Ph.D. THESES

CONTROL OF THE STRESS RESPONSES IN AUXIN HETEROTROPHIC AND AUTOTROPHIC TOBACCO TISSUE CULTURES

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INTRODUCTION

The requirement for auxin and cytokinin to maintain the growth of plant tissue cultures has long been known. Under certain conditions, however, proliferation can take place on medium containing no exogenous auxin and/or cytokinin; these hormone-independent cultures are referred to as autotrophic or habituated tissues, the process - in which the cells regain the hormone synthesizing capacity – is the habituation. Habituation defined as an epigenetic change, the sequence of DNA is not altered, but the DNA methylation patterns and expression of genes, posttranslation modifications can be different. It is extremely stable and heritable at cellular level.

The auxin-independent autotrophic and the auxin-dependent heterotrophic lines of tobacco calli may differ not only in their indoleacetic acid (IAA) synthetizing abilities and sensitivities to exogenous auxins, but also in their primary metabolic pathways. It was observed, that the autotrophic sugarbeet calli gradually lost their peroxidase activities and their organogenic capacity during the habituation, this would affect their stress tolerance either.

The activities of antioxidant enzymes such as catalase, superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), glutathione reductase (GR) , glutathione peroxidases (GSH-PX), glutathione S-transferase (GST) are generally increased in plants under stress conditions and correlate well with enhanced tolerance.

Several plant GST promoters have been found to contain *ocs* (octopine synthase) or *ocs*-like elements, which appear to be stress-inducible, they respond to a variety of electrophilic agents and can be induced by biologically active hormones and inactive hormone analogues. The promoter of the mannopine synthase *mas1'* also contains an *ocs*-like *cis*-acting element.

Heterotrophic and auxin autotrophic tissues have been established from protoplasts of *Nicotiana tabacum* SR1 plants containing *mas1'*:: GUS (β-glucuronidase reporter gene) gene fusion in order to investigate what the effect of changing the hormone metabolism and/or regulation of auxin is on several auxin- and ethylene-induced processes and on the regulation of key enzymes of stress responses.

OBJECTIVES

We have investigated the following questions:

1. What is the reason of the absence or presence of IAA biosynthesis in heterotrophic and autotrophic lines?

2. Why do the heterotrophic and auxin autotrophic tissues differ in their abiotic stress tolerance?

Oxidative stress and the formation of reactive oxygen species are common components among the parameters investigated.

3. As an example for abiotic stress, we have chosen a 100 mM NaCl treatment to answer the following questions:
   - Is there any difference between the H_2O_2 concentrations of the heterotrophic and autotrophic tobacco calli under NaCl stress?
   - How do the activities of the H_2O_2 -producing SOD and the H_2O_2-eliminating catalase, GPX enzymes change in the tissues?
   - How will the enzymes related to the glutathione homeostasis in the heterotrophic and autotrophic tobacco calli be activate under NaCl stress?
   - What differences are there between the two cultures in the activation of detoxification mechanisms, especially concerning the GST and GSH-PX activities?
   - What is the role of ethylene in the abiotic stress tolerance of the heterotrophic and auxin autotrophic tissues?
4. - What is the direct effect of the H_2O_2 -induced oxidative stress on the growth and the extractable GST, GSH-PX activities of the heterotrophic and autotrophic calli?
5. - How does the sensitivities of the two lines to exogenous auxins differ from each other?
   - What is the connection between the exogenous auxin-induced ethylene formation and the auxin/ethylene-regulated *mas1'* promoter activity in the heterotrophic and autotrophic tissues?

- What kind of connection is there in the exogenous 2,4-D-induced ethylene forming and in the auxin/ethylene regulated GST activity in the heterotrophic and auxin autotrophic cultures?

6. What are the possible explanations for the different auxin-ethylene interaction in the heterotrophic and auxin autotrophic calli?

MATERIALS AND METHODS

2-3-week-old microcolonies originating from protoplasts of *Nicotiana tabacum* SR1 plants were transferred onto MS medium containing 2 μM kinetin, 17.4 μM IAA and 0.45 μM 2,4-D and onto the same medium but without IAA and 2,4-D, in order to obtain the auxin heterotrophic and autotrophic cultures. These subcultures were plated separately and after 3 passages the calli growing well on auxin-free medium were selected. The auxin requiring cultures were grown on a solid MS medium containing growth regulator kinetin and auxins, the autotrophic cultures were transferred onto the same medium without auxin, they were kept in a growth chamber at 25 ºC and under 8.4 Wm^{-2} warm white fluorescent light (Tungsram F29 lamps, Hungary).

Investigation of aldehyde oxidase in native gel

The poliacrilamide gel electrophoresis was carried out using 1.5-mm-thick slabs of 7.5 % acrylamide gel in Laemmli buffer system in the absence of SDS at 4 ºC. The gels were loaded with 100 mg protein. After electrophoresis the enzyme was detected with activity staining using IAAd (indole-3-acetaldehyde) and IAld (indole-3-aldehyde) as substrates.

Analysis of ion contents

Two-week-old NaCl-treated callus tissues were dried at 70 ^{o}C for 72 h. Concentrations of Na^{+}, K^{+}, Ca^{2+}, Mg^{2+} and total Fe were analyzed after acidic digestion of the dry material

at 200 ^{o}C for 3 h and the samples were measured with a Hitachi Z-8200 Zeeman polarized atomic absorption spectrophotometer.

Ethylene production

Ethylene was determined after closing the glass flasks hermetically for 24-h. A 2 ml gas sample was injected into a gas chromatograph (Hewlett-Packard 5890 Series II). To investigate the effect of auxin-induced ethylene and the ethylene receptor inhibitor NBD (2,5-norbornadién), the 100 ml jars containing the calli were sealed with sterile serum caps, through which 2 µl liquid NBD/100 ml air was injected onto the wall of flasks.

Activity measurements of antioxidant enzymes

Superoxid dismutase activity was determined by measuring the ability of the enzyme to inhibit the photochemical reduction of Nitro Blue Tetrazolium (NBT) in the presence of riboflavin in the light. Catalase activity was measured spectrophotometrically by following the decrease of H_2O_2 and guaiacol peroxidase activity was determined by measuring the increase in absorbance at 470 nm during oxidation of guaiacol. Glutathione reductase activity was determined by measuring the absorbance at 412 nm when DTNB (Ellmann reagent) was reduced by glutathione (GSH), generated from the glutathione disulfid (GSSG). GST activity was determined spectrophotometrically by using the artificial substrate CDNB, measuring the amount of GSH-conjugated product. Gluthatione peroxidase activity was measured with cumene hydroperoxide substrate, detecting the decrease in NADPH, used to recover GSH from GSSG. GSSG is forming during conversion of the lipid hydroperoxides catalyzed by the GSH-PX. The level of lipid peroxidation was assayed using a thiobarbituric acid method and the H_2O_2 content was measured by spectrofluorometer.

The protein contents of the extracts were determined by the method of Bradford.

The *mas* promoter activity was followed by fuorogenic assay of GUS activity.

RESULTS AND DISCUSSION

Auxin autotrophic callus tissues from the leaf protoplasts of transgenic *Nicotiana tabacum* SR1 plants involving *mas1′*::GUS gene fusion were generated by transferring microcolonies onto MS medium without IAA and 2,4-D. After several passages the calli growing well on auxin-free medium were selected as auxin autotrophic calli.

The growth of the autotrophic cells exhibits a longer logarithmic, “rapid growth” phase: the fresh weight of the tissues growing on auxin-free medium were less than that of the heterotrophic calli growing in the presence of IAA and 2,4-D, but at the end of the 3rd week the difference disappeared. Their sensitivity to the auxin content in the medium had been changed; the exogenous auxin inhibited their growth

Our results can be summarised as follows:

1. The investigation of changes in the IAA metabolism the activity staining for aldehyde oxidase (AO) after native PAGE separation revealed, that the isoenzyme pattern of AO was different in the two types of calli. AO2 exhibited a very strong staining in the heterotrophic tissues using indole-3-aldehide (IAld) as a substrate, suggesting that in the IAA degradation pathway the oxidation of IAld catalysed by AO2 has an important role.

   This enzyme could be involved also in the IAA biosynthetis. Using the indole-3-acetaldehyde substrate we demonstrated, that this activity is present in the heterotrophic calli either¸ which means that the lack of the IAA synthesis of the cultured cells is not due to the limiting AO activity.

   However, in the autotrophic calli a new isoenzyme (AO1) could be detected, which had considerable activity toward IAAld substrate. It is possible, that this newly activated enzyme – which is regulated possibly in a different way – is involved in the synthesis of IAA and in the auxin-independent growth of the autotrophic tissues
2. With the increasing temperatures, the growth rate was elevated at a higher degree in the auxin autotrophic calli than in the heterotrophic cultures: while the heterotrophic

calli was already damaged at 35ºC, the fresh weight of autotrophic tissues was significantly higher and, compared to the 30ºC, they grew even better. This indicates, that the autotrophic and heterotrophic lines of tobacco calli differ not only in their hormone metabolism, but also in their stress tolerance.
Supplying the medium with different NaCl and KNO_2 concentrations resulted significant differences in the growth of the two cultures and veryfied the enhanced abiotic stress resistance of the auxin autotrophic lines.

The oxidative stress and the enhanced production of active oxigen species is common component of these different types of abiotic stresses. For further investigation of the stress tolerance of the autotrophic tissues, we have choosen 100 mM NaCl treatment, which did not influence the growth of the autotrophic cells very much after 2 weeks, but resulted a significant, ca. 30 % growth inhibition in the heterotrophic ones.

3. Determination of the Na^+ concentrations of the 2-week-old calli resulted in similar contents in the two types of tissues and indicated, that the Na^+ uptake of the autotrophic tissues was not inhibited. The levels of K^+, Mg^{2+}, and Ca^{2+} was higher in the autotrophic cultures, but the total Fe, which has a role in the generation of $OH\cdot$ radicals, was lower. NaCl treatment elevated the differences between the cultures. These results suggested, that processes leading to the altered ion homeostasis were activated in the autotrophic calli, which could maintaine the growth of the cells even under stress conditions.

4. Comparison of the superoxid dismutase (SOD) and catalase (CAT) activities in the two lines revealed about 50 % lower activities in the autotrophic tissues than in the heterotrophic lines, their H_2O_2 content was lower either. After NaCl stress the activity of the H_2O_2 scavenging catalase enhanced only in the heterotrophic calli; the autotrophic tissues probably activate other antioxidant mechanisms, which could led to even lower H_2O_2 level after salt stress.

Similarly, the activity of GR is induced by NaCl in the heterotrophic calli, while in the autotrophic lines we could measure lower values and there were no significant changes after 2 weeks of salt treatment.

5. The guajacol peroxidase activity was higher in the autotrophic tissues. One of the most important roles of peroxidases in plant cells is to catalyze the cross-linking of cell wall components and in this way to restrict the expansion of the cells. The differences in cell wall-bound peroxidase activity may be responsible for the different growths of the heterotrophic and autotrophic lines. The covalently bound peroxidase activity increased about 4 times in the presence of 100 mM NaCl in the heterotrophic tissues, but not in the autotrophic cells. The elevated enzyme activity may explain why the auxin autotrophic line grew slowly, but the growth inhibition caused by NaCl was smaller in the autotrophic than in the heterotrophic tissues.
   Determination of the activity of peroxidases with IAA substrate resulted in similarly higher activities in the autotrophic tissues, and this was induced further by NaCl treatment. It suggests, that this activity is under the control of the stress-induced elements rather than the endogenous level of IAA.

6. The glutathione S-transferase (GST) activity was higher in the autotrophic line. The GSTs play an important role in the detoxification mechanism by catalyzing the GSH conjugation of toxic substances under stress. Some isoenzymes of GSTs have been shown to exhibit considerable GSH-PX activity. The plant GSH-PXs are induced by different stresses and their function can be the reduction of alkyl hydroperoxides, such as fatty acid hydroperoxides, and in this way to maintain the membrane integrity.

7. Determination of GSH-PX activities with cumene hydroperoxide substrate resulted 5 times higher GSH-PX activities in the autotrophic than in the heterotrophic calli and it was induced during the salt stress. The level of MDA, a lipid peroxidation product in the tissues, was less in the autotrophic line and the amount of this product decreased further on NaCl treatment. These results indicate, that the autotrophic

calli do posses a very effective acclimation mechanism, in which the elevation of the activities of the peroxidases, the GST and especially the GSH-PX play an important role.

8. The activities of the peroxidases and the GSTs could be influenced by ethylene. The heterotrophic tissues evolved much more ethylene with an early maximum on an auxin-containing culture medium and the cells increased the ethylene production in the presence of the salt, which can be important e.g. in the induction of peroxidases after NaCl treatment. The ethylene formation was much less in the autotrophic cultures, and its level did not change significantly on NaCl treatment. Because the peroxidase activity was higher in this type of calli, it seems, that the role of the ethylene is different in the two cultures.

9. The activation of the defence mechanisms by the generation of AOS including H_2O_2, which is an important part of the abiotic stress response, was the earliest proved in the case of the *GST* genes.
   In order to reveal the sensitivities of the two lines to exogenous H_2O_2, the calli were transferred onto fresh MS medium containing 0.1-10 mM H_2O_2. The growth of the auxin heterotrophic calli decreased significantly at lower H_2O_2 concentrations. This proves that the autotrophic calli really do posses a more effective H_2O_2-scavenging mechanism, but the concentration that activated the cell death at the two lines was the same.
   There was a considerable induction in the GST activity of the heterotrophic tissues in the presence of 0,1-5 mM exogenous H_2O_2, the activation of the GSH-PX was not so definite. The activities of the investigated enzymes was higer in the autotrophic calli, but with the increasing H_2O_2 concentrations the enzymes did not induced as much.

   Several plant GST promoters have been found to contain *ocs* (octopine synthase) or *ocs*-like elements, which can be induced either by different kind of stresses and biologically active hormones, inactive hormone analogues.

10. The activities of wound- and auxin-inducible *mas1′* promoter-driven GUS and GSTs was compared in our transgenic heterotrophic and auxin autotrophic lines growing on medium containing different exogenous IAA or 2,4-D concentrations. The autotrophic calli according to their growth proved to be more sensitive either to IAA or 2,4-D. The GST activity in the autotrophic calli was significantly higher than that in the heterotrophic tissues. The induction was much higher in the autotrophic calli and it was more pronounced following 2,4-D treatment. Comparison of the GUS activities in the heterotrophic and auxin autotrophic lines revealed lower *mas1′*::GUS activities in the autotrophic calli, external auxin usually elevated the GUS activities.Comparing to the control, the induction of the GUS activity was 3-5 times higher in the presence of 2,4-D in the autotrophic tissues.

    Supraoptimal auxin concentrations in the medium might induce ethylene production.

    2,4-D, the synthetic auxin elevated the ethylene evolution in both of the cultures.

11. The GST activity of heterotrophic calli is under the control both of auxin and ethylene. GST activity was increased by different auxin concentrations; the presence of NBD increased further both the GST and *mas1′*::GUS activities. This indicates that ethylene and auxin may counteract in the control of GST and GUS activities in the heterotrophic tissues. According to these results we can conclude, that the exogenous 2,4-D elevated the synthesis both of the ethylene and GST, but among the effect of ethylene is the decreasing of expression of specific genes, including *GST* genes.

    However, in the autotrophic calli the presence of NBD had no significant effect on the GST activity. The higher GST activity in the autotrophic calli could be the result of e.g. changes of auxin-ethylene interaction on their expression processes, if the shift in the synthesis or degradation of the transcription factors involved in the regulation of auxin-induced genes.

    Blocking the ethylene receptors in the autotrophic tissues resulted in an even lower *mas1′*::GUS activity which did not exhibit a 2,4-D concentration dependence.

This finding allows the conclusion that ethylene is primarily responsible for the induction of the *mas1′* promoter activity in the auxin autotrophic tissues. It seems that the activity of the *mas1′* promoter in the autotrophic lines is predominantly under the control of ethylene, while in the heterotrophic lines it is much more under the influence of auxin.

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