Influence of Mannose on the Apoplastic Retrieval Systems of Source Leaves

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ABSTRACT

Experiments were conducted in which D-mannose was supplied to mature Beta vulgaris L. (sugar beet) leaves, via the transpiration stream, to perturb photosynthetic carbon allocation by sequestering cytosolic Pi. Biochemical and enzymic analyses conducted on this tissue indicated that mannose-6-P was present, that it was only slowly metabolized, and that after a 24-h pretreatment sugar metabolism was slightly perturbed. However, sucrose retrieval by the mesophyll tissue was greatly impaired in 24-h mannose-pretreated tissue, a response which was due in part to mannose acting as an osmoticum. Inhibition of glucose, fructose, and arginine uptake into mannose-treated sugar beet leaf discs indicated that mannose may elicit a general perturbation of all membrane transport processes. This conclusion was supported by our finding that sucrose efflux was increased from mannose-treated tissue. Analysis of adenine nucleotide levels showed that whereas these levels declined over the first 3 to 6 hours of the mannose treatment, by 24 h they had recovered to near control values. Similar experiments conducted on Nicotiana rustica indicated that whereas mannose-6-P was present in mature leaves, it remained at a much lower level than that found in sugar beet. Sucrose uptake into N. rustica was insensitive to mannose pretreatment. However, glucosamine treatment, which is also thought to sequester cytosolic Pi, inhibited sucrose uptake in both N. rustica and B. vulgaris. Further, experiments conducted on N. tabacum L. var Xanthii showed that mannose caused an inhibition of sucrose uptake, indicating that a range of sensitivity to mannose exists between closely related species. These results are discussed in terms of possible mechanisms of inhibition.

In a recent study on the regulation of sucrose transport across the plasmalemma of sugar beet mesophyll cells, we used mannose to alter the partitioning of carbohydrates between the cytosol and the chloroplast. In sugar beet, mannose did not reduce the sucrose level in the manner expected from reports in the literature (5, 10, 13, 14); however, it did cause a reduction in exogenous [14C]sucrose influx. In addition, mannose pretreatment prevented, in some way, the change in partitioning of exogenously supplied [14C]sucrose, which was found to occur following the removal of sink activity (31). These findings suggested that further studies, using mannose as an experimental treatment, may provide information on both the regulatory mechanisms involved in transport at the plasma membrane and carbohydrate allocation in relation to phloem loading.

It is generally accepted that in species lacking mannose isomerase, mannose is able to reduce cytosolic inorganic phosphate (Pi) levels by phosphorylation to mannose-6-P, which is then only slowly metabolized (10, 16, 23). Since the transport of Pi between the vacuole and cytosol is thought to be slow (23, 27; but see Ref. 16), the addition of mannose affects a sequestering of cytosolic Pi (5, 13; but cf. Ref. 16). Inhibition of sugar transport by mannose has been previously documented, e.g. glucose uptake into (15) and sucrose storage in corn scutellum (9) are both inhibited by mannose treatment. The work conducted on corn scutellum indicated that mannose treatment reduced the ATP level in this tissue, and Humphreys postulated that this may have a significant role in the inhibitory response of the tissue to mannose (see also Ref. [28]).

In the present study, we further investigated the effect of mannose on the transport of sucrose, glucose, fructose, and arginine into mature leaf tissue of sugar beet. Our results indicate that the inhibition of plasmalemma transport is not limited to sucrose as the influx of these other substrates was also reduced. Furthermore, the inhibitory aspects of mannose cannot be explained by a reduction in adenine nucleotide levels alone since, after a 24 h mannose pretreatment, these levels recovered to near-control values. Since tobacco has been reported to be able to metabolize exogenously supplied mannose (5, 12, 13, 20), we selected two species of Nicotiana, N. rustica and N. tabacum, to form a comparative basis for this study. We found that whereas sucrose influx into N. rustica was completely insensitive to mannose pretreatment, transport into N. tabacum was inhibited. Glucosamine pretreatment inhibited sucrose influx into N. rustica and sugar beet. Collectively, these studies implicate a role of cytosolic Pi in the regulation of transport across the plasmalemma.

MATERIALS AND METHODS

Plant Material. Beta vulgaris L. (var SSBN1, USH10, Lot 3102 or USH11, Lot 82313) seeds were planted in a mixture containing vermiculite, peat moss, and sand (2:1:1 by volume) and grown in a controlled-environment chamber under a 27°C day/17°C night temperature regime and a 16 h photoperiod (250 μmol m−2 s−1). The plants were watered daily and fertilized twice weekly with Hoagland solution. The third pair of leaves (after the cotyledons) of 4-week-old sugar beet plants were used. Nicotiana tabacum L. var xanthii and Nicotiana rustica L., were planted in the soil mixture described above and grown under greenhouse conditions. Plants were fertilized during the third, fifth, and seventh weeks with ammonium phosphate. Experimental plants were 9 weeks old.

Kinetic Studies. Kinetic uptake studies on B. vulgaris were performed using the method of Maynard and Lucas (21) as modified by Wilson et al. (32) and Wilson and Lucas (31). Radioactive sugars (D-[U-14C]sucrose, -fructose, and -glucose) were obtained from International Chemical and Nuclear Corp. (Irvine, CA). Radioactive arginine (L-[U-3H]arginine) was obtained from NEN Research Products (Boston, MA). The specific activities of the uptake solutions for all sugar and arginine...
experiments were 1.78 × 10^3 Bq mmol⁻¹. In Nicotiana experiments, a segment of the mature tobacco leaf was excised and placed on a rubber pad located in a dish containing a solution of 20 mM CaCl₂ and 60 mM Mes buffer (pH 5.0). Leaf discs, 4 mm in diameter, were cut and then pretreated for 30 min on a solution containing 20 mM CaCl₂, 120 mM Mes (pH 5.0), and enough mannitol to adjust the total osmolality to 200 mosm; sugars at the concentration specified for the particular experiment were also included. This pretreatment was followed by a 30-min uptake period, which was terminated by using three 7-min washes in media containing 20 mM CaCl₂, 60 mM Mes (pH 5.0), and the appropriate sugar at 40 mM. Radioactivity within the sugar beet and tobacco leaf discs was quantitated using a Beckman 9800 scintillation spectrometer (Beckman Instruments Inc.) as previously described (22).

**ATP Extraction and Determination.** Leaf discs that had been treated using the same protocol employed for kinetic experiments were quickly frozen using liquid N₂. These discs were then lyophilized for 18 h in a DURADRY freeze-dryer equipped with a bulk tray dryer (FTS Systems, Stone Ridge, NY). Lyophilized samples were weighed and then ground, using a ground glass homogenizer, in 2.0 ml of ice-cold 1.0 m HClO₄, 2 mg ml⁻¹ of Polyclay AT (Polysciences, Warrington, PA) was also added to the extraction medium (18). The homogenate was rinsed with 1.0 ml of 0.05 M phosphate buffer (pH 7.5), and after allowing 20 min for complete extraction and protein denaturation, the rinse and the extract were combined. The mixture was then centrifuged at 25,000 g for 30 min at 4°C. The supernatant was neutralized to pH 7.5 with 5 N KOH. After waiting 20 min to allow for the precipitation of potassium perchlorate, the extract was again centrifuged for 30 min at 25,000 g. The supernatant was stored at −70°C until assayed (2–3 d).

A modification of the procedures of Yoshioka and Tamura (33) and Preston (26) was employed to determine AMP, ADP, and ATP levels (32). Chromatography was performed using a Beckman Series 344 Gradient Liquid Chromatograph equipped with a Ultrasphere IP column (4.6 mm diameter, 250 mm long). The eluting peaks of the adenine nucleotide 1,N⁶-ethenoadenosine-derivatives (32) were detected using a fluorescence detector (Beckman model 157) and analyzed on an Altex Chromatopac C-R1B data processor (Beckman Instruments).

Peaks were identified by using retention times of standards. Further confirmation of the peaks in the extracts was obtained enzymically. Addition of pyruvate kinase and adenylate kinase (Sigma) or pyruvate kinase alone, to extracts, resulted in the disappearance of the peaks which were identified as AMP and ADP, respectively, while the ATP peak increased. Addition of hexokinase or calcium phosphate to the extract resulted in the loss of the putative ATP peak.

**Sugar, Starch and Mannose 6-P Analysis.** Sugars and starch were extracted from leaf tissues and analyzed using the procedures and protocols described by Wilson and Lucas (31). Mannose 6-P was extracted according to the procedure of Herold et al. (14). The extraction procedure was similar to our sugar protocol (31), except that 20 leaf discs were used and the ethanol extractions were followed by two extractions using hot water. The extracts were evaporated to dryness under a stream of nitrogen gas and stored at −70°C until assayed (1–2 d).

To assay the extracts for mannose 6-P, the samples were first rehydrated to 0.5 ml, and 50 μl of this sample was added to a 13 × 100 mm culture tube containing 2.82 ml of 50 mM HEPES-NaOH buffer (pH 7.6), 100 μl of NADP (12 mM), and 10 μl each of glucose 6-P dehydrogenase (G6PDH, 340 units ml⁻¹), phosphoglucone isomerase (PGI, 580 units ml⁻¹), and phosphomannose isomerase (PMI, 140 units ml⁻¹). This mixture was incubated for 1 h by which time the stoichiometric production of NADPH for each mannose 6-P molecule was complete. The resulting NADPH was measured spectrophotometrically at 366 nm against a blank containing 10 μl distilled water in place of phosphomannose isomerase. (G6PDH from Bakers yeast, PGI from Bakers yeast type III, and PM1 from yeast along with NADP were obtained from Sigma.)

**Mannose and Glucosamine Pretreatments.** Three h into the photoperiod, the leaf-petiole complex of the third pair of leaves (after the cotyledons) were excised from the base of the crown while the sugar beet plant was submerged underwater. The leaf-petiole complex was then either placed in distilled water (control) or a 10 mM mannose (or glucosamine) solution before being returned to the controlled-environment chamber. After predetermined intervals, leaf discs (4 mm diameter) were punched from the leaf and used for sugar analyses and kinetic experiments (same protocol was followed for tobacco plants). When appropriate, 10 mM mannitol (or sorbitol) was substituted for mannose.

**[14C]Sucrose Efflux Experiments.** As in sucrose uptake experiments, the third pair of leaves (after the cotyledons) from 4-week-old sugar beet plants were used in our efflux studies. Leaves were cut from the plant and submerged in a 20 mM CaCl₂ solution while the lower epidermis was peeled away. Leaf discs were excised and then pretreated, for 30 min, on a medium containing 20 mM sucrose, 120 mM Mes (pH 5.0), and mannitol added to adjust the osmolality to 500 mosm. Discs were then transferred to an identical medium containing 20 mM [14C]sucrose. After a 30-min loading period, the discs were transferred to fresh pretreatment medium. At predetermined times, discs were again transferred to fresh media, and aliquots of the efflux solution were analyzed for radioactivity by liquid scintillation counting. At the end of the experiment, the discs were analyzed for residual radioactivity. Efflux data were analyzed according to Pitman (25) and Cram (6), the results being interpreted in terms of an apoplastic and two intracellular compartments (vacuole and cytosol) arranged in series. In certain instances some of the leaf discs were analyzed for ethanol-soluble sugars as previously described. Radioactivity remaining after ethanol extraction followed by cold water extraction was attributed to starch.

**RESULTS**

**Inhibitory Effect of Mannose on Sugar and Amino Acid Transport.** Wilson and Lucas (31) recently showed that pretreatment of sugar beet leaves with mannose results in a progressive decline in the capacity of these leaves to retrieve exogenously supplied [14C]sucrose. This inhibition by mannose may have been due to its osmotic rather than biochemical properties; this possibility was tested by substituting mannitol (or sorbitol) for mannose. After a 24-h mannitol treatment, the capacity of the leaves to take up [14C]sucrose was slightly decreased, whereas a similar treatment with mannose caused a strong inhibition of sucrose influx (Fig. 1). This suggests that mannose has effects independent of its osmotic properties. (An equivalent inhibition was obtained with either sorbitol or mannitol.)

Since mannose had dramatic effects on sucrose influx, apart from a small osmotic effect, we decided to examine its influence on the transport of other sugars and amino acids. As in the case of sucrose, mannose pretreatment resulted in a decrease in the transport of both [14C]fructose (Fig. 2A) and [14C]glucose (Fig. 2B). However, in contrast with sucrose, the uptake of these sugars, after mannose pretreatment, was mediated solely by the first-order kinetic component.

We extended our investigation on the specificity of the mannose effect by measuring the influence of mannose on amino acid transport. Dual-label ([3H]arginine and [14C]sucrose) experiments were performed in these studies. Control experiments showed that arginine was taken up via the combined effect of a saturable and a first-order kinetic system (see also Ref. 22), with
INHIBITION OF SUCROSE UPTAKE

Fig. 1. Effect of mannose and mannitol on net sucrose influx into B. vulgaris leaf discs. Petioles were excised at the base, while the crown of the plant was submerged underwater. The petioles were then placed in either distilled water (C), 10 mM mannitol (D), or 10 mM mannose (A) before being returned to the controlled-environment chamber. After 24 h, kinetic studies were performed. A 30-min preincubation period in unlabeled sucrose was followed by a 30-min incubation in [*4C]*sucrose. Uptake solutions were adjusted to 500 mOsM with mannitol. Each point represents the mean ± SE for two sets of six replicates.

Fig. 2. Effect of mannose pretreatment on net fructose (A) and net glucose (B) influx into B. vulgaris leaf discs. Experimental techniques were as described in Figure 1. Points for both the fructose control (C), mannose-treated (A), and glucose control (C), and mannose-treated (B) represent the means ± SE for two sets of six replicates. Sucrose control (C) is included for reference.

The major contribution occurring via the first-order kinetic system. As with sugar influx, mannose pretreatment resulted in an inhibition of both the first-order and saturable components of [*4C]*arginine uptake (Fig. 3). Hence, the inhibitory influence of mannose appears to be related to either a general cellular- or membrane-related phenomenon.

Adenine Nucleotide Levels Affected by Mannose Pretreatment. It has been suggested that mannose may reduce sugar transport into cells through its sequestration of cytosolic Pi. This removal of Pi would reduce the level of ATP available for membrane transport. To investigate this hypothesis, we analyzed the levels of adenine nucleotides in mannose-treated and control tissue over a 24-h period (Table I). We found that leaves which had been pretreated with mannose for 3 h contained lower levels of AMP, ADP, and ATP compared with control leaves. However, after 24 h, the adenine nucleotide levels in the mannose-treated leaves had recovered to near-control values while, during this period, membrane transport activity continued to decline. It would appear from these data that mannose is acting by some other means to inhibit membrane transport.

Mannose 6-P Levels in Mannose-Pretreated Leaves. It is possible that the mannose-induced inhibition of membrane transport is related to either the sequestration of cytosolic Pi or the subsequent formation of mannose 6-P, per se. To examine this possibility, we sampled control and mannose-treated leaves at various intervals over a 24-h experimental period and determined the mannose 6-P levels. We were unable to detect mannose 6-P in control tissue, but in mannose-treated leaves, the mannose 6-P levels progressively increased throughout the light period and then decreased during the subsequent dark period (18 h) (Table II). During the following light period, the mannose 6-P level returned to the pre-dark value. These data indicate that Beta vulgaris is capable of metabolizing mannose 6-P, although the rate appears to be too low to prevent its accumulation in this tissue.

Effect of Mannose on Nicotiana. Having characterized the

We furthered our investigations on tobacco by examining the
levels in *S. rustica* and thereby elicit a decrease in sucrose influx. Herold et al. (13) have suggested that glucosamine sequesters Pi in tobacco leaves. Glucosamine pretreatment of *N. rustica* leaves resulted in an inhibition of both the saturable and linear components of uptake, with the saturable system being the most affected (Fig. 5). However, the extent of glucosamine-induced inhibition was not as severe as that observed with sugar beet (Fig. 5).

**Effect of Mannose on Sucrose Efflux.** Herold (11) suggested that mannose had marked effects on the membrane permeability of spinach beet leaf tissue. We performed efflux studies in order to determine if sucrose efflux was also affected in sugar beet. The effect of a 24-h mannose pretreatment on \[^{[14]}C\]sucrose efflux is presented in Fig. 6. In control tissue the half-time for \[^{[14]}C\]sucrose exchange across the plasmalemma was 40.5 min, whereas in mannose-treated tissue this half-time was reduced to 18.8 min. Mannose also affected the distribution of \[^{[14]}C\]sucrose in the cytosolic and vacuolar compartments. In control leaves, only 5% of the \[^{[14]}C\]sucrose was present in the cytosol, while in mannose-treated tissue this value increased to about 25%. Thus, it would appear that mannose can affect intracellular transport processes in addition to perturbing the fluxes crossing the plasmalemma.

**DISCUSSION**

In the present study, we used mannose and glucosamine to probe the regulatory processes involved in sugar transport into mature leaf tissues of sugar beet and tobacco. Mannose has been used in various plant systems as a tool to investigate the role of cytosolic Pi in regulating various aspects of photosynthetic carbon metabolism (8, 10, 13, 30). It is currently accepted that mannose sequesters cytosolic Pi through its reaction with Pi to produce mannose 6-P, via hexokinase. Many plant tissues either lack phosphomannoisomerase, or this enzyme is compartmentalized such that mannose 6-P is metabolized very slowly (10, 23). In our experiments, mannose pretreatment caused a dramatic perturbation of sugar influx into mesophyll tissue of sugar beet plants. Mannose pretreatment resulted in a progressive decrease in sucrose uptake over a 24-h period (31). Further studies revealed that uptake of both glucose and fructose was also reduced, indicating that the mannose effect may be a consequence of a more general phenomenon. This interpretation is supported by the observation that arginine uptake was similarly affected.

Consistent with this view is the observation that mannose also influenced sucrose efflux from sugar beet leaf discs. Our experiments indicate that both the plasmalemma and tonoplast are affected by mannose, as the half-times for sucrose efflux from both cytosolic and vacuolar compartments were decreased relative to control values. Although some caution is needed in the interpretation of the efflux data obtained in this manner (4), we
Table II. Mannose 6-Phosphate Levels in Mannose-Treated Sugar Beet and Tobacco Leaves

Leaves were excised at the base of the petioles when the crowns of the plants were submerged underwater. The petioles were either placed in 10 mM mannose or distilled water (control) before being returned to the controlled-environment chamber for the times specified. Experimental treatments were started 3 h into the photoperiod; the controlled-environment chamber was programmed for a 16-h light:8-h dark cycle. Mannose 6-P levels were determined by enzymic assay.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Time (h)</th>
<th>Mannose 6-P (μmol mg⁻¹ Chl ± se)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar beet</td>
<td>0</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>Tobacco</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>(N. rustica)</td>
<td>24</td>
<td>0.7 ± 0.1</td>
</tr>
</tbody>
</table>

*Not detected.

feel our interpretation of the data as representing efflux from a three-compartment model is justified (at least to a first approximation) by a comparison of plots of log Q and log Q₅₀ versus time as suggested by Cram and Laties (7) and further emphasized by Kochian and Lucas (17).

By extrapolating the efflux curves to t = 0, we were able to estimate the relative amounts of radiolabel present in the vacuolar and cytosolic compartments. At the end of a 30-min uptake period only 5% of the label resided in the 'cytosolic compartment' of sugar beet source leaves. This result is similar to the findings of Thom et al. (29) for their study of efflux of sugars from cultured sugar cane suspension cells. They reported that while most of the label was in the cytoplasm following the initial minutes of uptake, the majority of the label was subsequently located in the vacuole. However, after mannose pretreatment, 25% of the [¹⁴C]sucrose was located in the cytosolic compartment of the sugar beet leaf. Evidently, mannose may either directly or indirectly affect the partitioning of sucrose between the vacuole and the cytosol.

An important aspect of the mannose effect on sugar uptake was the difference in the pattern of inhibition displayed by sucrose, glucose, and fructose. Although uptake of sucrose was reduced by mannose pretreatment, the resulting kinetic profile demonstrated that influx was still mediated by both the first-order and saturable components. However, for fructose and glucose, mannose pretreatment completely inhibited the saturable transport component; influx was mediated solely by the first-order component. Perhaps there exists a basic difference in the regulation of the various sugar-transport systems.

Previous investigators have documented reduced synthesis of ATP upon the addition of mannose and have used this finding as a basis to explain their observations (9, 28). In the present study, we found that mannose addition reduced adenine nucleotide levels when measured after 3 h and that this correlated with decreased sugar influx. However, after 24 h, adenine nucleotide levels recovered to near control values, while sugar transport activity continued to decline. Based on this finding, we feel the explanation for our observed mannose response must lie elsewhere.

An interesting outcome of this study was the discovery that two closely related species of Nicotiana differed widely in their response to mannose. N. rustica seemed very tolerant to mannose pretreatment even after 24 h, while N. tabacum var. xanthii reacted in a manner similar to that observed with sugar beet. Differences in phosphomannosiosemerase activity or compartmentalization might account for the observed differences. Although N. rustica appeared to be tolerant of mannose pretreatment, we were able to inhibit sucrose influx by substituting glucosamine for mannose. Since glucosamine is reported to sequester Pi in tobacco, our results imply that changing cytosolic Pi levels may be important in the regulation of sugar transport across the plasmalemma in tobacco as well as sugar beet.

On a speculative note, it may be that both mannose and glucosamine elicit a general perturbation of membrane transport by interrupting a phosphate-dependent component of the phosphatidylinositol cycle (1). Support for this hypothesis can be found in the plant literature, insofar as data is now available that the phosphatidylinositol cycle is present in plant cells (2, 24). Further, lithium, an inhibitor of phosphatidylinositol reincorporation into the plasma membrane (1), also inhibits sugar transport. Carlier and Thellier (3) reported that lithium treatment
Table III. Influence of Mannose and Glucosamine on Sugar and Starch Content in Mature Leaves of N. rustica

Leaf petioles were excised at the base while the crown of the plant was submerged underwater. The leaf-petiole complex was either placed in 10 mM glucosamine or 10 mM mannose when appropriate or distilled water (control) and then returned to the controlled-environment chamber for 24 h.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sugar Content</th>
<th>Starch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sucrose</td>
<td>Glucose</td>
</tr>
<tr>
<td>Control (untreated)</td>
<td>4.9 ± 0.3</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>Mannose-treated</td>
<td>6.0 ± 0.6</td>
<td>5.6 ± 0.4</td>
</tr>
<tr>
<td>Control</td>
<td>5.4 ± 0.2</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>Glucosamine-treated</td>
<td>10.5 ± 0.9</td>
<td>6.5 ± 0.5</td>
</tr>
</tbody>
</table>

Fig. 5. Effect of glucosamine pretreatment on net [14C]sucrose influx into N. rustica (▲) and B. vulgaris (●) leaf discs. Experimental techniques were as described in Figure 1, except 10 mM glucosamine was substituted for mannose. The points for controls (Δ, ○) and glucosamine-treated (▲, ●) leaves represent the means ± SE for six replicates.

Fig. 6. Kinetics of [14C]sucrose efflux from control (A) and mannose-treated (B) leaf discs of B. vulgaris. Leaf discs were loaded for 30 min in 20 mM [14C]sucrose. The efflux solution contained 20 mM sucrose, 120 mM Mes (pH 5.0) and enough mannitol to achieve 300 mOsm. The data are plotted as the logarithm of cpm remaining in the tissue (Q) versus time. The lowest effluxing component (vacuole) was extrapolated to zero and subtracted from the overall curve. The insets represent cytoplasmic efflux from control (A) and mannose-treated (B) tissues. The half-times for exchange of the cytoplasmic compartment was 40.5 and 18.5 min for control and mannose-treated tissue, respectively.

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