (IAA) content was detected in the rubbed internode and the upper one. Lignin synthesis in tomato plant measured 14 days after mechanical stress application was significantly stimulated in the rubbed internodes (n°4) as compared with the control. Fourier transform infrared (FTIR) analyses revealed that lignin synthesized in response to mechanical elicitation displayed a distinct structure, substantially enriched in syringyl (S) units, as compared to constitutive lignin. Taken together, our results suggest that the decrease in rubbed internode length is as a result of IAA oxidation, increases in enzyme activities (PAL and POD) and cell wall rigidification induced by lignification process.

Key words: Mechanical stimulation, *Solanum lycopersicum,* indole-3-acetic acid (IAA), Fourier transform infrared (FTIR), lignin.

INTRODUCTION

In their environment, plants are constantly subjected to several stimuli such as wind, rain and wounding. The growth response of plants to such stimuli was termed thigmomorphogenesis and was observed in a wide range of plants (Braam and Davis, 1990; Hofmann, 2009; Monshausen and Simon, 2009). The most common thigmomorphogenetic response is a retardation of tissue elongation accompanied by an increase in thickness (Biro et al., 1980). The plant response to mechanical perturbbation is mainly restricted to the young developing internode, since no influence can be detected when the internode has reached its final length (Erner et al., 1980; Dépège et al., 1997). These plant growth modifications, which characterize thigmomorphogenesis, are related to biochemical events associated with lignification process (De Jaegher et al., 1985) and ethylene production (Boyer et al., 1983).

An almost ubiquitous feature of plant responses to mechanical stress, compatible pathogens or to elicitors (Massala, 1987) is the activation of phenylpropanoid metabolism in which phenylalanine ammonia-lyase (PAL) catalyses the first committed step of the core pathway of general phenylpropanoid metabolism. Branch pathways lead to the synthesis of compounds that have diverse functions in plants, notably in defence, such as cell wall strengthening (Cabané et al., 2004; Cai et al., 2006).

To counteract mechanical damages, plants create a physical barrier including the synthesis of lignin and suberin (Walter et al., 1990; Grabber et al., 2004). Lignin is a complex polymer composed of phenyl propane units derived from three cinnamyl alcohols (monolignols), p-

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Abbreviations: FTIR, Fourier transform infrared; **IAA,** indole-3-acetic acid; **PAL,** phenylalanine ammonia-lyase; **POD,** peroxidase; **MIS,** mechanically-induced stress; **HPLC,** high performance liquid chromatography; **G-POD,** guaiacol peroxidase; **CAT,** catalase; **EDTA,** ethylene diamine tetraacetic acid; **S-POD,** syringaldazine-peroxidase; **SOD,** superoxide dismutase.

coumaryl, coniferyl and sinapyl (Whetten et al., 1998; Boudet, 2000). After mechanical stimulation, the increase in lignification processes was correlated with the enhancement of PAL and peroxidases (POD) activities (De Jaegher et al., 1985). In several plants subjected to environmental injury, increase in POD activity was inversely related to plant growth. It was shown that peroxidases are involved in several metabolic plant processes such as auxin catabolism, bridges formation between components of the cell wall and oxidation of the cinnamyl alcohols before their polymerisation during lignin and suberin formation (Quiroga et al., 2000; Delannoy et al., 2003; Passardi et al., 2004). Thus, stress induced stimulation of peroxidase activities is thought to be involved in the defence mechanism of plants (Castillo, 1992; Cipollini, 1997).

With evidence of growth inhibition in the rubbed plant internode, and the suggestion that lignification and IAA oxidation may be involved, we investigated here the changes in lignin content in the rubbed tomato internodes and its relation to PAL and POD activities.

MATERIALS AND METHODS

Plant material and growth conditions

Tomato plants (*Solanum lycopersicum* var. Ventura) were raised from seeds in moistened filter paper for 6 days at 25°C in the dark. At cotyledon stage, plants were transferred to plastic beakers (6 L capacity, 6 plants per beaker) filled with continuously aerated nutrient solution (Morizet and Mingeau, 1976). Plants were grown in a controlled environment room equipped with incandescent lamps delivering a photon flux density of about 250 µmol m^{-2} s^{-1} for 16 h at 24°C. During the 8 h dark period, the temperature was 18°C. The relative humidity varied between 70 and 80%. The nutrient solution was replaced every 2 days and pH was checked and adjusted daily to 5.5 to 5.6.

Application of mechanical stress

Mechanical stimulus was applied to 3-week-old plants with six developed internodes. The young growing internode 4 (N4) from the bottom of the plants was held between the thumb and forefinger and rubbed back and forth as previously described by Boyer et al. (1983). Twenty four hours after mechanical stimulation, plants were harvested and the forth (N4) and fifth (N5) internodes from the shoot bottom of each plant were sampled and used for all subsequent analyses. Internode lengths of control and rubbed plant were measured 14 days after mechanical stimulation.

Protein determination

Protein quantity was determined using protein assay reagent from Bio-Rad (Munich, Germany) with bovine serum albumin (BSA) as standard according to Bradford (1976).

Enzyme assays

Phenylalanine ammonia-lyase (PAL)

Crude enzyme extracts were prepared according to Westcott and Henshaw (1976) by homogenising 5 g of frozen tissues powder with 10 ml of 0.1 M sodium borate buffer (pH 8.8). The reaction mixture which contained 0.8 ml supernatant and 1.2 ml of 50 mM L-phenylalanine in sodium borate buffer (200 mM, pH 8.8) was incubated at 37°C for 90 min. The reaction was finished by adding HCl (6N). PAL activity was assayed by measuring the absorbance of transcinnamic acid at 290 nm. Enzyme activity was calculated as nmol of trans-cinnamic acid produced from L-phenylalanine per min per mg of protein. The experiment was carried out in triplicate in three independent sets of experiments.

Peroxidases (POD)

Peroxidase enzymes were extracted from 5 g of frozen tissue powder with 10 ml of sodium phosphate buffer (200 mM, pH 6.0) as described by Sitbon et al. (1999). The incubation mixture for the determination of guaiacol-peroxidase (G-POD) activity consisted of 50 mM sodium phosphate buffer (pH 6.0), 5 mM gaiacol, 5 mM H_2O_2 and 50 µl of tissue extract. The increase in absorbance at 470 nm at 30°C was recorded for 2 min. One unit of G-POD activity was defined as the amount of enzyme which caused a change of 0.01 in absorbance per minute under the specific conditions and the data are expressed on a protein basis.

Syringaldazine-peroxidase (S-POD) activity was assayed in a reaction medium containing 50 mM phosphate buffer (pH 6.0), 0.05 mM syringaldazine, 0.2 mM H_2O_2 and 50 µl of tissue extract. One unit of S-POD activity was defined as the amount of the enzyme which caused a change of 0.01 in absorbance at 530 nm per minute at 30°C under the specified conditions and the data are expressed on a protein basis. It was repeated four times.

Catalase

Plant material was extracted in 50 mM potassium phosphate buffer (pH 7.0) containing 5 mM sodium ascorbate and 0.2 mM ethylene diamine tetraacetic acid (EDTA). The homogenate was centrifuged at 13 000 g for 15 min. The resulting supernatant was used for assays of catalase (CAT) activities as described by Aebi (1984).

Auxin analysis

Control and rubbed internodes were homogenized in liquid nitrogen and extracted with 4 ml of phosphate buffer (pH 6.5). Fifty microliter (50 µl) butylated hydroxytoluene (BHT) as antioxidant and 30 µl IAA^3H as internal standard were added to the homogenate. After 1 h in darkness, the samples were centrifuged for 10 min at 14.000 g, the supernatants were filtered with Whatman GF/C filter and washed with 4 ml of phosphate buffer (pH 6.5) before depositing on Bond-Elut C18 columns activated and conditioned to pH 6.5. The eluates were acidified to pH 2.5 with 2.8 M phosphoric acid and then applied to C18 columns activated and conditioned to pH 2.5. The columns were washed with 2 ml distilled water and 2 ml of acetic acid/ethanol mixture (ethanol : acetic acid : water, 20:2:78, v/v/v). Auxin was eluted from the second column with 100 µl of absolute methanol and 500 µl of methanol (80%, v/v). Fifty microliter was injected in a fully automated Merck-Hiitachi HPLC system. The high performance liquid chromatography (HPLC) column was a Merck Lichrocart 100RP18, 12.5 cm long, 5 µm particle sizes, solvent and column temperatures were 30°C, and the mobile phase was acetonitrile/acetic acid/water (10:2:88, v/v/v). The eluate was monitored with a fluorescence detector (excitation at 292 nm; emission at 358 nm); the elution patterns were similar to those shown by Nordstrom et al. (1991).

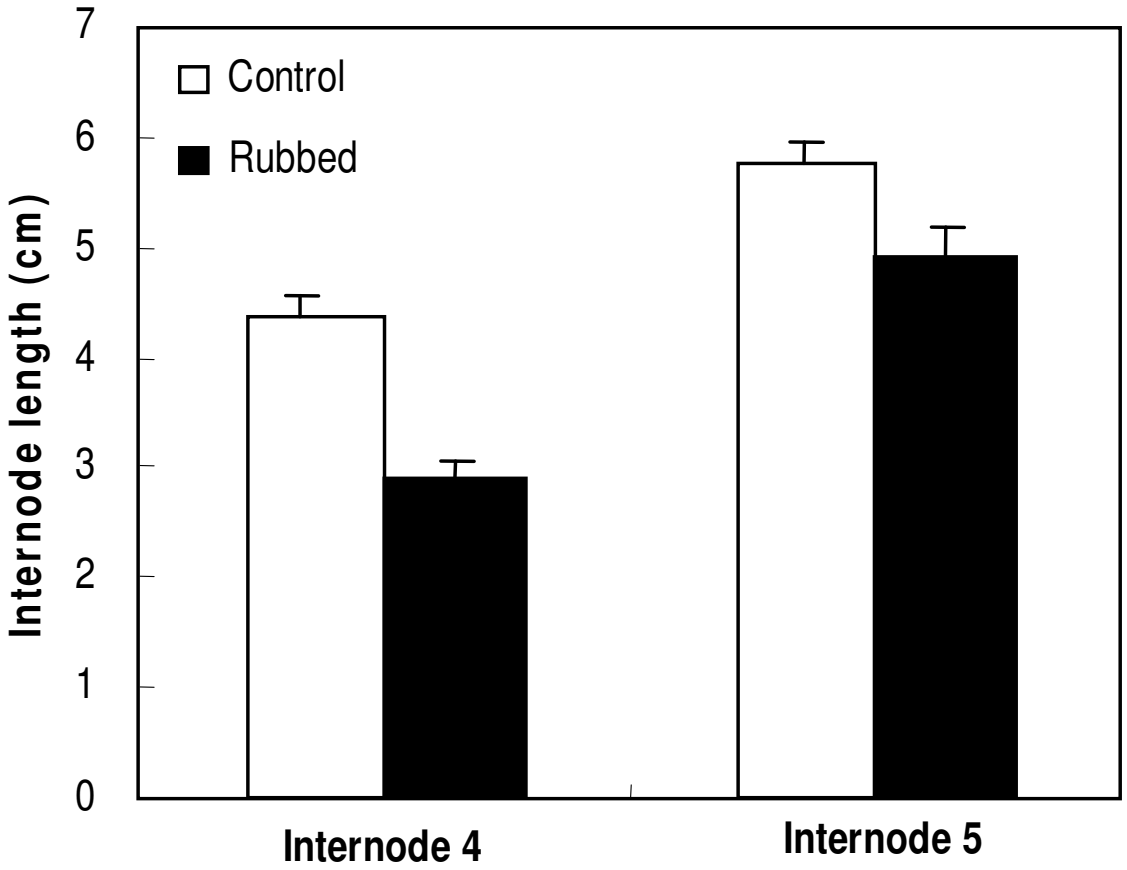

Figure 1. Internode lengths of control and rubbed plants measured 14 day after mechanical stress applied to the fourth internode. Standard errors are indicated by vertical bars.

Lignin analysis

The internodes 4 and 5 of control and rubbed tomato plants were collected, dried and ground before exhaustive extraction in a Soxhlet apparatus by ethanol-toluene (2:1, v/v) mixture, ethanol and water. The extract-free samples were dried before lignin analysis. The determination of lignin content of extract-free samples was performed by the gravimetric Klason method (Dence, 1992).

Fourier transform infrared (FTIR) analysis

The internodes 4 and 5 of control and rubbed tomato plants were collected, dried and ground before spectroscopy FTIR analysis. An area of 50 mm^2 was selected for spectral collection. Spectra were collected using a ThermoNicolet Nexus spectrometer (Madison, WI) with a continuum microscope accessory. Fifty interferograms were collected in transmission mode with 8 cm^{-1} resolution and coadded to improve the signal-to-noise ratio of the spectrum. Spectra were then baselined and normalized as described by Robin et al. (2003). Student's t test was applied as described by Mouille et al. (2003).

Statistical analysis

Statistical analyses were carried out with STATISTICA for windows using Student's test with $p < 0.05$.

RESULTS

Effect of mechanical stimulation on internode elongation

The length of internodes 4 (N4) and 5 (N5) was measured 14 days after rubbing of the fourth internode. Results reported in Figure 1 show that rubbing led to a significant reduction of elongation of the stressed internode (N4) (decrease of N4 length from 4.3 cm in the control plant to 2.9 cm in the rubbed one). This effect was not limited to the rubbed area but also affected the elongation of the neighbouring internodes (N5) that were shorter in rubbed plants than in control ones.

Enzyme activities

Catalase and peroxidase activities were investigated in internodes 4 and 5, 24 h after rubbing of the fourth internode. Results show that catalase activity was significantly enhanced by mechanical stress application in both internodes N4 and N5 (Figure 2), as compared

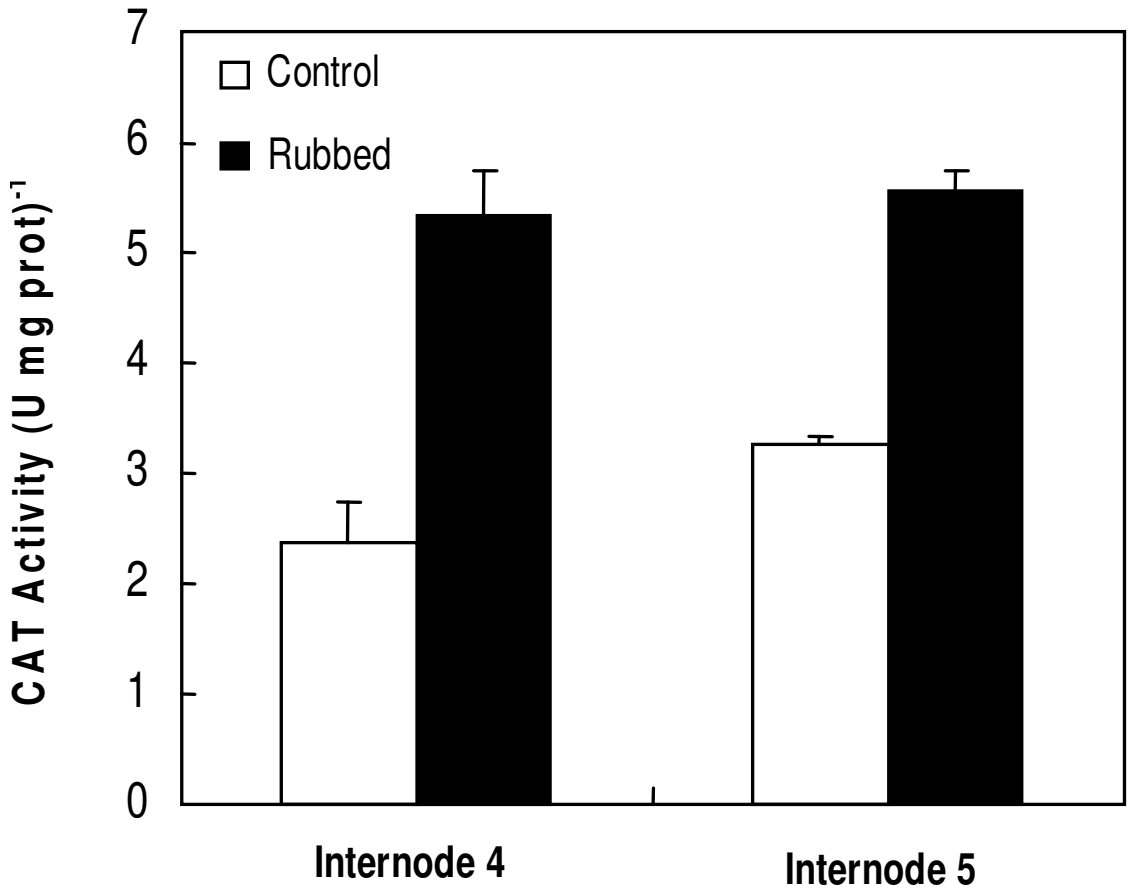

Figure 2. Catalase (CAT) activity of internode 4 and 5 in control and rubbed plants 24 h after rubbing of the fourth internode. U = 1 unit OD min^{-1}. Standard errors are indicated by vertical bars

with corresponding controls. Guaiacol peroxidase (G-POD) activity was strongly increased in the rubbed internode N4 (80,72U) when compared to control tomato internodes (30,80U) (Figure 3). Further, G-POD activity in N5 was also enhanced in the rubbed internode (26,35U) as compared with the control (13,05U).

Figure 4 shows that syringaldazine peroxidase activity was enhanced in the rubbed internode N4 and the upper one, N5. In the same way, PAL activity was increased in internode N4 and N5 with respect to control (Figure 5).

Auxin analysis

Indole-3-acetic acid (IAA) was quantified in control and rubbed plant internodes 24 h after rubbing of the fourth internode. Results reported in Figure 6 shows that in control sample and as expected, the content of indole-3-acetic acid was found to be higher in the younger internode (N5) as compared to the older one (N4). Rubbing led to a significant decrease in IAA levels in N4 (5.06 nmol g/FW) as compared with corresponding controls (7.27 nmol g/FW). Similar results were observed in internode 5, where IAA content was reduced from 16.52 nmol g/MF in control internode to 12.35 nmol g/FW in the rubbed internode (Figure 6).

Lignin analysis

For lignin analysis, N4 and N5 were harvested 14 days after rubbing the fourth internode (N4) and subjected to an exhaustive solvent extraction in order to recover a cell wall residue without any extractives that could interfere with the lignin determination. The lignin content of the extract-free internodes was determined by the gravimetric Klason procedure (Dence, 1992). While the Klason lignin levels of the youngest internode N5 were found to be similar (Table 1), the cell walls of the rubbed internode N4 was found to be significantly enriched in lignin by the mechanical treatment (increase from 7.6% in the control to 10.0% in the mechanically stressed samples, Table 1).

FTIR analysis

There are many studies that have identified specific

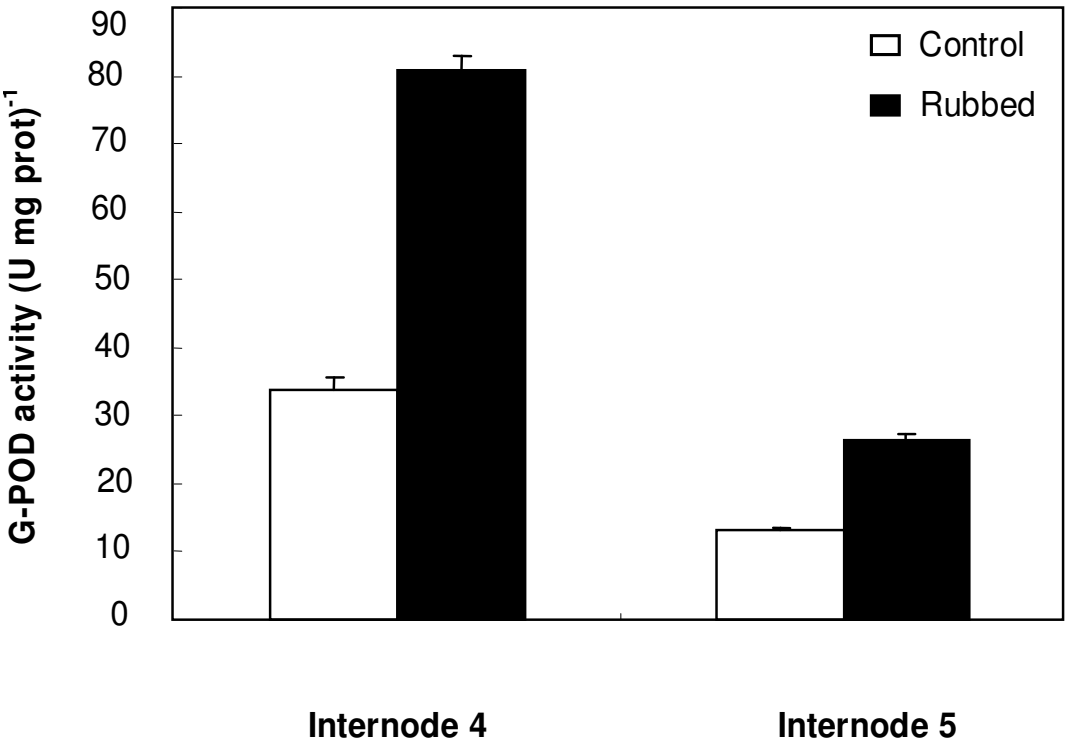

Figure 3. Guaiacol-POD (G-POD) activity of internode 4 and 5 in control and rubbed plants 24 h after rubbing of the fourth internode. U = 1 unit OD min^{-1}. Standard errors are indicated by vertical bars.

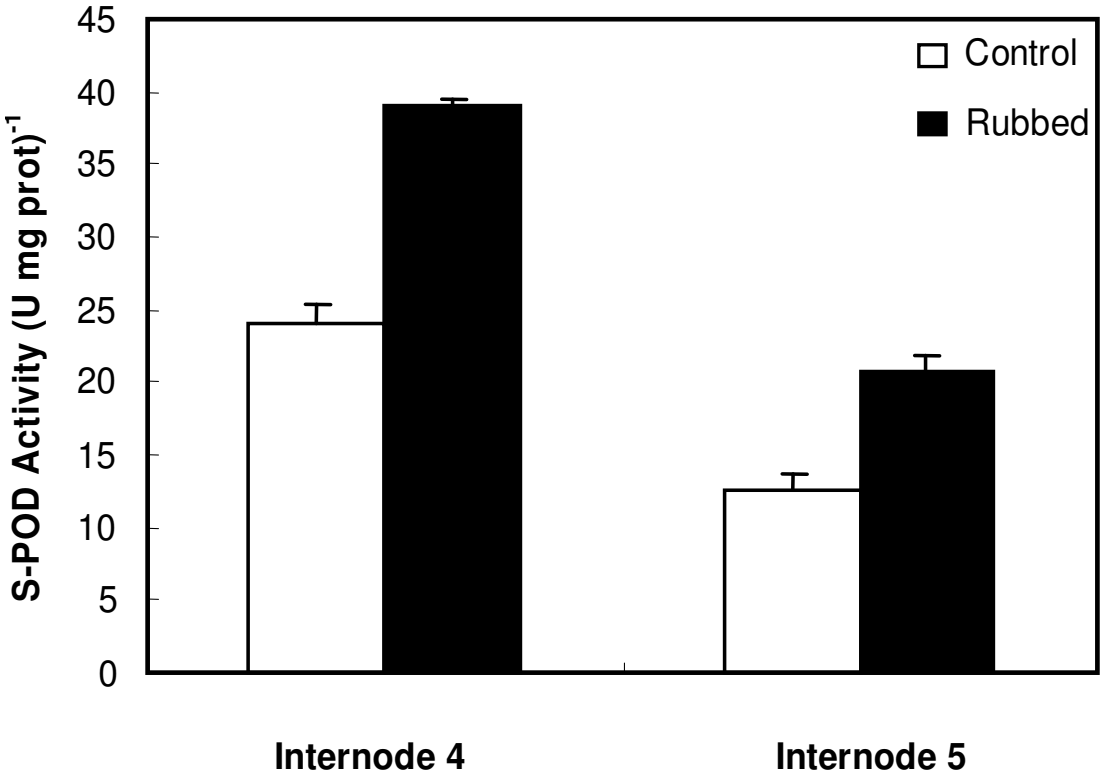

Figure 4. Syringaldazine-POD (Syr-POD) activity of internode 4 and 5 in control and rubbed plants 24 h after rubbing of the fourth internode. U = 1 unit OD min^{-1}. Standard errors are indicated by vertical bars.

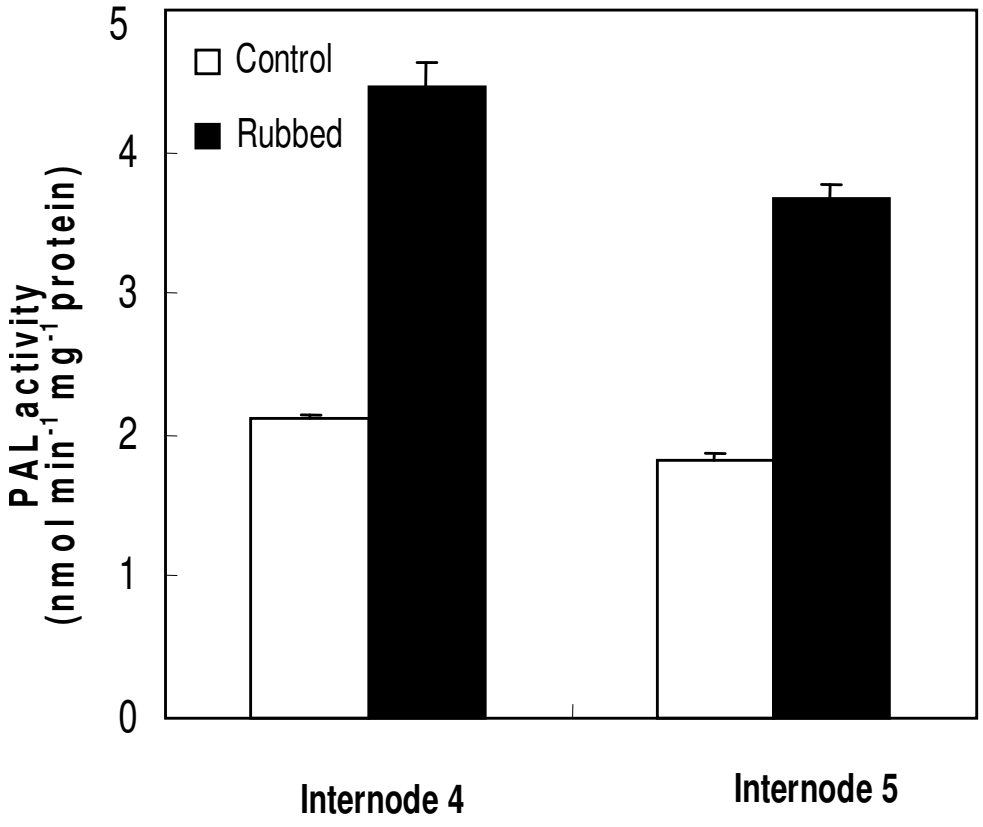

Figure 5. PAL activity of internode 4 and 5 in control and rubbed plants 24 h after rubbing of the fourth internode. Standard errors are indicated by vertical bars.

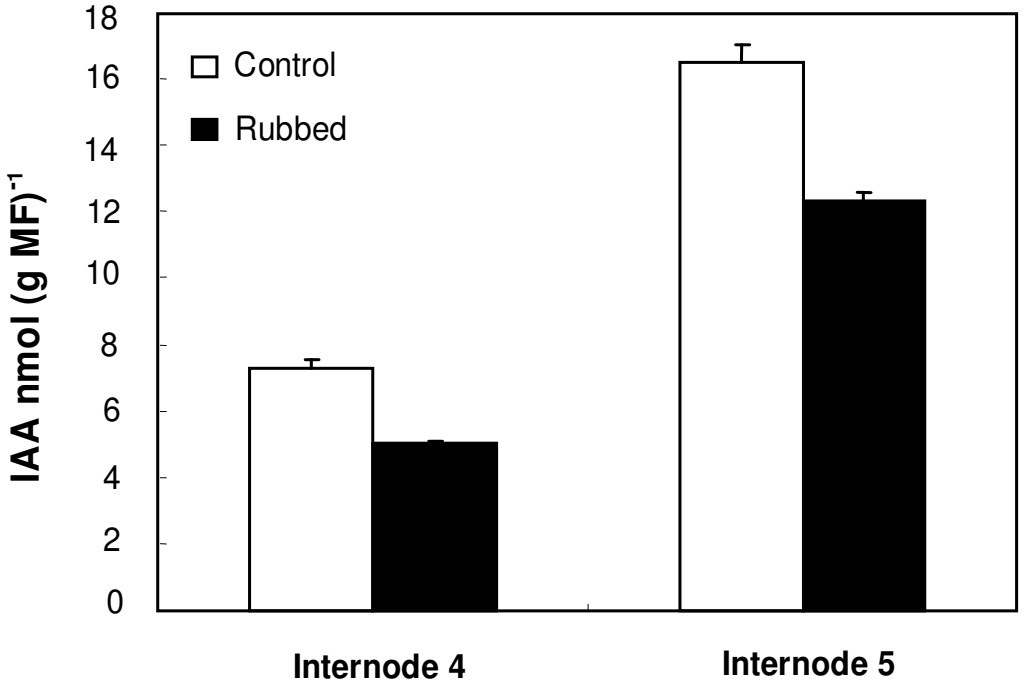

Figure 6. IAA level of internode 4 and 5 in control and rubbed plants 24 h after rubbing of the fourth internode. Standard errors are indicated by vertical bars.

Table 1. Lignin analysis of control and rubbed tomato internodes. The Klason lignin (KL) content is expressed as weight percentage of free and dry sample extracts. The data are mean values ± SE of duplicate or triplicate analyses.

Sample	KL (% by weight)
Control (Internode 4)	7.68 ± 0.06
Treated (Internode 4)	10.08 ± 0.02
Control (Internode 5)	10.48 ± 0.13
Treated (Internode 5	9.13 ± 0.02

infrared vibrations that can be assigned to individual cell wall components or to combination of components (Blaschke et al., 2004). Cell wall chemical changes induced by mechanical rubbing were analysed in N4 and N5 of tomato plants by FTIR spectroscopy.

Difference in spectra modifications between control and rubbed internodes were analysed within the range of 1800 to 830 cm^{-1} (Figure 7). Tissue absorbance indicated that difference in spectra from 1288 to 1234 cm^{-1} are consistent with an induced lignification in the rubbed tomato internode (N4) as compared with the control. Absorbance at these wave lengths was not found in the upper tomato internode (N5). FTIR spectra of N4 exhibit a notable increase in absorbance centred at 1349 cm^{-1}. Absorbance in this region can be assigned to syringyl (S) units (Leplé et al., 2007), suggesting an enrichment of new lignin synthesis in the rubbed internode, with S lignin units. By contrast, no clear-cut effect could be evidenced for internode N5 (Figure 7).

DISCUSSION

The results reported here establish an evident correlation between growth limitation of the rubbed internode and their degree of lignification, the increase in enzymes activities and auxin degradation after mechanical stress application.

Elongation was inhibited in the rubbed internode (N4) and in the upper one (N5). Hence, the morphological response induced by mechanical stress affected young organs in which elongation of the cell wall was still possible. Young tissues seemed to be especially sensitive to mechanical stimulation even when they were not directly handled. Similar results were obtained in beans and tomato plants subjected to mechanical stress (Coutand et al., 2000). Inhibition of internode 5 elongation suggests that the signal received by the stressed internode was translocated from the rubbed site to the upper part of the plant. Coutand and Moulia (2000) show that mechanosensing is local and scattered through the stem of tomato plant submitted to a basal binding. In beans, Erner et al. (1980) proposed the involvement of a translocable factor, of either hormonal or electrical origin. In *Bidens pilosa*, mechanical signal is rapidly transmitted over all the plant via the induction of an electric depolarization wave (Frachisse et al., 1985). In contrast, in *Bryonia dioica*, the morphological response was exclusively detected in the rubbed internode (Broyer, 1983). Differences in plant structure and morphology could explain the heterogeneity of response outside the stimulated site.

Auxin seems to be involved in thigmomorphogenesis (Erner and Jaffe, 1982). It was proposed that mechanically-induced stress (MIS) has opposite effects on auxin levels in the two species studied to date, *Phaseolus vulgaris* (Erner and Jaffe, 1982) and *Bryonia dioica* (Hofinger al., 1979). Auxin level as measured by bioassay, increased in *P. vulgaris* following rubbing of the stem (Erner and Jaffe, 1982). It was proposed that a build up of auxin may result from the reduced polar transport of IAA at the rubbed internode, causing a build up of IAA in the stem tissue. Exogenous indole-3-acetic acid (IAA) did not reverse the MIS inhibition of growth in *P. vulgaris* and high levels of IAA retarded growth in non-stressed plants (Erner and Jaffe, 1982). Thus, retardation of extension growth in *P. vulgaris* may have been caused by high levels of endogenous auxin.

In *Bryonia dioica*, auxin catabolism was accompanied with changes in both soluble and ionically bound cell wall basic peroxidases (Grambow and Langenbeck-Schwich, 1983) and the appearance of an additional peroxidase. This suggests that auxin catabolism is hastened by mechanical stimulated peroxidase. In addition, Boyer et al. (1983) reported that lithium pre-treatment prevents both thigmomorphogenesis and appearance of specific cathodic isoperoxidase in Bryonia plants subjected to MIS. This gives further credence to the possibility that the peroxidase-auxin system is involved in Bryonia thigmomorphogenesis. In addition, ethylene increases peroxidase activity which reduces the auxin content in the tissue to a level low enough not to support normal growth. We have evidence that decrease of auxin level contribute to the mechanism leading to tomato internode inhibition subjected to mechanical stress.

PAL, a key enzyme of phenylpropanoid pathway, was

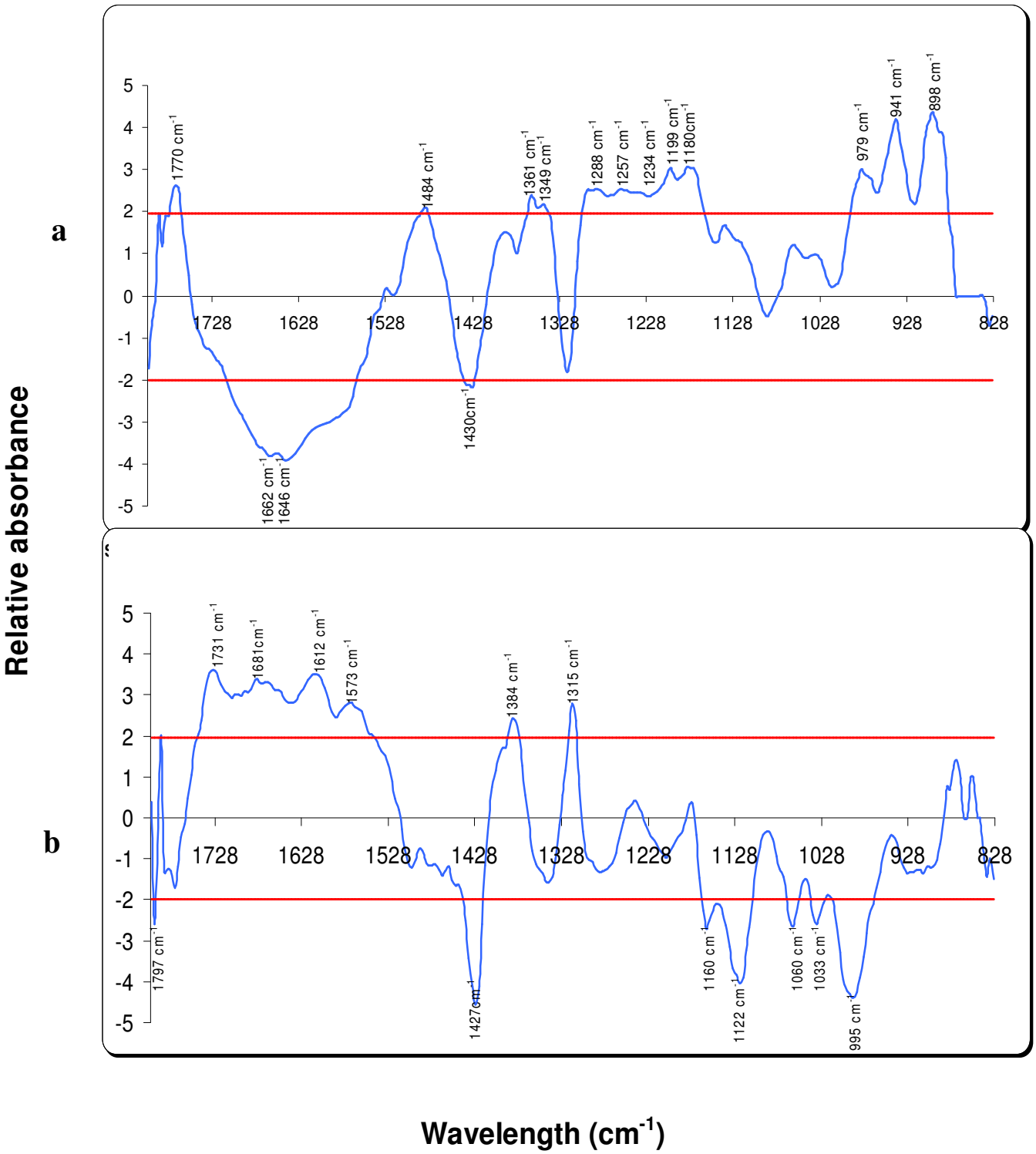

Figure 7. Vibration spectra from control and rubbed tomato internodes. a: Internode 4; b: Internode 5.

activated by mechanical stress application. It was shown that PAL is generally stimulated in plant tissues exposed to several environmental stresses (Jaffe, 1973). The same authors indicated that PAL enhancement in these stress conditions is due to H_2O_2 generation, which occurs as primary reaction in response to stress. So, it seems that, in tomato internode, the enhancement of PAL activity could be related to the implication of this enzyme in the plant response to mechanical stress.

Growth inhibition has been suggested to be the result

of tissues lignification (Dépège et al., 1997). As the initial enzyme in the monolignol biosynthesis pathway, PAL has a direct influence on lignin accumulation (Campos et al., 2004). The characteristics of lignin differ among cell wall tissues and plant organs (Grabber et al., 2004). It comprises polyphenolic polymers derived from the oxidative polymerization of different monolignols, including p-coumaryl, coniferyl and sinapyl alcohols via a side pathway of phenylalanine metabolism leading to lignin synthesis (Whetten and Sederoff, 1995). The increase in lignin content in the rubbed tomato internode could be a response mechanism to mechanical stress. It is known that plants create a natural barrier that includes lignin and suberin synthesis, components directly linked to support systems (Vance et al., 1980; Aquino and Mercado, 2004).

Mechanical stress-induced membrane depolarization would generate different species of free radicals and peroxides (Monshausen et al., 2009), which in turn initiate lipid peroxidation (Dépège et al., 2000). Such damages could be mitigated and repaired by antioxidative enzymes like catalase (CAT), superoxide dismutase (SOD) and peroxidases (Ben Rejeb et al., 2004). The degradation of cell membranes is suggested to bring about rapid changes in ionic flux (Weerasinghe et al., 2009), especially release of K^+ which would result in an enhanced endogenous Ca/K ratio and in leakage of solutes, among them are electron donors such as ascorbic acid and phenolic substances. The increased intracellular relative calcium level activated secretion of basic peroxidases (Van huystee and Esnault, 1995) into the free space where, in association with the electron donors and possibly with the circulating IAA, they eliminate the peroxides and facilitate the binding of basic peroxidases to membrane structures allowing the role of 1-aminocyclopropane-1-carboxylic acid (ACC)-oxidases.

The resulting IAA and ACC oxidase-mediated changes in ethylene production (Biro and Jaffe, 1984) would further induce the activity of PAL and peroxidases (this time through the protein synthesis machinery). The resulting lignification and cell wall rigidification determines the growth response of tomato internode to the mechanical stress.

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