Оригинальные работы

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SALMINE AND THE HOMEOTIC INTEGRITY OF EARLY EMBRYOS OF NORWAY SPRUCE

Salmine, an arginine-rich protamine, is explored for its concentration-dependent potential to restructure the genome and remodel the homeotic development of Norway spruce embryos expressing monozygotic cleavage polyembryony (MCP). In controls and at low salmine, two protein fractions on SDS-PAGE gels were associated with cells responsible for generating the basal plan for early embryogenesis. With high salmine, embryonal initials no longer differentiated into embryonal tubes. Embryos having embryonal tubes no longer enucleated and differentiated into embryonal suspensors. Biomass and amino acid N declined. Nuclear and cytoplasmic organization was disrupted and nucleoli were highly vacuolated. The transcription of the two protein fractions, PCNA (cyclin) activity and MCP were blocked. Cellular proteins were turned over by proteasomal ubiquitination and others released into the culture medium. Biomass loss and gluconeogenesis of amino acids led to the accumulation of free arginine N. No evidence was obtained with salmine for the remodeling of cells into gametes.

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Introduction. Storage proteins in Norway spruce (*Picea abies* [L.] Karst.) seeds were historically regarded as rivaling the protamines of fish sperm as a rich source for arginine, but the importance of arginine-rich proteins in conifers was difficult to explain [49, 63]. Salmine is found only in the sperm of salmonoid fishes [1, 26]. It takes part in generating haploid germ cells for reproduction. When highly phosphorylated, salmine interacts with somatic chromatin and initiates histone replacement. Subsequent dephosphorylation completes nucleoprotamine condensation and inactivates the genome [4]. These properties make salmine a candidate for displacing proteins, reprogramming cells, and restructuring the homeotic development of early embryos. Moreover, the turnover of salmine to amino acids would contribute to nutrition and the generation of metabolic energy of embryos in suspension cultures in darkness when sucrose in the culture medium is the main energy source.

The basal plan for the homeotic differentiation of an axial tier from "embryo initials" in Norway spruce and other conifers is described by the USE algorithm [9, 11, 67]. The primary early embryonal group (E) establishes an axial tier with apical-distal polarity by differentiating embryonal tube cells (Et). Distal Et cells then differentiate into embryonal suspensors (S). In seed embryos, the suspensor system derives from two sources, an upper tier (U) and from Et cells [10]. In suspension cultures, the U tier does not contribute to the formation of the suspensor system. Norway spruce seed embryos were first classified as not showing MCP [9]. MCP was subsequently demonstrated in cell suspension cultures [3, 17, 33].

A wide range of salmine concentrations are examined as an amino acid source for biomass growth and development. Amino acids are carefully regulated by cells to meet the requirements for protein, nucleic acid and plant hormonal synthesis [49]. This study examines which amino acids promoted biomass recovery, became inhibitory or accumulated in the soluble N pool over a wide range of salmine concentrations.

Second, salmine, being a low mw basic protamine [55], is explored for its potential to bind and displace acidic proteins, and restructure the homeotic basal plan of early embryos. During axial tier development, Et cells undergo enucleation with the release of nucleoli for the differentiation of embryonal suspensors [35, 36]. The TUNEL

(terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling) assay is used to detect DNA degradation in cells along the axial tier [34]. PCNA is required for DNA synthesis and repair [50, 73]. Anti-PCNA (cyclin) [37] and anti-ubiquitin immunofluorescence assays [16] are employed as markers for cell cycling and protein turnover by proteasomal ubiquitination. Proteins having an amino-terminal Lys are turned over to amino acids by ubiquitination [2, 48]. PCNA [6, 46, 61, 72] and salmine [55, 68], both not having an amino-terminal Lys residue, would be protected from turnover by this mechanism [2, 60]. PCNA, and other acidic cellular proteins, may be inactivated by binding with highly basic salmine.

Third, Stains-all reactions [31, 32], having a wide range of distinct colors for the cytochemical detection of many types of proteins, are used to locate transcribed proteins in cells along the axial tier of the basal plan. Soluble proteins, separated and assayed on gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [44], were reacted with Stains-all for comparisons with proteins in cells along the axial tier and released into the culture medium. Control over basal plan development is aimed at improving the quality, uniformity and clonal scale-up of elite Norway spruce genotypes for tree improvement programs employing MCP [17]. Modifying the basal plan with a protamine aims at silencing development for the initiation of gamete formation from somatic cells in tree breeding programs and for the study of mechanisms contributing to the alternation of generations.

Materials and methods. An embryogenic cell line (KJ1) from an elite genotype of Norway spruce from Finland, and showing MCP [40], was scaled up for this study. Cultures, were initiated with one cc packed cell volume in 100 ml 0.5 LP medium, containing 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 5 µM N6-benzyladenine (BA) and 3.5 mM L-glutamine in one-liter nippled flasks rotated at 1 rpm in darkness (23 ± 2 °C). Sucrose in the culture medium was the main energy source. Salmine sulfate (mw 6000 to 7000, Sigma) was added in eight concentrations (g/100 ml) in duplicated treatments (Table). Two flasks, without added salmine, served as controls. After 14 d, early embryos were separated from the culture medium by sedimentation and filtration (Millipore AW03 047 00) under aseptic conditions for cytochemical staining, protein extraction, and free amino acid analyses. The clear and filtered culture medium from each treatment was mixed with 50/50 (v/v) with cold acetone and refrigerated at 4 °C overnight for the recovery of precipitated proteins.

Free Amino Acid Analyses. Triplicated samples of embryo biomass were extracted five times in 70 % ethanol ensuring that all ninhydrin positive materials were recovered. Extracts were dried under a jet of N before being dissolved in 2.2 Na citrate buffer for free amino analysis. Free amino acids (Table) were determined within ± 3 percent using an automated amino acid analyzer designed for the analysis of physiological fluids (LKB Model Alpha-1) [8]. Pearsonrs linear coefficients of correlation [69] were calculated (n = 9) for amino acid N content and biomass recovery.

Cytochemical Stains. Cells were observed with an epi-fluorescence microscope equipped with UV (excitation at 113 360 nm; emission ⩾420 nm) and FITC filters (blue light; excitation at 450–490 nm; emission ⩾114 520 nm). Cellular autofluorescence at ex 365 nm, em 420 nm was weak. Acetocarmine staining 115 [64], which distinguishes chromosomal constituents from other organelles, was used as a red-116 orange marker for totipotent cells having the potential for MCP [15, 17]. Cationic Stains-all [31, 32] at pH 4.1 to 4.3 was used to locate proteins along the axial tier of early embryos. No fewer than twenty slides from each treatment were stained in darkness before exposure to weak light for photography. Proteins stain red to red-orange, less acidic proteins are pink [5]. Nuclear proteins stain purple, and phosphoproteins blue. Mucoproteins stain various colors including pink and blue. The UV fluorescence of stained cells along the axial tier was compared with proteins separated and stained on polyacrylamide gels. Salmine treatments between 2.0 to 8.0 g/100 ml (Table) were used to correlate cytochemical changes with axial tier development, biomass recovery and amino acid N profiles.

Protein Separations and Gel Staining. Soluble proteins in biomass were extracted with Tris-HCl buffer (6.25 mM, pH 6.8) containing sodium dodecyl sulfate (SDS) (2.5 % w/v), dithiothreitol (DTT) (2 % w/v) and glycerol (10 % v/v). The clear filtrate was mixed with 50/50 with cold ace-

tone to recover proteins released into the culture medium and refrigerated at 4 °C overnight before analysis. Proteins in both fractions were separated by filtration and examined with a discontinuous system using SDS-PAGE [44].

Total protein was estimated by Lowry's method [45] with bovine serum albumin a standard. Gels (stacking gel 4 %, separating gel 12.5 %) were run in a Protean II Slab System (Bio-Rad). Proteins were loaded in each well and gels run using Bio-Rad recommendations (stacking: 25 mA/gel, 200 V.H (volts × hours); separating: 35 mA/gel, 1600 V.H). For Stains-all reactions (Figs 3 and 4), gels were fixed in 10 % acetic acid and 50 % ethanol (overnight) and 15 % glutaraldehyde for one hour. Gels were rinsed in distilled water for 2 hours and stained overnight in darkness with a solution of 0.005 % Stains-all in 0.05 % barbital (w/v). Proteins from culture media were loaded in equal volumes on the gels for comparison with those recovered from tissues. Each run was triplicated and kept in darkness to avoid photo bleaching before photography in UV and white light.

*TUNEL, Anti-Ubiquitin and Anti PCN*A. The TdT-mediated dUTP-biotin nick-end labeling (TUNEL) reaction as a marker for apoptosis [28] was modified for conifers [35]. In this assay, DNA degradation by endonuclease activity in nuclei is detected by a terminal deoxynucleotidyl transferase (TdT). TdT labels the 3' OH ends of DNA generated by DNA fragmentation by nicking with biotin-conjugated dUTP for visualization with sulforhodamine as a red fluorescent marker. Counterstaining with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) distinguished the blue non-apoptotic from red apoptotic nuclei.

Ubiquitin (9.6 kDa) antiserum (Sigma lot 100H8860) was conjugated to keyhole limpet hemo-

Salmine g/100 ml	**Nil**	**0.1**	**1.0**	**2.0**	**4.0**	**6.0**	**8.0**	**10.0**	**100**
Gel Lanes	**1, 9**	**4, 12**	**5, 13**					**6**	**7**
N % Total soluble N									
Alanine *	46.0	41.0	42.2	40.6	36.5	33.3	10.2	4.4	nil
Arginine *	2.9	3.9	4.5	10.0	10.8	18.7	25.5	40.9	66.2
Asparagine	4.4	4.6	4.7	4.7	4.8	4.0	4.1	3.6	nil
Aspartate *	0.5	0.6	0.6	0.6	0.9	0.9	0.5	0.2	nil
GABA	3.8	5.3	5.9	6.0	5.2	4.0	3.2	nil	nil
Glutamate *	5.8	3.3	3.2	3.1	3.5	3.9	4.0	4.9	nil
Glutamine	18.9	15.5	12.8	13.6	12.6	16.6	33.6	23.3	nil
Glycine *	2.7	3.1	3.2	3.0	2.9	2.6	1.3	3.4	12.4
Histidine *	1.9	3.3	3.3	3.6	3.6	3.5	3.4	2.0	nil
Isoleucine*	0.8	0.8	0.9	0.9	0.9	0.9	0.6	0.6	nil
Leucine *	0.5	0.8	0.8	0.9	0.9	0.9	1.0	0.9	nil
Lysine *	2.4	3.0	3.1	3.1	4.0	3.1	2.5	2.2	nil
Methionine	0.1	0.1	0.1	0.1	0.1	0.1	0.1	nil	nil
Phenylalanine	0.7	0.8	0.8	0.9	0.9	1.0	1.5	1.1	nil
Proline *	3.4	2.0	1.9	2.0	2.6	3.1	3.0	2.1	nil
Serine *	1.9	2.7	2.7	2.7	2.8	2.9	2.3	7.4	19.1
Threonine *	1.7	1.7	1.7	1.7	1.8	1.5	1.0	1.8	2.3
Tyrosine	0.3	0.4	0.4	0.4	0.4	0.4	0.5	0.6	nil
Valine *	1.3	1.3	1.3	1.4	1.4	1.3	1.2	nil	nil
µM N/g f wt	27.9	60.3	70.8	58.2	55.1	49.7	36.1	4.1	57.1
g Fresh wt	5.5	7.7	8.8	7.2	6.7	5.8	1.5	0.7	0.5

Note. The N in each free amino acid as a percent of the total soluble N (µM N/g f wt) in 746 cellular biomass after 14 d in response to salmine added to the culture medium. Amino acids (*) 747 reported in salmine. GABA is γ-Aminobutyric acid. Underlined values for fresh weights are 748 based on moribund biomass with dead early embryos. Treatments are numerically coded to lanes 749 in SDS-PAGE gels (Figs 3 and 4). Salmine between nil to 1.0 g/100 ml improved biomass 750 recovery and doubled the total soluble N. Higher salmine levels greatly reduced both biomass 751 recovery and the total soluble N.

cyanin as the immunogen. The secondary antibody was anti-rabbit IgG peroxidase conjugate (Sigma). Whole mounts of early embryos were permeabilized by a 1 h exposure to the following enzyme mixture (w/v): 1.0 % cellulysin, 1.0 % pectinase, and 0.5 % macerase in half-strength culture medium. They were rinsed in phosphate buffered saline (PBS), pH 7.0, three times and incubated for 1.5–2 hrs at room temperature with ubiquitin antiserum, diluted 1:100 in PBS. They were again rinsed three times with PBS and treated with a FITC (fluorescein isothiocyanate-labeled anti-rabbit antibody (Sigma) for 1.5–2 hrs at room temperature. At the 164 end of incubation, slides were rinsed in PBS three times and mounted in 50 % glycerol/water for microscopic examination.

PCNA is an acidic non-histone nuclear protein with an apparent mol. wt of 33–36 kDa as determined by SDS-PAGE [46, 72]. For PCNA detection [37], permeabilized embryos were quickly rinsed in 100 % methanol and in PBS with 0.1 % Triton X-100 for 10 min. Embryos were incubated with primary antibody for PCNA for 30 min followed by anti-mouse IgG FITC conjugate (Sigma) diluted in PBS (1:60) and bovine serum albumin (1:70) for 10 min. Preparations were washed twice in PBS for 10 min and mounted on slides for fluorescent microscopy. Controls were prepared without primary and/or secondary antibodies.

Observations. *Amino Acid N and Biomass*. Nil to 1.0 g salmine/100 ml increased biomass recovery by 40 % and more than doubled the total soluble N in biomass over 14 d (Table). Biomass recovery was positively correlated to free alanine (0.9754) and glutamate N (0.8981) and negatively only for arginine N (–0.0553).

Thereafter, salmine concentrations up to 8.0 g/100 ml reduced biomass recovery (8.8 to 1.5 g) and blocked axial tier development. The total soluble N declined by nearly 50 % (70.8 to 36.1 μM N). It was characterized by high percentages of arginine alanine and glutamine N. Compared to the control, the percent free arginine N increased five-fold (4.5 to 25.5 %).

Moribund biomass (10 to 100 g salmine/100 ml), reduced to 0.5 g f wt, was now sticky and comprised of dead embryos. The total soluble N was comprised of 41 to 66 % arginine N. Three other amino acids, which are known residues in salmine, accounted for the remaining soluble N.

Axial Tier Cytochemistry and SDS-PAGE. The limited availability of biomass protein for triplicated assays required that gels were the loaded with half the amount of proteins for treatments compared to controls (Figs 3 and 4, lanes 2 to 7, 10 to 13). Insufficient protein precluded assays for the 10 and 100 g salmine treatments. For the culture medium, protein was recovered only for assays of the control (lane 9), and for salmine treatments between 0.1 to 1.0 g (lanes 10 to 13).

The initial asynchrony in early embryo development had the advantage that all stages of axial tier development could be observed in each treatment and in response to salmine. With acetocarmine, embryo initials of the early embryonal group (E) stained red-orange (Fig. 1. All figures look a pasting-in at the end of number). Enucleated embryonal tubes and the elongated suspensors showed little or no acetocarmine staining. SDS-PAGE gels, stained with acetocarmine, revealed a complex, thick and diffuse red-orange band of low mw proteins and accounted for most of the acetocarmine staining in whole mounts.

Stains-all more completely differentiated all three tiers of the basal plan (Fig. 2). E and Et cells stained red-orange. Embryonal suspensors stained blue. Staining patterns corresponded with proteins separated and stained on SDS-PAGE gels (Fig. 3, lanes 1 to 7). Two protein fractions were associated with cells that generated the basal plan for early embryogenesis. A low mw pink fraction corresponded to the acetocarmine red-orange band (Fig. 3, lane 1, ca. 4 to 6 kDa). A second and stronger red-orange fraction (ca. 18–20 kDa) corresponded to staining in embryonal initials and in the Et tier.

Low salmine stimulated biomass recovery with small changes in protein fractions (Fig. 3, lanes 2 to 5). In controls, blue proteins >97 kDa associated with elongating suspensors were released as conditioning factors in the culture medium (lane 9). With increased salmine more purple nuclear proteins from the E and Et tiers appeared in the culture medium (lanes 9 to 12).

High salmine (>1.0 g/100 ml, lanes 6, 7) blocked axial tier differentiation and reduce red-orange staining proteins (lane 1 control) in the E and Et tiers. Purple nuclear proteins <97 kDa (E tier) were increased and released in the culture medium. Blue cytoplasmic proteins were found mainly in embryonal suspensors (S tier) (Fig. 2). Salmine migrated

at the top of the low mw pink and blue proteins (6 to 7 kDa, lanes 6 and 7). Increases of proteins in lanes 6, 7 and 10 to 13 were due to the binding of salmine and to the denaturation cellular proteins.

UV fluorescence brought out more details (Fig. 4). In moribund cultures, proteins (35–36, 19–25 and 14 kDa) which stained red-purple (Fig. 3, lanes 6 and 7) gave a yellow or bright red-orange fluorescence. Their molecular masses corresponded with conifer histones, nonhistone chromosomal proteins, and ribosomal proteins [56–59]. Histone F1 and F2a1 are observed at 25 and 11kDa respectively, and F3 is 14 kDa [57, 58]. Nonhistone chromosomal proteins (NHCP) occupy a wider kDa ranges than histones and are more heterogeneous [59]. Conifer ribosomal proteins have molecular masses between 10 to 82 kDa [55].

Subcellular Organization. Cytoplasm and nuclei (E tier) were disrupted by salmine >1 g/100 ml (Fig. 5). Nucleoli became highly vacuolated. The binding of acidic proteins to salmine resulted in irregular purple patches in the cytoplasm. A band of congealed nucleoproteins was observed around nuclear membranes of most cells (Figs 5, 6, 7). Chromosomal proteins at a metaphase plate (* Figs 6, 7), were purple and fluoresced red-orange with Stains-all.

Figs 8 and 9 compare the Stains-all reactions in white and under UV light (4.0 g salmine/100 ml). High mw blue proteins on gels and in embryonal suspensors gave a red fluorescence under UV light. Low mw blue proteins on gels (e.g., Fig. 3, lanes 5 and 6) fluoresced yellow and were associated with spent nuclei. Red proteins associated with the enucleation of Et cells were degraded by an assembly of proteasomes (*) (Fig. 8). The red cytoplasm in three small cells matched with cytoplasmic proteins being degraded by proteasomes.

In moribund biomass (10 and 100 g salmine), low mw proteins fluoresced yellow and accumulated on SDS-PAGE gels (Fig. 4, lanes 6 and 7) but not in the culture medium. Red and orange fluorescent proteins increased in the culture medium when nuclei (yellow fluorescence) became spent (4 to 8 g salmine, Fig. 9).

Sequential Blocks in Axial Tier Differentiation. Basal plan development was not visibly altered by salmine treatments less than 1 g/100 ml. The TUNEL reaction and release of free nucleoli into the cytoplasm of Et cells were useful markers for apoptosis (Fig. 10). Nuclei in the E tier continued to show a bright blue fluorescence with DAPI.

In co-joined embryos showing MCP, salmine (2.0 g/100 ml) inhibited Et and S formation (Fig. 11). With Stains-all, the protodermal cells in the early embryonal group now stained brown-yellow rather than the characteristic red-orange of controls. Two small early embryos, one attached to each flank of a large central and primary early embryo, aborted embryonal tube formation and prematurely stained blue.

E tiers, not having yet differentiated embryonal tubes, increased in size and showed increased dark-purple staining between cells (Fig. 12). These proteins were also found in the culture medium (Fig. 3). At high salmine concentrations >6 g/100 ml (Fig. 13), embryonal initials started to turn from a dark purple to brown-black. Embryos became disorganized and moribund.

PCNA, Ubiquitination, Proteins in the Culture Medium. When Et formation was blocked by salmine (Fig. 14), PCNA was detected as specs of green-yellow fluorescence on the surfaces of early embryonal initials. The differentiation of embryonal suspensors required that Et cells enucleate and release their nucleoli, one of which (n) is shown in Fig. 15. Green-yellow anti-PCNA fluorescence occurred throughout the enucleated cytoplasm. Degraded chromatin (chromosomes $n = 12$) was lit up by anti-PCNA fluorescence (Fig. 16). In elongated embryonal suspensors, the ubiquitination of residual proteins were located by FITC anti-ubiquitin fluorescence. Acetone precipitates from the culture medium (lanes 9 to 13, Figs 3 and 4) contained high mw blue proteins, derived mainly from embryonal suspensors, and a wide range of purple nuclear and cytoplasmic proteins

Discussion. *Amino Acid Profiles and Biomass Recovery*. During seed-embryo development, the arginine-rich proteins, unlike salmine, are synthesized, compartmentalized, protected and stored in protein bodies [20]. Protein bodies contain storage, matrix, and crystalloid proteins, ranging between 15, 20, 37, 38, 39, 40 and 71–212 kDa [34]. With imbibition and germination, the contents of protein bodies are mobilized for the provision of amino acids for growth and development. As protein bodies empty, their membranes coalesce to form vacuoles [14, 20, 21]. A protein argi-

nine kinase, which *N*-phosphorylates Arg residues in proteins, serves as an epigenetic factor for the utilization of storage proteins during germination [56, 59].

L-Glutamine, added to the culture medium, contributed amide N and glutamic acid as a substrates for transaminations providing an array of protein amino acids for biomass gain. The larger the soluble N pool, the more biomass was recovered (Table). Low levels of salmine increased biomass recovery and arginine N in the soluble N pool. Between 1 and 2 g salmine, biomass declined (8.8 to 7.2 g); arginine N doubled (4.5 to 10.0 %) and axial tier development was now being blocked (Figs 11, 12, 14).

As biomass became moribund, amino acids underwent gluconeogenesis to glucose for the provision of energy. At the highest salmine treatments, arginine N now comprised 41 to 66 % of the total soluble N (Table). Only four of the thirteen amino acids (arginine, glycine, serine, threonine), reported in salmine [7], remained in the soluble N. The other amino acids were oxidized by catabolism to carbon dioxide and water via the citric acid cycle and respiratory chain.

Salmine and Protein Interactions. When highly phosphorylated, protamines in salmonoid fishes interact with somatic chromatin and initiate histone replacements for soma-to-germline transformations [4]. Dephosphorylation by protein phosphatases completes nucleoprotamine condensation and inactivates the genome. When chromatin is remodeled, transcribed enzymes covalently modify the histones and use ATP to reposition nucleosomes on DNA [50].

In Norway spruce nuclei, the Arg residues of salmine would invade the minor groove of the DNA duplex forming hydrogen bonds with the DNA backbone and compete with DNA-binding proteins for DNA-sequence preferences. Invasion would disorganize the chromatin normally regulated by constituent histones and NHCPs. Histone and NHCP displacements were evident by changes in SDS-PAGE gels and supported by acetocarmine and Stains-all reactions for nuclei. The phosphorylation of assimilated salmine was unlikely because salmine inhibits phosphorylases and substrate phosphorylation [42, 46].

Protamines are used in cation-exchange chromatography to displace proteins [29]. In the embryogenic embryonal initials (E tier), salmine displaced cytoplasmic proteins and disrupted subcellular organization (Figs 5 and 6). The increased purple staining around the nuclear membrane and in the cytoplasm indicated the congealment of displaced nuclear proteins with cytoplasmic proteins. In controls, these cells normally stain red-orange and not purple (Fig. 2).

Tyrosine-phosphorylated proteins [38] and a filamentous network of proteins in the nuclear lamina underlay the inner nuclear membrane [30]. They became disorganized by salmine as judged by the heavy purple staining concentrated around nuclear membranes (Fig. 5). These proteins normally bind to DNA, interact with chromatin via histones, and anchor chromatin fibers to the nuclear periphery. Disorganized nuclear networks, due to the displacement of nuclear proteins, were characterized by the residual yellow fluorescence of spent nuclei (Fig. 9) and by an increase in yellow fluorescent proteins on gels (Fig. 4, lanes 6 and 7).

The accumulation of purple stain around nuclear envelopes represented blocks in the formation and traffic of ribosomes, tRNA and mRNA. Large complexes, responsible for the synthesis and cleavage of rRNA precursor, would be inactivated. Increased nucleoli vacuolation (Fig. 5) is a biomarker for blocks in nucleolar RNA and ribosomal protein processing. Time-lapse studies with jack pine cells fed D-glutamine showed an increase in the size and number of nucleolar vacuoles when protoplasmic streaming completely stopped [18]. In barley and wheat, salmine became bound to cell surfaces and inhibited root elongation [48]. Histones and polylysine were not growth inhibitory.

Transcription Factors, PCNA and Ubiquitination. No single transcription factor has yet been identified as initiating or controlling homeotic development and MCP. Red-orange staining with acetocarmine and Stains-all revealed two main protein fractions specifically associated with embryonal initials responsible for generating the basal plan. One was a low mw acetocarmine-reactive fraction (Fig. 3, lane 1, ca.) in embryonal initials showing MCP (Fig. 1). With Stains-all and at 4 to 6 kDa, this fraction was red-orange (Fig. 2, Fig. 3, lane 1). Under UV light, it gave a weak yellow-orange fluorescence. In the presence of salmine, it corresponded to the yellow fluorescence of proteins released from spent nuclei.

A second and stronger red-orange fraction (ca. 18–20 kDa) was brought out by Stains-all but poorly with acetocarmine. It may comprise transcription factors reported for the control of spruce embryogenesis [54, 70]. Salmine altered the Stains-all color reactions of nuclear proteins and their distribution in cells. This occurred in embryonal initials when axial tier differentiation was blocked (Figs 5 to 7).

Leucine, isoleucine and valine are highly abundant residues in ubiquitin [60, 61] and occur in salmine [1, 7]. Acetolactate synthase is responsible for the biosynthesis of these amino acids. Chlorsulfuron, an inhibitor of acetolactate synthase, disrupted the development of axial tiers in a similar way to salmine [24**].**

In white spruce (*Picea glauca*) somatic embryos, regulatory proteins, having Leu residues, are transcribed by a homeodomain leucine-zipper gene (*HD-Zip*) [70]. HD-Zip transcription factors recognize two distinct 9 bp pseudopalindromic sequences in DNA [64]. These factors are also encoded by *REVOLUTA (REV)* genes that initiate the formation of lateral meristems [53]. In Norway spruce, the initiation of meristems in laterally flanking early embryos, co-joined by MCP, may also require transcription factors from *HD-Zip*.

Norway spruce nuclei (E tier), exposed to high chlorsulfuron disabling axial tier differentiation, reacted to antibodies for the human transcription factor p53 (monoclonal, amino acids 371–380) and to the cell cycle inhibitor p21 (mouse, amino acids 2–159) [24]. In humans and mice, the disabling of p53 function improved the efficiency of inducing and producing pluripotent stem-cells even when DNA damage was present [43]. Pluripotency required p21 as an effector. By analogy, the nuclear proteins in Norway spruce, containing amino acid sequences common to p53 and p21, when disabled may comprise a mechanism in embryonal initials enabling axial tier differentiation and the expression of MCP.

Before enucleated embryonal suspensors are differentiated from Et cells, lamins may be solubilized [51]. In Et cells, PCNA accumulates in nucleoli late in the G1 and early S phase. During mitotic exit, PCNA is down-regulated by ubiquitination [46, 50] and free nucleoli are released into the cytoplasm (Fig. 10) [35]. Residual chromatin strands, having anti-PCNA fluorescence, were observed during enucleation and before the transition of Et cells to embryonal suspensors (Figs 8, 9, 15–17).

Salmine blocked Et cells from differentiating into embryonal suspensors (Figs 11, 14). The binding of salmine to PCNA and other acidic proteins disrupted cellular organization, stopped transcription, congealed the cytoplasm, and contributed to the release of proteins into the culture medium. Specks of PCNA were found on the surfaces of embryos without embryonal suspensors (Fig. 14). PCNA and salmine, both lacking a Lys N-terminal amino acid, would not be degraded by proteasomal ubiquitination [50]. Ubiquitin ligases are the last enzymes in the ubiquitin-protein-conjugation pathway. Proteasomes are proteases which initiate the ATP-dependent degradation of ubiquitin-protein conjugates [62].

Fourth fifths of the N-terminal groups in salmine are proline [55]. The rest are arginine, serine, and glycine. An ubiquitin ligase in *Arabidopsis* mediates the degradation of proteins having N-terminal Glu or Asp residues by their substitution with Arg [27]. This is accomplished by a tRNA-protein transferase which transfers arginine from Arg-tRNA to the N-terminus of target proteins having Glu or Asp residues [25, 69]. Salmine, not having N-terminal Glu, Asp and Lys, would not be turned over by this mechanism. It would also not be turned over by the N-terminal Lys rule for ubiquitination. Core histones, having N-terminal in Lys and Arg, would be susceptible to degradation and account for some of the amino acid changes in the soluble N pool. Salmine inhibits the proteolytic activity of arginine-specific cysteine proteases [41]. Inhibition may account for some of the undegraded proteins in the culture medium (Figs 3 and 4, lanes 9 to 13).

Hormonal Control and Embryogenesis. Basal plan development is governed by the transcription of a cascade of homeobox proteins which determine physiological and hormonal gradients [74]. Proteins along the axial tier and on SDS PAGE gels (Figs 3, 4), when color-coded to cells, delineated the alteration of transcripts associated with the blocking of homeotic progression. In Norway spruce, polar auxin transport and the expression of a putative protein coding *WUSCHEL-related homeobox 2 gene (WOX2)*, were highest at the earliest stages of somatic embryo development [54].

Auxins provide positional information by means of their compartmentalization, distribution and gradients in tissues. Hormone signaling pathways connect to proteasomal ubiquitination [62]. Together with F-box proteins, the ubiquitin ligases have been shown to provide specificity and function as receptors for some plant hormones. In *Arabidopsis*, they also contribute to the auxin-dependent de-repression of transcription.

Nitric oxide (NO) is now being recognized as a plant hormone [62]. Protodermal cells of embryonal initials produce NO from arginine and oxygen by a putative nitric oxide synthase (NOS) [17, 22]. Low levels of NO establish apical-distal polarity and orient plant roots to gravity [39]. NOSs represent important transcription factors. The disruption of cells by salmine would elevate NO levels and damaging nitrosative and oxidative stresses [22]. High levels of NO may have contributed to the browning of protodermal and other cells in E tiers showing MCP (Fig. 11).

Internal hormonal gradients arising from 2,4-D and BA in the culture medium were suppressed by salmine as judged by blocks in axial tier development (Figs 11 to 13). In clonal procedures, abscisic acid is used to separate embryos co-joined by MCP and enhance embryo maturation [3, 17]. The ubiquitin-proteasomal pathways [62] may have blocked abscisic acid activity so that the early embryos remained co-joined.

SmRNAs were first detected in conifers as products of ^{32}P-labeling but their role in embryogenesis was not clear [23]. Small noncoding RNAs (smRNAs) and microRNAs (miRNAs) are now recognized as molecular switches with hormones to down regulate gene expression [50]. Down-regulation is achieved by reducing the translation and/or the stability of mRNAs. This shuts down early developmental patterns and allows for later ones to proceed. Specific smRNAs and miRNAs are required for the silencing of gene expression in somatic and zygotic embryos of loblolly pine [52]. Genes are silenced by degrading mRNA [66]. In Norway spruce, RNA synthesis in nucleoli and RNA-dependent molecular switches were blocked at the nuclear membrane by congealment of salmine with acidic proteins.

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САЛЬМИН И ГОМЕОЗИСНАЯ ЦЕЛОСТНОСТЬ ЗАРОДЫШЕЙ ЕЛИ ОБЫКНОВЕННОЙ НА РАННИХ СТАДИЯХ

Сальмин, богатый аргинином протамин, вовлечен в реструктуризацию генома и изменение гомеозисного развития зародышей ели обыкновенной, проявляющих монозиготную расщепленную полиэмбрионию благодаря его зависимому от концентрации потенциалу. В контрольных экспериментах и при низкой концентрации сальмина с помощью гель-электрофореза в присутствии додецилсульфата натрия обнаружено, что две белковые фракции ассоциированы с клетками, ответственными за развитие самых начальных этапов раннего эмбриогенеза. При высоких концентрациях сальмина эмбриональные инициалии не дифференцировались в эмбриональные трубки. Зародыши, имеющие эмбриональные трубки, не теряли ядро и дифференцировались в эмбриональные суспензоры. Биомасса и содержание аминокислотного азота при этом уменьшались. Ядерная и цитоплазматическая организация нарушались, а ядрышки были сильно вакуолизированы. Транскрипция двух белковых фракций, активность циклина PCNA и монозиготная расщепленная полиэмбриония блокировались. Часть клеточных белков посттрансляционно убихитинилировалась, а часть выделялась в культуральную среду. Потеря биомассы и глюконеогенез аминокислот приводили к накоплению свободного аргинина. Не получено доказательств участия сальмина в ремоделировании клеток в гаметы.

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САЛЬМІН ТА ГОМЕОЗИСНА ЦІЛІСНІСТЬ ЗАРОДКІВ ЯЛИНИ ЗВИЧАЙНОЇ НА РАННІХ СТАДІЯХ

Сальмін, багатий аргініном проталін, залучений в реструктуризацію геному та зміни гомеозисного розвитку зародків ялини звичайної, що проявляють монозиготну розщеплену поліембріонію завдяки його залежному від концентрації потенціалу. В контрольних експериментах і при низькій концентрації сальміну за допомогою гель-електрофорезу у присутності додецилсульфату натрію виявлено, що дві білкові фракції асоційовані з клітинами, що відповідають за розвиток початкових етапів раннього ембріогенезу. При високих концентраціях сальміну ембріональні ініціалії не диференціювались в ембріональні трубки. Зародки, що

мали ембріональні трубки, не губили ядро і диференціювались в ембріональні суспензори. Біомаса та вміст амінокислотного азоту при цьому знижувались. Ядерна та цитоплазматична організація порушувались, а ядерця були сильно вакуолізовані. Транскрипція двох білкових фракцій, активність цикліну PCNA та монозиготна розщеплена поліембріонія блокувались. Частина клітинних білків посттрансляційно убіхітинувалась, а частина виділялась в культуральне середовище. Втрата біомаси та глюконеогенез амінокислот приводили до накопичення вільного аргініну. Не одержано доказів участі сальміну в ремоделюванні клітин у гамети.

REFERENCES

1. *Ando T., Yamasaki M., Suzuki K.* 1973. Protamines. Springer Verlag, New York.
2. *Bachmair A., Finley D., Varshavsky A.* In Vivo Half-Life of a Protein is a Function of its Amino Terminal Residue. *Science*, vol. 234, pp. 179–186.
3. *Boulay M.P., Gupta P.K., Krogstrup P., Durzan D.J.* Development of Somatic Embryos from Cell Suspension Culture of Norway Spruce (*Picea abies* Karst.). *Plant Cell Repts*, 1988, vol. 7, pp. 134–137.
4. *Brewer R.L., Corzett M., Balhorn R.* Protamine-Induced Condensation and Decondensation of the Same DNA Molecule. *Science*, 1999, vol. 286, pp. 120–123.
5. *Campbell K.P., MacLennan D.H., Jorgensen O.* Staining of the Ca-Binding Proteins, Calsequestrin, Calmodulin, Tropinin C, and S-100, with the Cationic Carbocyanine Dye «Stain-all». *J. Biol. Chem.*, 1983, vol. 258, pp. 11267–11273.
6. *Citterio S., Sgorbati S., Levi M., Colombo B.M., Sparvoli E.* PCNA and Total Nuclear Protein Content as Markers of Cell Proliferation in Pea Tissues. *J. Cell. Sci.*, 1992, vol. 102, pp. 71–78.
7. *Corfield M.C, Robson A.* The Amino Acid Composition of Salmine. *Biochem. J.*, 1953, vol. 55, pp. 517–522.
8. *Dilley K.J., Rocek R.M.* Physiological Fluid Analysis Using the LKB 4400 Amino acid Analyzer with a Lithium Buffer System. 1982, *LKB Protein Chemistry Note*. No. 11. LKB Biochrome Ltd., Cambridge, U.K.
9. *Dogra P.D.* Morphology, Development and Nomenclature of Conifer Embryo, *Phytomorphology*, 1979, vol. 28, pp. 307–322.
10. *Dogra P.D* The Embryology, Breeding Systems and Seed Sterility in Cupressaceae-A Monograph. In: *Glimpses in Plant Research*, ed. Nair, P.K.K., 1984, Vikas House, New Delhi, vol. 6, pp. 1–126.
11. *Doyle J.* Proembryogeny in *Pinus* in Relation to That in Other Conifers – A Survey. *Proc. Royal Irish Acad.*, 1963, vol. 62B, pp. 181–216.
12. *Durzan D.J.* Nitrogen Metabolism of *Picea glauca*. I. Seasonal Changes of Free Amino Acids in Buds, Shoot Apices and Leaves, and the Metabolism of Uniformly Labeled 14C-L-527 Arginine by Buds during the Onset of Dormancy. *Canadian J. Botany*, 1968, vol. 46, pp. 909–919.
13. *Durzan D.J.* Nitrogen Metabolism of *Picea glauca*. IV. Metabolism of Uniformly Labeled 14C-L-Arginine, [Carbamyl-14C]-L-Citrulline, and [1,2,3,4–14C]-?-Guanidinobutyric Acid during Diurnal Changes in the Soluble and Protein Nitrogen Associated with the Onset of Expansion of Spruce Buds. *Canadian J. Biochemistry*, 1969, vol. 47, pp. 771–783.
14. *Durzan D.J.* Metabolism of Tritiated Water during Imbibition and Germination of Jack Pine Seeds. *Canadian J. Forest Research*, 1983, vol. 13, pp. 1204–1218.
15. *Durzan D.J.* Metabolic Phenotypes in Somatic Embryogenesis and Polyembryogenesis. In *Genetic Manipulation of Woody Plants*. Eds, Hanover J., Keathley D.E., Plenum Press, N.Y., 1988, pp. 293–312.
16. *Durzan D.J.* Protein Ubiquitination in Diploid Parthenogenesis and Early Embryos of Norway Spruce. *Intl. J. Plant Science*, 1996, vol. 157, pp. 17–26.
17. *Durzan D.J.* Monozygotic Cleavage Polyembryogenesis. *Cytology and Genetics*, 2008, 42:159–173.
18. *Durzan D.J., Bourgon G.* Growth and Metabolism of Cells and Tissue of Jack Pine (*Pinus banksiana*). 7. Observations on Cytoplasmic Streaming and Effects of L-Glutamine and its Analogues on Subcellular Activities. *Canadian J. Botany*, 1976, vol. 54, pp. 507–517.
19. *Durzan D.J., Jokinen K., Guerra M., Santerre A., Chalupa V., Havel L.* Latent Diploid Parthenogenesis and Parthenote Cleavage in Egg-Equivalents in Norway Spruce. Intl J. Plant Science, 1994, vol. 155, pp. 677–688.
20. *Durzan D.J., Mia A.J., Ramaiah P.K.* The Metabolism and Subcellular Organization of Jack Pine Embryos (*Pinus banksiana*) During Germination. *Canadian J. Botany*, 1971a, vol. 49, pp. 927–938.
21. *Durzan D.J., Mia A.J., Wang B.S.P.* Effects of Tritiated Water on the Metabolism and Germination of Jack Pine Seeds. *Canadian J. Botany*, 1971b, vol. 49, pp. 2139–2149.
22. *Durzan D.J., Pedroso M.C.* Nitric Oxide and Reactive Nitrogen Oxide Species in Plants. *Biotechnology and Genetic Engineering Reviews*, 2002, vol. 19, pp. 293–337.
23. *Durzan D.J., Pitel J., Ramaiah P.K.* Acid Soluble Nucleotides and Ribonucleic Acids from Germinating Jack Pine Seeds. *Canadian J. Forest Research*, 1972, vol. 2, pp. 206–216.
24. *Durzan D.J., Santerre A., Havel L.* Effects of Chlorsulfuron on Early Embryo Development in Norway Spruce Cell Suspensions. In: *Cell biology and Instrumentation:*

UV Radiation, Nitric Oxide, and Cell Death in Plants. Eds, Blume, Y., Durzan, D.J., Smertenko, P., NATO Advanced Research Workshop, Yalta, Ukraine, Sept 8 to 11, 2004, IOS Press, Amsterdam. 2006, pp. 262–273.
25. *Elias S., Ciechanover A.* Post-Translational Addition of an Arginine Moiety to Acidic NH2 Termini of Proteins is Required for Their Recognition by Ubiquitin-Protein Ligase. *J. Biol. Chem*. 1990, vol. 265, pp. 15511–15517.
26. *Felix K.* Protamines. *Advances in Protein Chemistry*, 1960, vol. 15, pp. 1–109.
27. *Garzón M., Eifler K., Faust A., Scheel H., Hofmann K., Koncz C., Yephremov A., Bachmair A.* PRT6/At5g02310 Encodes an *Arabidopsis* Ubiquitin Ligase of the N-End Rule Pathway with Arginine Specificity and is Not the CER3 Locus. *FEBS Lett*., 2007, vol. 581, pp. 3189–3196.
28. *Gavrieli Y., Sherman Y., Ben-Sasson B.* Identification of Programmed Cell Death In Situ via Specific Labeling of Nuclear DNA Fragmentation. *J. Cell Biol*. 1992, vol. 119, pp. 493–501.
29. *Gerstner J.A., Cramer S.M.* Cation-Exchange Displacement Chromatography of Proteins with Protamine Displacers: Effect of Induced Salt Gradients. *Biotechnology Progress*, 1992, vol. 8, pp. 540–545.
30. *Gotzmann J., Foisner R.* Lamins and Lamin-Binding Proteins in Functional Chromatin Organization. *Crit. Rev. Eukaryote Gene Expression*, 1999, vol. 9, pp. 257–265.
31. *Green M.R., Pastewka J.V.* Simultaneous Differential Staining by a Cationic Carbocyanine Dye of Nucleic Acids, Proteins and Conjugated Proteins. *J. Histochem. Cytochem*. 1974, vol. 22, pp. 774–781.
32. *Green M.R., Pastewka J.V., Peacock A.C.* Differential Staining of Phosphoproteins on Polyacrylamide Gels with a Cationic Carbocyanine Dye. *Analytical Biochemistry*, 1973, vol. 56, pp. 43–51.
33. *Gupta P.K., Durzan D.J.* Plantlet Regeneration via Somatic Embryogenesis from Subcultured Callus of Mature Embryos of *Picea abies* (Norway Spruce). *In Vitro* Cellular and Development, vol. 22, pp.685–688.
34. *Hajduch M., Nahálková J., Hrib J., Vooková B., Gemeiner P.* An Electrophoretic Analysis of the Seed Protein Body Proteins from *Pinus nigra*. *Biol. Plantarum*, 2001, vol. 44, pp. 137–140.
35. *Havel L., Durzan D.J.* Apoptosis during Diploid Parthenogenesis and Early Somatic Embryogenesis of Norway Spruce. *Intl J. Plant Science,* 1996a, vol. 157, pp. 8–16
36. *Havel L., Durzan D.J.* Apoptosis in Plants. *Botanica Acta*, 1996b, vol. 109, pp. 268–277.
37. *Havel L., Scarano M.T., Durzan D.J.* Xylogenesis in Cupressus Callus Involves Apoptosis. *Adv. Hort. Sci*., 1997, vol. 11, pp. 37–40.
38. *Henning O., Dreger M., Bengtsson L., Hucho F.* Identification of Tyrosine-Phosphorylated Proteins Associated with the Nuclear Envelope. *FEBS J*., 2001, vol. 268, pp. 420–428.
39. *Hu X., Neill S.J., Tang Z., Cai W.* Nitric Oxide Mediates Gravitropic Bending in Soybean Roots. *Plant Physiol*., 2005, vol. 137, pp. 663–667.
40. *Jokinen K., Durzan D.J.* Properties of Rescued Embryonal Suspensor Masses of Norway Spruce (*Picea abies* L. Karst.) Determined by the Genotype and the Environment in Vitro, *Silva Fennica*, 1994, vol. 28, pp. 95–106.
41. *Kontani M., Amano A., Nakamura T., Nakagawa I., Kawabata S., Hamada S.* Inhibitory Effects on Proteolytic and Adhesive Activities of *Porphyromonas ginivalis. Infection and Immunity*, 1999, vol. 67, pp. 4917– 4920.
42. *Krebs E.G.* 1954. The Effect of Salmine on the Activity of Phosphorylase. *Biochim. Biophys. Acta*, 1954, vol. 15, pp. 508–515.
43. *Krizhanovsky V., Lowe S.W.* The promise and perils of p53. *Nature*, 2009, vol. 460, pp. 1085–1086.
44. *Laemmli U.K.* 1970, Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* (London), 1970, vol. 227, pp. 680–685.
45. *Lowry O.H., Rosebrough N.J., Farr A.L., Randall N.T.* Protein Measurement with the Folin Phenol Reagent. *J. Biol. Chem*. 1951, vol. 193, pp. 265–275.
46. *Madsen N.B., Cori C.F.* The Interaction of Phosphorylase with Protamine. *Biochim. Biophys. Acta,* 1954, vol. 15, pp. 516–525.
47. *Mathews M.B., Bernstein R.M., Franza B.R., Garrels J.I.* Identity of the Proliferating Cell Nuclear Antigen and Cyclin. *Nature* (London), 1984, vol. 309, pp. 374–376.
48. *McLaren A.D., Bradfute O.E.* Inhibition of Plant Root Growth by Enzymes and Histones. *Physiol. Plantarum*, 1966, vol. 19, pp. 1094–1100.
49. *Mothes K.* Physiologische Untersuchungen Über das Asparagin und das Arginin in Coniferen. *Planta*, 1929, vol. 7, pp. 585–649.
50. *Nelson D.L., Cox M.M. Principles of Biochemistry*. 5th ed., 2008, W.H. Freeman, New York.
51. *Oberhammer F.A., Hochegger K., Fröschi G., Tiefenbacher R., Pavelka M.* Chromatin Condensation is Accompanied by Degradation of Lamin A+B Without Enhanced Activation of cdc2 Kinase. *J. Cell Biol*., 1994, vol. 126, pp. 827–837.
52. *Oh T.J., Wartell R.M., Cairney J., Pullman G.S.* Evidence for Stage-Specific Modulation of Specific MicroRNAs (miRNAs) and miRNA Processing Components in Zygotic Embryo and Female Gametophte of Loblolly Pine (*Pinus taeda*). *New Phytologist*, 2008, vol. 179, pp. 67–80.
53. *Otsuga D., DeGuzman B., Prigge M.J., Drews G.N., Clark S.E.* REVOLUTA Regulates Meristem Initiation at Lateral Positions. *Plant J*., 2001, vol. 25, pp. 223–236.
54. *Palovaara J., Hakman H.* Conifer WOX-Related

Homeodomain Transcription Factors, Developmental Consideration and Expression ofWOX2 During *Picea abies* Somatic Embryogenesis. *J. Integrative Plant Biology*, 2008, vol. 50, pp. 860–867.
55. *Phillips D.M.P.* The N-Terminal Groups of Salmine. *Biochem. J.*, 1955, vol. 60, pp. 407–409.
56. *Pitel J.A., Durzan D.J.* Ribosomes and Ribosomal Proteins of Dry and Germinating Conifer Seeds. *Phytochemistry*, 1977, vol. 16, pp. 1475–1480.
57. *Pitel J.A., Durzan D.J.* Chromosomal Proteins of Conifers. 1. Comparison of Histones and Nonhistone Chromosomal Proteins from Dry seeds of Conifers. *Canadian J. Botany*, 1978a, vol. 56, pp. 1915–1927.
58. *Pitel J.A., Durzan D.J.* Chromosomal Proteins of Conifers. 2. Tissue-Specificity of Chromosomal Proteins of Jack Pine (*Pinus banksiana* Lamb.). *Canadian J. Botany*, 1978b, vol. 56, pp. 1928–1931.
59. *Pitel J.A., Durzan D.J.* Chromosomal Proteins of Conifers. 3. Metabolism of Histones and Nonhistone Chromosomal Proteins in Jack Pine (*Pinus banksiana* Lamb.) during Germination. *Physiol. Plantarum*, 1980, vol. 50, pp. 137–144.
60. *Pollmann L., Wettern M.* The Ubiquitin System in Higher and Lower Plants-Pathways in Protein Metabolism. *Botanica Acta*, 1989, vol. 102, pp. 21–31.
61. *Rechsteiner M.* Ubiquitin-Mediated Pathways for Intracellular Proteolysis. *Annu. Rev. Cell Biol.*, 1987, vol. 3, pp. 1–30.
62. *Santner A., Estelle M.* Recent Advances and Emerging Trends in Plant Hormone Signaling. *Nature* (London), 2009, vol. 459, pp. 1071–1078.
63. *Schulze E.* Über die beim Umsatz der Proteinstoffe in den Keimpflanzen einiger Coniferen Arten entstehanden Stickstoffverbindungen. *Zeit. physiol. Chem.* 1896, vol. 22, pp. 435–448.
64. *Sessa G., Morelli G., Ruberti I.* DNA-Binding Specificity of the Homeodomain-Leucine Zipper Domain. *J. Mol. Biol.*, 1997, vol. 274, pp. 303–309.
65. *Sharma A.K., Sharma A.* Chromosome Techniques, Theory and Practice, Norfolk, Frankenham Press Ltd., 1980, p. 121.
66. *Siomi H., Siomi M.C.* On the Road to Reading the RNA-interference code. *Nature* (London), 2009, vol. 457, pp. 396–404.
67. *Singh H.* Embryology of Gymnosperms, Berlin : Gebrüder, Borntraeger, 1978.
68. *Smalle J., Vierstra R.D.* The Ubiquitin 26S Proteasome Proteolytic Pathway. *Annu. Rev. Plant Biol.*, 2004, vol. 55, pp. 555–590.
69. *Spremulli L.* Protein Synthesis, Assembly and Degradation. In: *Biochemistry & Molecular Biology of Plants*. Eds, Buchanan BB, Gruissem W, Jones RL. American Society Plant Physiologists, Rockville, Md. 2000, pp. 412–454.
70. *Steel R.G.D., Torrie J.H.* Principles and Procedures of Statistics. New York, McGraw-Hill, 1960.
71. *Tahir M., Belmonte M.F., Elhiti M., Flood H., Stasolla C.* Identification and Characterization of PgHZ1, a Novel Homeodomain Leucine-Zipper Gene Isolated from White Spruce (*Picea glauca*) Tissue. *Plant Physiol. & Biochem.*, 2008, vol. 46, pp. 1031–1039.
72. *Takasaki Y., Fishwild D., Tan E.M.* Characterization of Proliferating Cell Nuclear Antigen Recognized by Antibodies in Lupus Sera. *J. Exp. Med.*, 1997, vol. 59, pp. 981–992.
73. *Toschi L., Bravo R.* Changes in Cyclin/PCNA Distribution during DNA Repair Synthesis. *J. Cell Biol.*, 1988, vol. 107, pp. 1623–1628.
74. *Willemsen V., Scheres B.* Mechanism of Pattern Formation in Plant Embryogenesis. *Annu. Rev. Genetics*, 2004, vol. 38, pp. 587–614.

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