Thigmomorphogenesis in *Solanum lycopersicum*: Morphological and biochemical responses in stem after mechanical stimulation

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**Abstract**

*Solanum lycopersicum* Mill. growth responses to mechanical stimulation showed that rubbing applied to the fourth tomato internode inhibit elongation. This morphological change was correlated with an enhancement in lignification process. Stress lignins synthesized in response to this mechanical elicitation displayed a distinct structure, relative to constitutive lignins. They were found substantially enriched in syringyl (S) units.

Peroxidase (EC 1.1.1.7) activity investigated in the rubbed internode was induced 24 h after stress application. In order to get more information about peroxidase implication in growth restriction observed in internode 4, protein isoform purification was performed using concanavalin and cation exchange chromatography. The Western blot analysis of the purified protein extract from rubbed internode revealed the appearance of a novel basic peroxidase isoform. Moreover, an increase in the basic isoform activity was observed in rubbed internode as compared with the control one.

Increased activities of cinnamyl alcohol dehydrogenase (CAD) (EC 1.1.1.197) and of peroxidase (EC 1.1.1.7), enzymes designated as markers of lignification process were associated with a higher lignin content in the rubbed internode. Cell wall rigidification as a result of changes in enzyme activities and accelerated lignification could explain the reduced elongation of treated tomato internodes.

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**1. Introduction**

Environmental stimuli affect numerous aspects of plant growth and development process. Generally, many of these stimuli induce specific responses. Mechanical stress (MS) such as touch, wind or vibration usually induces morphological growth changes in stressed plants [1–4]. The most common response to mechanical stress found in a wide variety of plant species is a delay in elongation [5]. The growth response of plants to such stimuli was termed thigmomorphogenesis by Jaffe [6], and was documented in all types of plants, shrubs, trees, grasses, and dicot herbs.

Biochemical responses to mechanical stress are complex. Specifically, at the cellular level it has been shown that mechanical stress affect the cell wall by both a decrease in cell expansion and an increase in stem monolignol polymerisation [7]. In young rubbed *Bryonia dioica* internodes, inhibition of elongation has been shown to be correlated with a rapid rise in peroxidase activity, which in turn induces an increase in lignin content [8,41]. Peroxidases are ubiquitous enzymes that catalyse the oxidation of a variety of organic and inorganic substrates using hydrogen peroxide as oxidant. These enzymes have been involved in several physiological and biochemical processes, such as cell growth and expansion [9,10], auxin catabolism (Grambow et al., 1983), and lignification process [11–13], as well as abiotic and biotic stress responses.

Cipollini [14] demonstrated the induction of soluble peroxidase, cinnamyl alcohol dehydrogenase activities, and lignification in leaves of 7-day-old bean seedlings by short daily periods of non-injurious wind-induced mechanical perturbation. Peroxidase induction may contribute to such cell wall toughening events by phenolic cross-linking and lignification, which can strengthen leaf and stem tissues against potential damage caused by mechanical perturbation [15–17]. Increase in peroxidase activity was observed in several plants subjected to environmental injury, thus, peroxidases have
often been used as biochemical markers of stress involved in the defence mechanism of plants [4,18].

The purpose of the present work was to determine to what extent the cellular and biochemical mechanisms leading to the inhibition of tomato internode elongation might result from cell wall rigidification through the lignification process. Perooxidase and CAD activities were investigated through biochemical methods and the level of lignification was determined in rubbed and control internodes. Perooxidase isoenzymes, representing the majority of the total activity in the forth internode were purified and analyzed by Western blot, in order to determine which peroxidase was involved in this process.

2. Methods

2.1. 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 filled with continuously aerated nutrient solution [19]. 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%.

2.2. Application of mechanical stress

Mechanical stimulus was applied to 3-week-old plants with six developed internodes. The young growing internode no. 4 was held between the thumb and forefinger and rubbed back and forth. Rubbing treatment was done once for 10 s as previously described [7]. Internode lengths of control and rubbed plant were measured 14 days after mechanical stimulation.

2.3. Enzymes assays

2.3.1. Peroxidases

Tissues of internodes 4 and 5 were ground in ice-cold extraction buffer containing 25 mM Bis–Tris, pH 7.0, 200 mM CaCl2, 10% glycerol (v/v), 4 μM Na-cacodylate, and 1/200 (v/v) protease inhibitors (Sigma L-9599) and blended for 5 min. The homogenates were centrifuged successively two times at 8 °C for 10 min at 4000 × g. The supernatant was additionally centrifuged two times at 8 °C, for 10 min at 4000 × g then for 60 min at 13,000 × g. The resulting crude extract was used for enzyme assays and chromatographic analyses.

Peroxidase activity was measured in 100 mM Tampon acetate citric acid, pH 6, in the presence of H2O2 0.06%. Tetra methyl benzidine (TMB) oxidation was monitored by the increase in absorbance at 450 nm, activity was expressed as U (mg protein)−1, with U = 1 unit OD min−1. Protein quantity was determined using protein assay reagent from Bio-Rad (Munich, Germany) with BSA as standard according to Bradford [20].

2.3.2. Cinnamyl alcohol dehydrogenase

Proteins extracts were assayed spectrophotometrically for alcohol dehydrogenase activity by oxidation of coniferyl alcohol. Assays were carried out at 25 °C for 60 min in 240 μL of 100 mM Tris–HCl (pH 8.8), NADP (10 mM), and 10 mM of coniferyl alcohol using a micro-ELISA plate. Five to 100 μg proteins from stem extracts were used for these reactions. Formation of coniferaldehyde was monitored at 405 nm as described by Goffner et al. [21]. An assay without NADP was used as a control. Resulting units are defined as the amount of activity that converts 1 nmol of coniferyl alcohol into the corresponding aldehyde per second (1 nKatal) per microgram of crude protein extract.

2.4. Chromatographic fractionation

The protein extract from internode 4 was first separated by concanavalin A chromatography and tested for peroxidase activity. The fractions that exhibited major peroxidase activities were further separated by a cation exchange chromatography.

2.4.1. Concanavalin A chromatography

The protein extract was loaded onto a column (0.5 cm × 3 cm) with 1 ml Con A-sepharose 4B (Sigma), previously equilibrated in 20 mM Tris–HCl, pH 7.4, containing 0.5 M NaCl. After washing the column with starting buffer, elution was carried out with the same buffer added with 0.2 M methyl-α-glucopyranoside. Seven fractions of 1 ml were collected, proteins were monitored in each fraction by measuring absorbance at 450 nm. Fractions 1, 2 and 3 that exhibiting peroxidase activity were pooled and dialysed against 0.25 mM Tris–HCl buffer, pH 7.4, in the presence of 0.015% Triton-X100, and 10% glycerol.

2.4.2. Cation-exchange chromatography

Pooled fractions from concanavalin A chromatography were fractionated by cation exchange chromatography on a column (0.5 cm × 20 cm) of CM sepharose (Amersham Pharmacia Biotech). The column, previously equilibrated in 20 mM Na-acetate, 0.015% Triton-X100, pH 5, was washed with several column volumes of buffer to remove unadsorbed material. Bound peroxidases were eluted with a linear NaCl gradient (0–500 mM) in starting buffer.

2.5. Protein gel blot analysis

Proteins were separated by standard SDS-PAGE conditions. SDS-10% polyacrylamide gel electrophoresis was carried out using a mini-Protean II slab gel system according to the supplier’s instructions (Bio-Rad). The samples were run under partially denaturing conditions in Laemmli [22] buffer containing 0.1% SDS. Standard protein markers for molecular mass estimation were obtained from Sigma.

Proteins were transferred onto 0.45-μm Hybond ECL membrane (Amersham Biosciences, Piscataway, NJ) by electroblotting. Protein detection was performed using polyclonal antibodies raised against peroxidases [23].

2.6. Lignin analysis

The 5-week-old internodes 4 and 5 of control and rubbed tomato plants were collected, dried and ground (<0.5 mm) before exhaustive extraction in a Soxhlet apparatus by ethanol/toluene (2/1, v/v), ethanol and water. The extract-free samples were dried before lignin analyses.

The determination of the lignin content of extract-free samples was performed by the gravimetric Klasson method [24]. The evaluation of lignin structure was investigated using the thioacidolysis [25]. The lignin-derived monomers were identified by gas chromatography–mass spectrometry (GC–MS) of their trimethylsilylated derivatives.

2.7. Statistical analysis

Statistical analyses were carried out with STATISTICA for windows using Student’s test with p < 5%.
3. Results

3.1. Effect of mechanical stimulation on internode elongation

The length of internodes 4 and 5 was measured 14 days after rubbing of the fourth internode. Results reported in Fig. 1 show that rubbing led to a significant reduction of elongation of the stressed internode. This effect was not limited to the rubbed area but affected also the elongation of the neighbouring internodes (no. 5) that were shorter in rubbed plants than in control one.

3.2. Lignin analysis

For lignin analysis, internodes 4 and 5 were harvested 14 days after rubbing the fourth internode 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 [24]. While the Klason lignin levels of the youngest internode no. 5 were found to be similar (Table 1), the cell walls of the rubbed internode no. 4 was found to be significantly enriched in lignin by the mechanical treatment (increase from 7.68% in the control to 10.08% in the mechanically stressed sample, Table 1).

Lignin structure was evaluated by thioacidolysis; the key reaction of thioacidolysis is the cleavage of the labile β-O-4 bonds that are the major interunit bonds in native lignins [26]. The p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin units specifically involved in β-O-4 bonds give rise to thioethylated H, G and S monomers respectively. Therefore, when expressed on the basis of the lignin content, the recovery yield of these diagnostic monomers is a reflection of the frequency of β-O-4 bonds in lignins. These monomers were analyzed by GC–MS of their trimethylsilylated derivatives. In the control samples and as expected, the yield of lignin-derived monomers was found to be higher in the older internode no. 4 relative to the younger one no. 5 (Table 1). This result confirms that lignins formed at the later stages of lignification are enriched in β-O-4 bonds [27].

The rubbed internode no. 4 released a significantly higher yield of thioacidolysis monomers and the frequency of S monomer was also significantly increased. By contrast, no clearcut effect could be evidenced for internode no. 5, except a slight enrichment in G thioacidolysis monomers (Table 1).

3.3. Enzyme activities

CAD and peroxidase activities were investigated in internodes 4 and 5, 24 h after rubbing of the fourth internode. Results show that mechanical stress application induces a strong increase of CAD activity in the rubbed internode with an approximately two-fold increase when compared to control tomato internodes (Fig. 2).

Peroxidase activities analysed in the rubbed and in the upper internode (internodes 4 and 5 respectively) were stimulated as compared to control plant (Fig. 1).

### Table 1

Lignin analysis of control and rubbed tomato internodes. The Klason lignin (KL) content is expressed as weight percentage of extract-free and dry samples. The thioacidolysis total yield (H + G + S) is expressed in μmol of lignin-derived H, G and S monomers per gram of Klason lignin and the frequencies of the H, G and S monomers are expressed on a molar basis. The data are mean values (standard error in parentheses) of duplicate or triplicate analyses.

<table>
<thead>
<tr>
<th>Samples</th>
<th>KL (% by weight)</th>
<th>Thioacidolysis results</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Total yield (H + G + S)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(μmol/g LK)</td>
</tr>
<tr>
<td>Control internode 4</td>
<td>7.68 (0.06)</td>
<td>1406 (207)</td>
</tr>
<tr>
<td>Treated internode 4</td>
<td>10.08 (0.02)</td>
<td>1592 (117)</td>
</tr>
<tr>
<td>Control internode 5</td>
<td>10.48 (0.13)</td>
<td>1150 (44)</td>
</tr>
<tr>
<td>Treated internode 5</td>
<td>9.13 (0.02)</td>
<td>987 (24)</td>
</tr>
</tbody>
</table>

Fig. 1. Peroxidase activity and internode lengths of control and rubbed plants measured respectively after 24 h and 4 days following the rubbing of the fourth internode. U = 1 unit OD min⁻¹ and standard errors are indicated by vertical bars.

Fig. 2. CAD activity of internodes 4, and 5 in control and rubbed plants 24 h after rubbing of the fourth internode. Standard errors are indicated by vertical bars.
3.4. Analysis of peroxidase isoforms

Results previously presented show an enhancement in peroxidase activity in internode 4 after mechanical stress application. In order to identify peroxidase isoforms induced in the rubbed tomato internode, protein extracts were subjected to chromatographic analysis.

Concanavalin A-sepharose chromatography showed that only the first three eluted fractions exhibit peroxidase activity using tetra methyl benzidine (TMB) as substrate (results not shown). These pooled fractions were further purified through a cationic exchange chromatography column. Peroxidase activity was eluted as one peak (Fig. 3).

Western blot analysis was performed on the purified proteins in order to identify cationic peroxidase isoforms in control and after the rubbing treatment. The patterns of peroxidase isozymes revealed, at least, four isoforms referred to as C1, C2, C3 and C4 in the rubbed internode (Fig. 4). Isoform C3 with a molecular weight of 59 kDa was not detected in control internode, when equivalent amounts of proteins were loaded on the gels. Moreover, an increase in the basic isoform C4 (55 kDa) activity was observed in rubbed internode as compared to the control one.

4. Discussion

The most common effect of mechanical stress was a reduction in stem extension growth, increased allocation to roots [28] and a reduction in petiole diaet [29]. This is known as the thigmomorphogenetic response and is reported in many plants [5]. The main morphological response of tomato plants to mechanical stimulation was an inhibition of internode elongation [30].

Under our experimental conditions, elongation was inhibited in the rubbed internode (no. 4) and in the upper one (no. 5). 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 [31,32].

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 [33] show that mechanosensing is local and scattered through the stem of tomato plant submitted to a basal binding. In beans, Erner et al. [31] proposed the involvement of a translocable factor, of either hormonal or electrical origin. In Bidens pilosa, mechanical signal is rapidly transmitted overall the plant via the induction of an electric depolarization wave [34]. In contrast, in B. dioica, the morphological response was exclusively detected in the rubbed internode (Broyer, 1979). Differences in plant structure and morphology could explain the heterogeneity of response outside the stimulated site.

Phenylpropanoid metabolism and lignin biosynthesis pathway are usually stimulated by diverse stresses [13,35,36]. Induced lignification is one of plant defence responses to mechanical stress [8], wounding [37], and fungal elicitor [36]. Thigmomorphogenetic response of tomato plants is accompanied with lignification of the rubbed internodes. Consequently, reduced elongation of mechanically stressed internodes can be related to the increase lignin accumulation. Lignin has been shown to be a growth plant inhibitor [17]. Cell wall rigidification as a result of accelerated lignification can induce the inhibition of elongation observed in tomato internodes after mechanical stress application.

The global compositional analyses revealed that the lignin enrichment was observed mainly in the rubbed internode and not in the upper one no. 5. However, it is possible that lignification was not completed in the younger internode no. 5 at the onset of the mechanical stress whereas this was not the case for the rubbed internode no. 4. In addition, thioacidolysis confirmed that the rubbing treatment mainly affected the lignification of the rubbed internode no. 4. The increased thioacidolysis yield and the higher S frequency suggest that the lignification stage of internode no. 4 is advanced by the mechanical treatment. Taken together, these results suggest that the rubbed internode undergo a faster ageing process and that its lignification is somehow accelerated by the mechanical rubbing. These results are in agreement with the finding of De Jaegher et al. [8] who showed the considerably increase of coniferyl and sinapinic alcohols in rubbed B. dioica internodes subjected to mechanical stress. An increase in syringyl content was also observed in the wood of both wild type and transgenic poplar trees subjected to mechanical stress [38]. It is...
known that plants create a natural barrier that includes lignin and suberin synthesis in order to counteract mechanical damage [39]. Peroxidase activities, enzymes designated as marker of lignification process, were significantly increased in the wounded internode after mechanical stress application. The hypothesis of peroxidase-catalysed lignification, presented for the first time by Freudenberg et al. [40], has now been well established [18]. H2O2 formation, a prerequisite for lignification, as well as lignification itself, is both catalysed within the cell wall by peroxidases [41]. Peroxidases involvement in mechanical stress could be explained by its catalytic role in the cross-linking of pectin and structural proteins in the cell wall and the polymerisation of the phenolic monomers of lignin and suberin [18].

Activity analysis and isoform purification show that the purified proteins were basic and glycosylated. Via Western blot analysis we can suggest that the increase of peroxidase activity of the fourth internode in stressed plant was due to activity of basic isoform C3, a novel isoform with a molecular mass of 59 kDa, which is not detected in control plant, and to the increase of C4 (55 kDa) activity. These results indicate a crucial role of these basic peroxidases in the polymerization of phenolic compounds during lignin synthesis. Previous reports have proposed the involvement of the acidic isoforms in the lignification processes [16]. Thus, it is possible that basic, rather than acidic peroxidases, are responsible for the polymerization of the monomeric compounds, as it has been proposed for maize pollen [42].

Lignification is known to decrease cell wall elasticity and growth capacity, a hyper lignification could explain the reduced growth, in addition, cell wall rigidification through crosslinks established between the different cell wall components catalysed by peroxidases [10] could contribute to reduced internode growth rate. Growth reduction seems to be a general trend in plants over expressing peroxidase [16].

This result is in agreement with previous studies on the role of peroxidases in thigmomorphogenetic response. In *B. dioica* and *B. pilosa* it was also shown that mechanical stimuli induce basic peroxidases [7,8] but their molecular mass were not determined. In tomato plant, Loukili et al. [43] have purified a neutral peroxidase with a molecular mass of 36 kDa, this isoform has the capacity to oxidize conifer alcohol and was proposed to be involved in lignin biosynthesis. In transgenic tomato plants, lignin content was increased under stress condition. Western blot analysis shows the presence of a new isoform corresponding to a band at 41 kDa that was absent in the wild tomato plant [44]. Moreover peroxidase analysis in tomato stem after wounding indicated the presence of a new basic isoform [45].

Difference in CAD activity between control and rubbed internodes is also in agreement with the concept of an accelerated lignification. CAD enzyme, catalysing the synthesis of cinnamyl alcohol dehydrogenase, is rapidly increased after rubbing. This is in agreement with the findings of Mitchell et al. [35] who demonstrated the induction of CAD activity in elicitor treated leaves of wheat plant. In conclusion, we show that the response of tomato plant to mechanical stress by the inhibition of internode elongation was related to the induction of CAD activity and peroxidase isoforms. A new isoform, only expressed in rubbed plant and the enhancement of a peroxidase already expressed in control plant, suggests a possible role for these isoforms in the lignification process, which is thought to be involved in growth inhibition. This can suggest the use of peroxidase as a biochemical marker of stress.

**References**


