

Concurrent Synthesis and Degradation of Alcohol Dehydrogenase in Elicitor-Treated and Wounded Potato Tubers¹

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ABSTRACT

The accumulation of alcohol dehydrogenase (ADH) in arachidonic acid-elicited potato (*Solanum tuberosum* L.) tuber discs was studied. In accordance with our previous report of the accumulation of *Adh* mRNA beginning 2 hours after elicitor treatment (DP Matton, CP Constabel, N Brisson [1990] Plant Mol Biol 14: 775–783), immunoprecipitation of ADH from *in vivo* labeled discs indicated that ADH synthesis occurred as early as 12 hours after treatment. However, levels of ADH activity and protein, as shown by enzyme assay and immunoblot, did not rise in parallel but decreased during the first 24 hours of treatment. After 24 hours, ADH activity and protein began to increase, reaching a several-fold increase at 96 hours after elicitation. Water-treated control discs showed a similar though delayed and less pronounced pattern. These results imply a turnover of ADH following elicitor treatment of potato tuber discs. As shown by nondenaturing gel electrophoresis, the synthesis and degradation involved the same ADH isozyme.

The expression of *Adh*, one of the best-studied genes of higher plants, is regulated in response to both environmental and developmental signals. Under anaerobic conditions, *Adh* is rapidly expressed in maize seedlings along with about 20 other genes (23). Many of these encode enzymes involved in glycolysis (3), enabling the plant to withstand short periods of flooding. *Adh* is also part of the normal developmental program of many plants under aerobic conditions and is found in seeds, young seedlings and pollen (3), root cap and stele (16), fruit (4), tubers (7), leaves (14), and the cambium of trees (15). While the role of ADH² in anaerobiosis is thought to be as a component of glycolysis, its function in plants growing under aerobic conditions is not known. Work with null mutants of maize suggests, however, that in such conditions a functional *Adh* gene is not essential (8).

We recently isolated and sequenced a cDNA corresponding to a potato (*Solanum tuberosum* L.) *Adh* gene. The *Adh* mRNA was shown to accumulate rapidly in potato discs treated with arachidonic acid (20, 22), a fatty acid capable of eliciting phytoalexin synthesis in potato tubers (5). This elicitor was first isolated from mycelial extracts of the potato

pathogen *Phytophthora infestans* (Mont.) de Bary. *Adh* mRNA accumulation in arachidonic acid-treated discs was shown to be rapid, reaching its maximum level in 16 to 24 h. In water-treated control discs, the transcript was also induced but to low levels. We further identified a number of other elicitor and stress treatments capable of inducing *Adh* in potato discs (22).

Since *Adh* gene expression had not been previously reported to be involved in elicitor-stressed plant tissues, our next objective was to confirm that ADH activity increases correspondingly following elicitor treatment. In this report, we describe the synthesis of ADH in arachidonic acid-induced potato discs, the kinetics of its accumulation, and its isozyme composition.

MATERIALS AND METHODS

Plant Materials

Potato tubers (*Solanum tuberosum* L. cv 'Kennebec') were obtained from the Québec Ministry of Agriculture "Les Buissons" Research Station. They were stored in the dark at 4°C and brought to room temperature 24 h before use.

Elicitor Treatments

Potato discs (2.5 × 25 mm) were prepared and treated with either 75 µg of arachidonic acid as a 0.1% (w/v) emulsion in H₂O, 75 µg of salicylic acid as a 0.1% (w/v) solution in H₂O, or 75 µL of a *Phytophthora infestans* homogenate (20, 22). The mycelial homogenate was prepared essentially as described previously (19). Anaerobic treatments were carried out by incubating the discs in air-tight containers in an atmosphere saturated with water and flushed with nitrogen. At the end of the treatment period, the discs were frozen in liquid nitrogen and stored at –70°C until used.

Extraction and Assay of ADH

Potato discs (a minimum of three discs per time point or treatment) were ground to a fine powder in liquid nitrogen and proteins extracted into a buffer containing 0.1 M Tris-HCl (pH 7.5), 15 mM β-mercaptoethanol, 0.2% PVP, and 5% PVPP. The brei was centrifuged at 12,000g for 10 min and the supernatant assayed directly for ADH activity. Assays were done in the alcohol-aldehyde direction as described by Hanson *et al.* (10).

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² Abbreviations: ADH, alcohol dehydrogenase (E.C. 1.1.1.1); PVPP, polyvinylpyrrolidone.

Immunoblots

Proteins were separated by SDS-PAGE using 10% acrylamide slab gels and electroblotted onto nitrocellulose membranes in 5 mM Tris, 38 mM glycine, and 20% methanol. The blots were treated with TBS (20 mM Tris-HCl [pH 7.5], 150 mM NaCl) containing 3% BSA for 1 h before a 1-h incubation in TBS with 1% BSA plus an antiserum (1:3000 dilution) raised against barley ADH (provided by A. Good, Plant Biotechnology Institute, Saskatoon, Canada). After five washes (5 min each) in TBS containing 0.05% Tween-80, the blots were incubated with goat anti-rabbit IgG linked to alkaline phosphatase (Stratagene) for 1 h. A further series of washes was performed, and the blot was developed in a solution of 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 5 mM MgCl₂ containing 0.3 mg/mL nitroblue tetrazolium and 0.15 mg/mL 5-bromo-4-chloro-3-iodyl phosphate according to the supplier's instructions.

Nondenaturing Gel Electrophoresis

Nondenaturing PAGE was carried out in 7.5% slab gels as described by Hanson and Jacobsen (11) except that the separating gel contained 20% sucrose (9). ADH activity was visualized by incubating the gel in the dark at room temperature in 0.1 M Tris-HCl (pH 8.8) containing 0.2 mg/mL nitroblue tetrazolium, 0.05 mg/mL phenylmethylsulfate, 0.3 mg/mL NAD and 80 μ L/mL ethanol (95%). For controls, replicate gels were incubated as above in the presence of pyrazole, a specific inhibitor of ADH.

Expression of Potato ADH in *Escherichia coli*

A cDNA clone containing the entire *Adh* coding region was shown to be in phase with the pBluescript β -galactosidase α -fragment sequence (22). The synthesis of an ADH-Lac Z fusion protein was thus obtained by inducing Lac Z gene expression with the inducer isopropyl- β -D-thio-galactopyranoside (IPTG) as previously described (22). A construction with the *Adh* cDNA in reverse orientation was used as a control. Induced cultures were centrifuged, and the bacterial pellet was ground with alumina in a buffer containing 100 mM Tris-HCl (pH 7.5) and 15 mM β -mercaptoethanol, clarified by centrifugation, and boiled in SDS Sample buffer (18) prior to SDS-PAGE.

In Vivo Labeling and Immunoprecipitation

Arachidonic acid-treated or water-treated control discs were labeled for the final 3 h of treatment with 100 μ Ci [³⁵S]Met in 50 μ L of a solution containing 2.7 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, and 150 mM NaCl. At the end of the labeling period, discs were rinsed three times in the above salt solution and frozen in liquid nitrogen. The discs were ground and extracted into TTBS (30 mM Tris-HCl [pH 7.5], 0.15 M NaCl, 0.2% SDS, 1.0% Triton X-100) containing 15 mM β -mercaptoethanol and 5% (w/w) PVPP. The brei was clarified by centrifugation as described above followed by an additional 30-min centrifugation at 100,000g in a SW50.1 rotor. The extracts were adjusted to equal TCA-precipitable cpm and stored at -20°C until analyzed further.

Immunoprecipitations were carried out by a procedure modified from Tomura and Koshiba (24). After incubating 1 mL of plant extract overnight at 4°C with 3 μ L ADH antiserum, 20 μ L of protein A Sepharose CL-4B (Pharmacia) was added (1:1 v/v solution in H₂O), and the extracts were incubated for 2 h with end-over-end agitation. The Sepharose-antibody complexes were washed on mini-columns made from 1-mL disposable pipette tips stuffed with a small amount of glass wool. Washes consisted of 5 mL TTBS, 2 mL 1 M NaCl, and 1 mL H₂O. The immunoprecipitate was eluted in 110 μ L SDS gel sample buffer (18) and analyzed directly by SDS-PAGE and autoradiography.

RNA Extraction and Slot Blot Hybridization

Total RNA was isolated from discs as described previously (22) and poly(A)⁺ RNA obtained by oligo(dT)-cellulose chromatography (1). Poly(A)⁺ RNA (0.25 μ g) was fixed to nitrocellulose paper in a slot blot apparatus, and RNA blot hybridization was performed with the [³²P]-labeled ADH cDNA insert as a probe (specific activity $\approx 5 \times 10^8$ cpm/ μ g).

RESULTS

We had previously demonstrated that a number of stress and elicitor treatments induce a rapid accumulation of *Adh* mRNA in potato discs (22). Here we report on the levels of ADH activity and protein following such stresses. Preliminary experiments indicated that a rise in ADH activity was not immediately apparent but could be detected only after some delay following stress treatment. Furthermore, a significant amount of ADH was already present in untreated potato tubers which made small changes in activity difficult to detect. We therefore followed ADH activity in a 5-d time course experiment with the elicitor arachidonic acid. Figure 1 shows that ADH activity increased several-fold in crude extracts of arachidonic acid-treated discs during the 120-h time period.

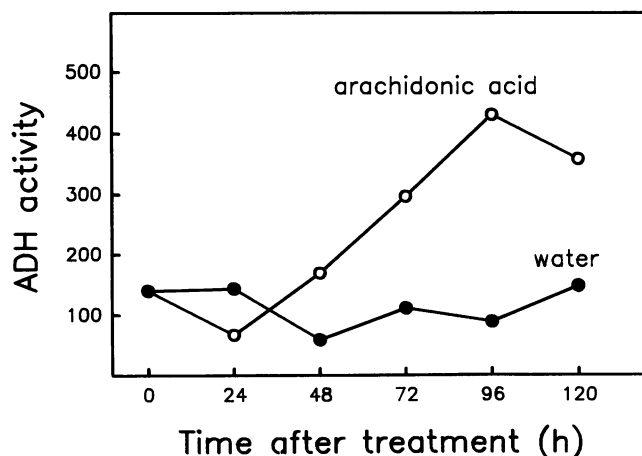


Figure 1. ADH activity in arachidonic acid-treated and water-treated potato discs over a 120-h time course. For each time point, three discs were extracted and assayed as described in "Materials and Methods." Each point is the mean of three assays. ADH activity is expressed as nmol acetaldehyde produced/min/mg protein. Standard deviations are obscured by the symbols.

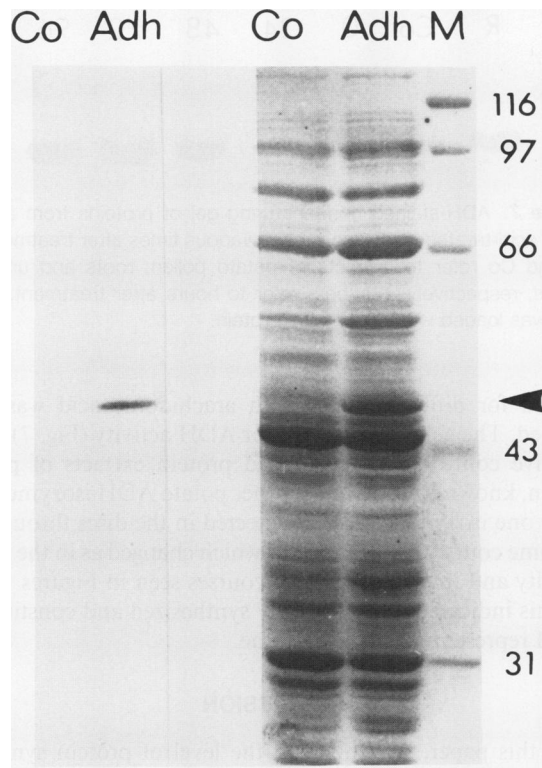


Figure 2. Expression of potato ADH cDNA in *E. coli*. Right panel: Coomassie brilliant blue-stained SDS-PAGE of proteins from IPTG-induced bacteria harboring the ADH cDNA clone in the sense (Adh) and antisense (Co) orientation. Left panel: ADH-immunoblot of SDS-PAGE gel as on right panel. M refers to molecular weight markers in kD. The arrow indicates the position of the ADH- β -galactosidase fusion protein. Each lane was loaded with 50 μ g protein.

However, during the first 24 h following arachidonic acid-treatment, a decline in ADH activity was observed. This decrease preceding the rise in activity was seen in all replicate experiments. In addition, further timed experiments with shorter intervals indicated that the low point in ADH activity during the time course was near 24 h (data not shown). In the control (H_2O) treatment, levels of ADH activity declined to a minimum at 48 h and then rose slowly (Fig. 1). In both arachidonic acid and control treatments, we observed a discrepancy between the rapid rise in *Adh* mRNA levels (maximum at 24 h) detected previously (22) and the delayed increase of ADH activity.

To enable us to monitor levels of ADH protein in tubers,

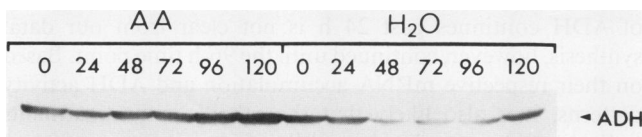


Figure 3. ADH immunoblot of protein extracts from arachidonic acid-treated and water-treated discs analyzed at various points during a 120-h time course. Total protein was separated by SDS-PAGE, electroblotted to nitrocellulose membranes, and incubated with a barley ADH antiserum. Each lane was loaded with 40 μ g protein.

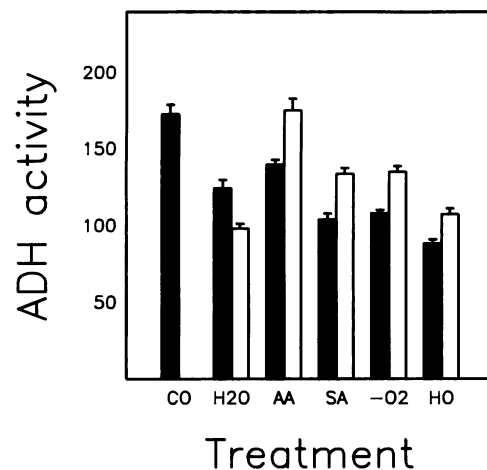


Figure 4. ADH activity in potato discs 24 h (solid bars) and 48 h (open bars) after different elicitor and stress treatments as described in "Materials and Methods." Enzymatic activity is given in nmol acetaldehyde produced/min/mg protein. Error bars represent one standard deviation. A minimum of three discs were analyzed per time point and treatment. Co, unsliced tuber tissue; H_2O , water; AA, arachidonic acid; SA, salicylic acid; $-O_2$, anaerobiosis; Ho, *P. infestans* homogenate.

we obtained an antibody raised against barley ADH. To verify that this antibody would also specifically react with the product of the *Adh* gene of potato, we carried out immunoblots of SDS-PAGE-separated extracts of *Escherichia coli* expressing the *Adh* cDNA. We had previously shown such extracts to have ethanol-oxidizing activity, which was not found in control extracts (22). A single band of the predicted size was recognized by the antibody in extracts of IPTG-induced *E. coli* harboring the *Adh* cDNA in the sense, but not in antisense, orientation (Fig. 2, left panel). This signal corresponded to a Coomassie brilliant blue-stained band seen only in *Adh*-expressing *E. coli* (Fig. 2, right panel). The results thus indicated that the antibody recognized potato ADH and would be useful in monitoring ADH levels in potato tubers.

We then used the antibody to perform immunoblots of extracts of arachidonic acid-treated discs at a number of time points following treatment. Figure 3 shows that ADH accumulation in both arachidonic acid-treated and control tuber discs followed the measured enzyme activities shown in Figure 1.

Having characterized the levels of ADH in arachidonic acid-treated discs, we then chose the 24 and 48 h time points to test the effect of salicylic acid and anaerobic treatments on ADH levels in potato. We had previously shown these also to induce a rapid accumulation of *Adh* mRNA in potato discs. In addition, we tested the effect of a *P. infestans* homogenate. Figure 4 shows that ADH activity increased between 24 and 48 h following all of the stress treatments. In these cases, however, there was a lower level of ADH activity at the 24 h time point than in the untreated (control) discs. In water-treated discs, a decrease in activity over the 48 h period was measured. Both these trends agree with the time courses seen in Figure 1. For comparison, the corresponding *Adh* mRNA

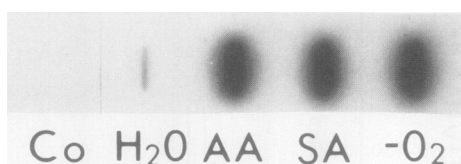


Figure 5. RNA slot blot analysis of *Adh* poly(A)⁺ RNA isolated from discs 24 h after treatment. Abbreviations as in Figure 4.

levels in discs 24 h after treatment are shown by slot blot hybridization (Fig. 5). It is evident that the changes in *Adh* mRNA and ADH activity are not well correlated following these other elicitor treatments as well: the substantial increase in *Adh* mRNA between 0 and 24 h is accompanied by a decrease in ADH activity during the same period.

The finding that the changes in ADH activity and protein level did not parallel the kinetics of *Adh* mRNA accumulation following any of the stress treatments prompted us to investigate actual rates of ADH synthesis by *in vivo* labeling experiments. Figure 6 shows the results of an experiment during which arachidonic acid-treated discs were labeled with [³⁵S]Met for the last 3 h of treatment, proteins extracted, and the ADH polypeptides immunoprecipitated followed by analysis on SDS-PAGE. Radiolabeled ADH could be seen as early as 12 h after elicitor treatment and reached high levels at 24 h. The rate of incorporation of [³⁵S]Met into total soluble protein did not vary significantly throughout the treatment period, with values of 9.9%, 8.5%, and 9.6% (TCA-precipitable cpm/total cpm of extract), for the 0, 12, and 24 h time points, respectively. Thus, ADH synthesis was rapidly and specifically initiated following elicitor treatment, in agreement with the rapid induction of its mRNA. The early synthesis of ADH suggests that in the enzyme assays and immunoblot time-course experiments, *de novo* ADH synthesis was masked by a net decrease in ADH levels. This situation was also seen in *in vivo*-labeled, water-treated discs: ADH synthesis was detected, albeit at a low level, 48 h after treatment (not shown), while the overall level of ADH was declining (Figs. 1 and 3).

It is possible that the constitutive and newly synthesized ADH do not represent the same ADH isozyme. To test this hypothesis, nondenaturing PAGE of protein extracts of discs

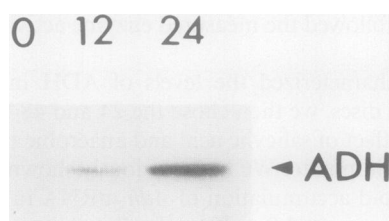


Figure 6. *In vivo* synthesis of ADH after arachidonic acid-treatment of potato discs. The radiolabel (100 μ Ci [³⁵S]Met) was applied for the last 3 h of the incubation period. Proteins were extracted and equal TCA-precipitable counts immunoprecipitated with ADH antiserum as described in "Materials and Methods." Immunoreactive peptides were analyzed by SDS-PAGE and fluorography. Numbers refer to h after treatment.

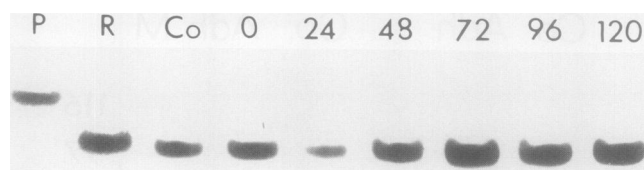


Figure 7. ADH-stained nondenaturing gel of proteins from arachidonic acid-treated discs analyzed at various times after treatment. P, R, and Co refer to extracts of potato pollen, roots and unsliced tubers, respectively. Numbers refer to hours after treatment. Each lane was loaded with 40 μ g total protein.

treated for different times with arachidonic acid was performed. The gels were stained for ADH activity (Fig. 7). As a positive control, we also loaded protein extracts of potato pollen, known to contain the other potato ADH isozyme (21). Only one isozyme band was detected in the discs throughout the time course, the intensity of which changed as in the ADH activity and immunoblot time courses seen in Figures 1 and 3. This indicated that the newly synthesized and constitutive ADH represent the same isozyme.

DISCUSSION

In this paper, we extend to the level of protein synthesis and accumulation our previous results on stress-induced accumulation of *Adh* mRNA in potato discs. *In vivo* labeling experiments demonstrated that the early appearance of *de novo* synthesized ADH polypeptides (Fig. 6) matched the rapid *Adh* mRNA accumulation in arachidonic acid-treated discs we reported previously (22). Time course measurements of total ADH activity and ADH immunoblots, however, gave a different pattern. In arachidonic acid-treated discs, we observed a decrease in ADH activity and protein during the first 24 h, after which both increased. In water-treated discs, a similar decrease followed by a slight increase occurred but these were neither as pronounced nor as rapid.

In both water- and arachidonic acid-treated discs total soluble protein levels remained constant following treatments (CP Constabel, N Brisson, unpublished results). Therefore, the decline in ADH observed was not the result of changes in overall protein content. The possibility that the initial decrease in ADH activity observed was caused by an endogenous inhibitor could also be eliminated, since the levels of actual ADH protein, as judged from immunoblots, matched levels of ADH activity.

Our results thus suggest that during the first 24 h after slicing and arachidonic acid treatment of potato discs, ADH was degraded as well as synthesized. Whether the degradation of ADH continues past 24 h is not clear from our data; synthesis, however, continued until the 96-h time point. Based on their respective mRNA accumulation and ADH activity patterns, it is also likely that the other elicitor treatments (salicylic acid, anaerobiosis, and *P. infestans* homogenate) had this effect. A simultaneous degradation and synthesis of ADH is surprising as part of the plant's response to stress, in particular because, according to results from nondenaturing gel electrophoresis, the ADH turnover does not result in a change in ADH isozyme composition. One possible explanation

tion is that degradation of ADH occurred in a different region or cell layer of the discs than *de novo* ADH synthesis. During wound healing, potato tuber cells divide and redifferentiate to form the wound periderm, a new cell layer distinct from the normal storage parenchyma (12). Our techniques would not have detected spatially separated changes in ADH levels within discs. Finer elucidation of these dynamics will require histochemical data.

Based on the diverse range of ADH-eliciting treatments that we had observed, we previously suggested that these elicitors act by enhancing the response of the tissue to damage caused by the slicing. The data presented here support such an interpretation. There is a basic similarity in the response of potato tubers to slicing (water treated) alone and slicing followed by arachidonic acid-treatment. In both cases, we observed rapid ADH mRNA induction and ADH synthesis with a simultaneous decrease in ADH activity and protein, followed later by an increase. Arachidonic acid seems only to accelerate and increase the degree of the response. Thus, one way to interpret the inductive effect of arachidonic acid and other elicitors on *Adh* expression in potato discs is as agents which damage the tissue and thus amplify the effects of damage from the preparation of the discs.

Anaerobiosis also strongly induces ADH in potato discs but is more difficult to interpret as an amplification of wounding. However, similar physiological changes may result from both types of stresses which could explain why *Adh*, a classic anaerobically induced gene, is also expressed following tissue damage. During anaerobiosis a build-up of lactic acid, a product of glycolysis, lowers the cytoplasmic pH, which in turn activates ethanol synthesis (6). This pH-based metabolic switch (the pH stat) prevents an excessive build-up of lactic acid and further cytosolic acidification. Interestingly, Kimmerer and Kozlowski (13) observed that certain chemical and physical stresses can also induce ethanol synthesis in plants. It was later suggested that these stresses did so by causing a drop in cytoplasmic pH (14). Ethanol synthesis may therefore occur as a response to the decrease in cytoplasmic pH rather than to anaerobiosis as such. It is possible that the induction of *Adh* in potato by the diverse treatments we used is also a response to a lowered cytoplasmic pH. Similar to anaerobiosis, the wound and elicitor treatments may provoke this drop in pH, which then serves as a signal for the induction of the *Adh* gene. There is evidence that pH is involved in pathogen and elicitor stress reactions in other plants. Kneusel *et al.* (17) showed that elicitation of parsley cells was accompanied by a decrease in cytoplasmic pH, and that this stimulates the activity of a phytoalexin biosynthetic enzyme. Using tobacco cell suspensions, Atkinson *et al.* (2) were able to demonstrate a drop in cell sap pH following elicitation with a bacterial pathogen. In potato discs, the physiological consequences of elicitation are not well understood. It would be interesting to test the effect of the treatments which we have used to induce accumulation of ADH on the cytoplasmic pH of potato tuber tissue.

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