QUALITATIVE CHANGES IN THE PROTEINS OF COTYLEDONS DURING COLD AND WARM STRATIFICATION OF *ACER PLATANOIDES* SEEDS

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ABSTRACT

In Norway maple (*Acer platanoides* L.) seeds, during dormancy breakage there appeared some qualitative changes in protein composition of the cotyledons. After 48h of soaking and in the first week of stratification, the number of different proteins decreased. This decrease in number of proteins was probably associated with the effect of hydration of embryo tissue. Protein patterns from imbibed seeds exposed to cold (3°C) and warm (15°C) treatments were not significantly different. The influence of temperature on protein patterns became visible in the third week of stratification, when a few new proteins appeared. One of them, designated B, appeared only during cold stratification before radicles protrusion. They were also present in seeds that had completed germination. These proteins are presumably associated with the process of dormancy breakage.

KEY WORDS: *Acer platanoides*, dormancy breakage, proteins, seed dormancy, stratification.

INTRODUCTION

After reaching physiological maturity, seeds of many trees, including Norway maple, enter a state of deep dormancy, and for germination to occur they require moist, cold treatment (stratification). Norway maple seeds need approximately 13 weeks of cold stratification at 1 to 5°C to emerge from deep dormancy and germinate.

Despite many years of research on tree seeds, the mechanisms of dormancy breaking at low temperature are virtually unknown. The process of seed dormancy breakage involves many parallel and concomitant pathways (Ross 1996). The theories include mechanisms mediated by endogenous growth regulators (Ross and Bradbeer 1971; Tomaszewska 1976; Szczotka and Tomaszewska 1979; Wareing 1982) and consequent quantitative and qualitative effects on gene expression (Slater and Bryant 1987; Taylor et al. 1993; Mullen et al. 1996; Jarvis et al. 1997). The changes in the cytosolic pentose phosphate pathway (Roberts and Smiths 1977) and phytochrome activity (Ross 1996) are also associated with dormancy breaking.

In comparison with physiological studies little is known about the molecular events associated with low-temperature dormancy breaking in seeds of forest species. Many years ago, Tuan and Bonner (1964) proposed that dormancy of seeds is associated with repression of the genome and that the genome is

unmasked as dormancy is broken. Slater and Bryant (1982, 1987), suggested that in *Acer platanoides* embryos during stratification the increased growth potential in embryonic axes was due to an increase in cellular machinery needed for the translation of mRNA, i.e. ribosomes.

In our earlier publications (Pawłowski and Szczotka 1997; Pawłowski et al. 1997) we presented qualitative and quantitative changes of protein synthesis in embryo axes of *Acer platanoides* seeds. We found by 2-DE two proteins associated and numerous unassociated with dormancy breakage. The highest increase of proteins number was observed after hydration. The proteins associated with dormancy breaking in last weeks of cold stratification appeared just before germination of seeds. Cotyledons seemed to be an interesting object of investigations because interaction between the embryo axis and the cotyledon plays an important role in the breaking of dormancy (Janerette 1978). Cotyledons restrained germination of isolated embryos in *Acer platanoides* seeds (Pinfield et al. 1987). Hance and Bevington (1992) also showed that in *Acer saccharum* seeds cotyledons play a role in imposing dormancy on embryo axes. The synthesis of specific proteins investigated by them in cotyledons was repressed during stratification.

The description of quantitative and qualitative changes of proteins in cotyledons was the aim of our investigation, similarly as in our previous work concerning the embryo axes proteins (Pawłowski and Szczotka 1997; Pawłowski et al. 1997), associated with dormancy breakage in cold stratification conditions.

Thomas et al. (1973) suggested that the dormancy of *Acer pseudoplatanus* seeds during the later stages of maturation was of phasic nature. The results of studies carried out in our labo-

Abbreviations:

DDT – dithiothreitol, IEF – isoelectric focusing, 2-DE – two-dimensional electrophoresis, PMSF – phenylmethylsulfonyl fluoride, SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

ratory confirm the phasic nature of changes of numerous metabolic indicators, during dormancy breaking of *Acer platanoides* seeds (Szczotka and Tomaszewska 1979). The phasic character showed protein synthesis dynamics (Pawłowski et al. 1997), the activity of elongation factor 1 (Korcz et al. 1986) and DNA synthesis level (Pawłowski et al. 1997). The first phase is related to imbibition and the second phase is due to low temperature associated with dormancy breaking. The third phase is related to germination. We suppose that this mechanics could result from successive changes in biological activity of the basic growth regulators, for example as discovered by Tomaszewska (1976). She found that dormancy in Norway maple seeds was broken by sequential action of cytokinins, gibberellins and auxins accompanied by the gradual decrease of free abscisic acid. A qualitative analysis of the proteins extracted from embryonic axes of *Acer platanoides* seeds showed that the process of dormancy release during stratification has also such a phasic character (Pawłowski and Szczotka 1997).

The present paper reports the results of investigations of qualitative changes in the protein content of *Acer platanoides* seed cotyledons during dormancy breakage. We have also verified the hypothesis that the dormancy release process has a phasic character.

MATERIALS AND METHODS

The studies were carried out on maple tree seeds collected in the autumn of 1996 in the Kórnik Arboretum. After collection, the seeds were dried at room temperature until they reached approximately 10% water content. Dried seeds were stored in plastic bags at -3°C .

After imbibition (48 h at room temperature) seeds were subjected to cold (3°C – the temperature which breaks dormancy) or warm (15°C – this temperature does not break dormancy) stratification without medium. Germination test was carried out on 50 seeds in 4 repeats. Analyses were carried out on the isolated cotyledons of these seeds. Germinated (with emerged radicles) seeds after cold stratification were also investigated.

Two-dimensional electrophoresis (2-DE) was performed by the combination of isoelectric focusing (first dimension) and SDS-PAGE (second dimension) as developed by Klose (1983). The IEF gels contained 4% w/v carrier ampholytes WITAllytes pH 2-11 (WITA, Teltow, Germany). The protein sample was applied to the anodic side of the gel. After IEF, gels were equilibrated for 10 min in a buffer containing 125 mM TRIS-phosphate, pH 6.8, 40% glycerol, 70 mM DTT, and 3% SDS. The IEF gel was applied to the SDS-PAGE gel, obtained by polymerisation of 15% w/v acrylamide and 0.4% bisacrylamide. The SDS-PAGE system of Laemmli (1970) was used. The separated proteins were later silver stained according to Heukeshoven and Dernick (1985).

Protein for 2-DE was prepared from 2 g cotyledons homogenised in liquid nitrogen in a mortar. The homogenate was thawed in a centrifuge tube and extracted with 10 ml buffer containing 0.5 M sucrose, 50 mM TRIS-HCl pH 8.0, 50 mM KCl, 5 mM magnesium acetate, 10 mM 2-mercaptoethanol, 1 mM PMSF, 0.1 μM pepstatin, 2.1 μM leupeptin. After centrifugation, ammonium sulphate was added to the supernatant (80%, 651 g/l), and this mixture was dialysed in a buffer containing 9 M urea, 25 mM TRIS-HCl pH 7.3, 50 mM KCl, 3 mM EDTA, 70 mM DTT and 2% carrier ampholytes WITAllytes pH 2-11 (WITA, Teltow, Germany). The concentration of the resulting protein determined by the modified (addition of 0.1 N HCl

removes the excess of urea) Bradford method (1976) ranged from 0.5 to 1.5 mg/ml. A sample containing 10 μg of the protein was applied to each isoelectric focusing gel.

The experiments were repeated twice with similar results.

RESULTS

The cold-treated seeds started germination in week 7 of stratification and reached 92% germination at week 13. The seeds stratified at 15°C remained in a state of deep dormancy and failed to germinate until the end of the experiment (Fig. 1).

Fig. 1. The germination of *Acer platanoides* seeds during cold and warm stratification.

Proteins which varied with week and temperature of stratification were marked on the picture with letters A-M (Fig. 2a-i).

Qualitative analysis of cotyledon proteins showed that during the first weeks of stratification the number of proteins distinguished by 2D electrophoresis and silver staining decreased. From 8 proteins (marked E-L) on gel of dry seeds (Fig. 2a) 5 disappeared at 3°C (present only E, I and L) and 6 at 15°C (present E and L) after 48 h of imbibition (Fig. 2b) and the first week of stratification (Fig. 2c, d). In the following weeks of stratification differences between temperatures of treatment became visible. In the third week, at 3°C (Fig. 2e) new proteins marked A, B, C and D appeared (molecular weight approx. 27 kDa). During this time there were no significant changes in the relative abundance of individual polypeptides of seeds stratified at 15°C . In the sixth week of stratification, at 3°C (Fig. 2g) the group of 4 proteins A-B was still present, whereas at 15°C (Fig. 2h) proteins C and D appeared.

In the cotyledons of germinated seeds (Fig. 2i) the whole group of proteins A-D and protein M were present. Protein M was present also during cold stratification, but only in week 4.

The protein marked B was present at 3°C , where dormancy was lost but not at 15°C . It appeared before visible germination while radicle protrusion occurred.

The proteins marked E-L appeared in the cotyledons of these seeds in an irregular way during cold and warm stratification.

DISCUSSION

The above data show that qualitative changes in the protein content of cotyledons of Norway maple seeds occur throughout

Fig. 2a (dry seeds) Fig. 2b (48 h)

Fig. 2c (1 w 3°C) Fig. 2d (1 w 15°C)

Fig. 2. Comparison of 2-DE patterns of cotyledons from *Acer platanoides* dry seeds (a), seeds hydrated for 48 h (b), hydrated seeds stratified at 3°C or 15°C for 1, 3, 6 weeks (c-h) and from germinated seeds (i). Proteins which varied with week and temperature of stratification were marked on the picture with letters A-M.

Fig. 2e (3 w 3°C)

Fig. 2f (3 w 15°C)

Fig. 2g (6 w 3°C)

Fig. 2h (6 w 15°C)

Fig. 2.

Fig. 2i (germinated seeds)

Fig. 2.

cold and warm stratification. After 48 h of imbibition and during the first week of stratification the number of proteins decreased. This was probably caused by hydration of embryo tissue and hydrolysis of some proteins in cotyledons. Differences between protein patterns from seeds exposed to cold and warm treatments in the first weeks were insignificant. In the third week of stratification proteins appeared, which we did not observe in dry seeds or in seeds soaked for 48 h. One of them, marked B, appeared only during cold stratification, before the beginning of radicle protrusion. It was also found in seeds that had completed germination. This protein is probably associated with the process of dormancy breaking.

In general, these results correspond with our previous investigations carried out on embryo axes of *Acer platanoides* seeds (Pawłowski and Szczotka 1997). We observed three phases of changes in protein composition. The first phase is associated with hydration of embryo tissue. The second is connected with appearance of new proteins, caused by the influence of temperature of stratification. In the third phase (the beginning of radicle emergence) proteins probably related to the breaking of the dormancy appeared. In cotyledons, during the first phase, the number of proteins decreased, in contrast to embryo axes (Pawłowski et al. 1997) where a few new proteins appeared. The appearance of new proteins in the second phase occurred in cotyledons a few weeks earlier than in embryo axes. The protein B which is probably associated with dormancy breaking appeared in embryo axes (Pawłowski et al. 1997) not in the second phase but in the third. The difference amounted to a few weeks. In cotyledons the third phase is not so clearly distinguished as in embryo axes. It seems probable that processes of dormancy breaking in cotyledons and in embryo axes are asso-

ciated in some way. It is conceivable that low temperature acts initially in the axis, triggering the movement of some agent(s) which travel to the cotyledons and diminish their inhibitory effect. It is also possible that in addition to whatever low temperature does in cotyledons, it may simultaneously change sensitivity of the axis to inhibition by the cotyledons. Janerette (1978) and Pinfield et al. (1987) observed the inhibitory influence of cotyledons on embryo axes elongation of Norway maple seeds. Hance and Bevington (1992) isolated from cotyledons of *Acer saccharum* the inhibitory proteins which keep seeds in the stage of deep dormancy. Under the conditions of stratification the proteins disappeared and seeds started to germinate.

Nevertheless, most of the new proteins that appeared over several weeks may not be related to the breaking of dormancy since they were observed at both stratification temperatures, including 15°C which does not promote seed germination.

Changes in number of proteins both in embryo axes and cotyledons do not correlate with dynamics of protein synthesis measured by leucine ^{14}C incorporation.

The qualitative changes in proteins, as shown in this paper and the previous investigations of protein synthesis (Szczotka and Tomaszewska 1979; Pawłowski and Szczotka 1997), seem to parallel the dynamics of biological activity of individual growth regulators in embryo axes which have previously been followed by Tomaszewska (1976). The appearance of a large number of new proteins during imbibition and at the start of stratification mirrors the decrease in biological ABA activity in pine seeds (Schneider and Gifford 1994). Also Nicolas et al. (1996) suggest that ABA and GA_3 could be involved in the regulation of the synthesis of two proteins that may play a role in the maintenance of dormancy in seeds of *Fagus sylvatica*. In seeds of Douglas fir Taylor et al. (1993) observed an increase in the level of polypeptides, which were the products of in vitro translation of RNA extracted from cold-treated seeds. Jarvis et al. (1997) showed that some of these proteins belong to group of the LEA (late embryogenesis abundant) proteins encoded by genes which were regulated during dormancy breakage by stratification. LEA transcripts were detected later in the megagametophyte than in the embryo. Ballard et al. (1996) found a few polypeptides in the axes and cotyledons of *Ambrosia trifida* seeds associated with dormancy breakage. However, most of the seed proteins described in literature were associated with maintenance of dormancy, e.g. glycine rich protein isolated from *Fagus sylvatica* seeds (Nicolas et al. 1996), protein SBP65 (Dehaye et al. 1997) and nuclear protein QP47 isolated from *Pisum sativum* (Chiatante 1993), unnamed proteins isolated from *Acer saccharum* (Hance and Bevington 1992) and *Pinus taeda* seeds (Gifford 1993). These proteins tend to disappear under conditions breaking seed dormancy. Such a behaviour is parallel with our results. At the beginning of stratification the number of proteins in cotyledons of *Acer platanoides* seeds also decreased.

We do not know exactly, in what degree quantitative and qualitative changes are the result of transcription de novo or the translation of stored mRNA. For example, Whitmore (1991) reported significant changes in transcription following stratification of *Pinus strobus* and concluded that stratification influences primarily the translation of stored mRNAs. Contrary to this report, recent studies indicated that large and consistent changes in transcription occur concomitantly with a change in the dormant state of *Pseudotsuga menziesii* seeds (Tranbarger et al. 1994). Misra (1994) suggested that different genes were being transcribed rather than being solely changes in translation

of stored RNA. An increase in the amount of specific mRNA transcripts was reported during stratification of *Pinus thunbergii* seeds, indicating that transcription of specific genes was activated during cold stratification (Masumori et al. 1992). It has also been suggested that seed dormancy may be controlled by blocking or unblocking of the synthesis of particular proteins involved in specific processes (Satoh and Esashi 1979).

In conclusion, the present results provide some support for the concept that gradual changes in protein content occur during chilling-induced breaking of seed dormancy. The protein B is probably associated with dormancy breaking both in embryo axes and cotyledons. However it should be stressed that changes take place first in cotyledons and then in embryo axes.

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ZMIANY JAKOŚCIOWE BIAŁEK LIŚCIENI
PODCZAS STRATYFIKACJI CHŁODNEJ I CIEPŁEJ NASION KLONU ZWYCZAJNEGO

STRESZCZENIE

W nasionach klonu zwyczajnego (*Acer platanoides* L.), podczas wychodzenia ze stanu spoczynku, pojawiły się ilościowe i jakościowe zmiany w obrazie białek liścieni. Po 48h namaczania i w pierwszym tygodniu stratyfikacji, liczba białek zmniejszyła się. Zmiana ta prawdopodobnie została spowodowana przez uwodnienie tkanek zarodka. Obrazy białek nasion poddanych chłodnej (3°C) i ciepłej (15°C) stratyfikacji nie różnią się znacząco. Wpływ temperatury uwidocznił się w trzecim tygodniu stratyfikacji, gdy pojawiły się nowe białka. Jedno z nich, oznaczone literą B, pojawiło się tylko w warunkach chłodnej stratyfikacji, zarówno w nasionach jeszcze nie kiełkujących jak i już skielkowanych. To białko prawdopodobnie związane jest z procesem ustępowania spoczynku.

SŁOWA KLUCZOWE: *Acer platanoides*, ustępowanie spoczynku, białka, spoczynek nasion, stratyfikacja.