agriculture

MDPI

Article

Insight into Hormonal Homeostasis and the Accumulation of Selected Heat Shock Proteins in Cold Acclimated and Deacclimated Winter Oilseed Rape (*Brassica napus* L.)

Julia Stachurska *, Iwona Sadura, Magdalena Rys, Michał Dziurka and Anna Janeczko

Polish Academy of Sciences, The Franciszek Górski Institute of Plant Physiology, Niezapominajek 21, 30-239 Krakow, Poland

* Correspondence: j.stachurska@ifr-pan.edu.pl

Abstract: The aim of the current work was to characterize disturbances in the hormonal balance and changes in the accumulation of the protective heat shock proteins (HSP) as a result of deacclimation in a few cultivars of oilseed rape. Samples for both analyses were collected from plants that had not been acclimated (before cold acclimation—control), cold acclimated (at 4 °C d/n, three weeks) and then deacclimated at 16/9 °C d/n (one week). The tested hormones included abscisic acid, jasmonic acid, salicylic acid, gibberellins, auxins and cytokinins (including their precursors, intermediates and conjugates). Unambiguous results were obtained for a stress hormone, abscisic acid, whose concentration increased in the leaves of all of the tested cultivars during cold acclimation while it strongly decreased during deacclimation. Deacclimation resulted also in an elevated level of the typical growth hormones. As a result of cold acclimation, the accumulation of protective proteins such as cytoplasmic HSP70 and HSP90 increased in three of the four tested cultivars. The HSP content most often decreased in the deacclimated plants compared to the cold-acclimated plants. The hormonal and protein changes are discussed relative to the frost tolerance changes of the tested cultivar.

Keywords: ABA; auxins; cytokinins; deacclimation; gibberellins; HSP70; HSP90; indole-3-carboxylic acid

check for updates

Citation: Stachurska, J.; Sadura, I.; Rys, M.; Dziurka, M.; Janeczko, A. Insight into Hormonal Homeostasis and the Accumulation of Selected Heat Shock Proteins in Cold Acclimated and Deacclimated Winter Oilseed Rape (*Brassica napus* L.). *Agriculture* **2023**, *13*, 641. https://doi.org/10.3390/agriculture13030641

Academic Editors: Mohd Asgher and Nafees A. Khan

Received: 10 February 2023
Revised: 2 March 2023
Accepted: 4 March 2023
Published: 8 March 2023

Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Cold acclimation (cold hardening) is a low-temperature-induced process that is especially important/characteristic for winter cultivars of crops because their vegetation season includes the autumn/winter months and, thus, requires specific metabolic adjustments. After a few weeks of growth at a temperature between +2 °C to +5 °C (in autumn), well cold-acclimated crop plants can survive temperatures that are as low as −20 °C, especially when they are under snow cover [1,2]. The process of cold acclimation of winter crops occurs in autumn. However, due to changes in climate conditions, in some regions where winter crops are cultivated, periods of warm breaks that interrupt the cold-hardening process are occurring more and more often. This phenomenon is called deacclimation (dehardening) and it disturbs the natural process of acquiring a high level of frost tolerance. Deacclimation can also occur in the middle of winter or in very early spring when the temperature starts to rise and plants begin to resume their growth and development, thereby losing their frost tolerance. In such a case, the sudden occurrence of even a light frost during that time is dangerous and could cause frost injuries. The negative effects of a seven-day deacclimation on a decrease in the frost tolerance of winter oilseed rape were characterized in detail in the work [3]. According to [4], deacclimation becomes "a crucial, but widely neglected" part of the problems that are associated with the winter survival of plants.

Cold acclimation triggers many biochemical and physiological changes in plant cells; for example, the most known are changes in the composition of fatty acids, changes in carbohydrate management and in the osmotic potential, the stimulation of the production

of protective proteins and an elevated level of stress hormones [5,6]. Deacclimation, on the other hand, is dangerous because it can reverse these metabolic adjustments. Although climate changes have led to more studies that are devoted to the detailed biochemical changes that accompany deacclimation, knowledge about these changes is still quite scarce.

One of the winter crops that is affected by deacclimation is oilseed rape—a plant that is mainly cultivated as the major source of vegetable oil. Our earlier studies confirmed that during cold hardening of this species, even one week of a warm break at a temperature of 16 °C/9 °C (d/n) had a reverse effect on metabolism. The chemical composition of the leaves, which was measured using FT-Raman spectroscopy, clearly confirmed that there were metabolic differences between the cold-acclimated and deacclimated plants [7]. Deacclimation increased the photosystem II efficiency that was suppressed by cold acclimation [3,7]. The content of soluble sugars was drastically decreased after deacclimation [7], which was accompanied by changes in the osmotic potential in a direction that was not beneficial from the point of view of frost tolerance. The leaf relative water content also increased after deacclimation [7]. Cold hardening also increased the accumulation of proteins BnPIP1 (aquaporin), while deacclimation decreased it [7]. The current work is a continuation of the studies of the deacclimation process of oilseed rape in which, as the next step, we are going to focus on a detailed analysis of hormonal homeostasis and any potential changes in the accumulation of the protective proteins from a group of heat shock proteins (HSP).

All metabolism of plants is controlled by hormones, and hormonal homeostasis is specifically linked to plant growth conditions. External factors such as temperature, light or water availability modify hormonal management, thereby allowing the metabolism to adapt to changing environmental conditions. Hormones, such as cytokinins, gibberellins, auxins, abscisic acid or brassinosteroids (and the interactions among them), play an important role in the growth/development of plants and in plants' reaction to various stressors [8–10]. Hormonal changes that occur during the cold acclimation of plants are important for the survival of plants in low temperatures. A higher level of ABA with a lower level of bioactive cytokinins, auxins and gibberellins was observed in wheat cultivars during cold acclimation [11]. In cold-acclimated oilseed rape leaves discs, the exogenous application of gibberellin GA_3 decreased the frost tolerance, while the application of ABA increased the frost tolerance [12]. The exogenous use of the auxin analogues TA-12 (calcium 4-(2-chloroethoxycarbonylmethyl)-1-naphthalenesulfonate) and TA-14 (ω-trialkylammonioalkyl ester of 1-napthylethanoic acid) on oilseed rape improved the winter hardiness of plants [13]. The exogenous application of jasmonate improved the freezing tolerance of *Arabidopsis thaliana* L. while blocking endogenous biosynthesis. and the signaling pathways of jasmonate caused plants to be hypersensitive to freezing stress [14]. While these are only a few examples, generally, there is a wealth of knowledge about the activity and significance of plant hormones in cold acclimation. However, the hormonal balance during deacclimation and its role in the changes in the frost tolerance of deacclimated plants is quite limited and relatively new. According to the literature, during the deacclimation of *A. thaliana* L., there was an overexpression of the genes related to the metabolism of auxins, gibberellins, brassinosteroids, jasmonate and ethylene [15]. Deacclimated plants of barley (*Hordeum vulgare* L.) were characterized by an increased level of hormones from growth-promoting groups such as indole-3-acetic acid (IAA), IAA methyl ester; the level of some gibberellins was also elevated, i.e., GA_6 or cytokinins (trans-zeatin and cis-zeatin), compared to cold acclimated plants [16]. In our earlier studies, the most abundant brassinosteroid (28-homocastasterone) in oilseed rape was accumulated in higher amounts in the cultivars that had maintained a better frost tolerance after deacclimation [3]. Interestingly, the accumulation of the transcript of BRI1 (which encodes the BR-receptor protein) decreased after cold acclimation, and in the more frost-tolerant cultivars, it remained low even after deacclimation [3].

Although heat shock proteins (HSP) are a group of proteins that are produced in plants especially as a reaction to heat stress [17], changing amounts of HSP are also found in

plants growing at room temperature or even cold-stressed plants [18,19]. There are many types of heat shock proteins that differ in their molecular weight from 10 to 200 kDa and perform various functions [20,21]. Among them, the HSP90 proteins are necessary for the proper functioning of all of the eucaryotic cells and assist other proteins in folding, maintaining and stabilizing the cytosolic proteins, including the proteins that are involved in cell cycle control and signal transduction [22]. Another family is HSP70, which stabilizes the precursor proteins and maintains them in an unfolded form [23]. Specific chloroplastic proteins HSP70 were also identified in plants [23]. For example, HSP70, which is found in the stroma, participates in the photoprotection and reparation of PSII during and after photoinhibition [24]; it is also necessary for heat tolerance [25].

As was mentioned earlier, although the heat shock proteins accumulate in plants that are growing under a high temperature stress, their expression increases under different abiotic stresses as well as during cold acclimation [18]; however, this has been much less studied. In grape plants (*Vitis vinifera* L. cv. Jingxiu), during, among others, cold acclimation stress, an increased level of HSP70 and small HSP17.6 was observed. Moreover, the synthesis of the HSP proteins was parallel to an increase in cold tolerance [18]. In oilseed rape plants, an increased level of HSP90 mRNA was observed in young plant tissues such as the shoot apices, as well as after exposure to high and low temperatures. During an exposure to 5 °C, there was a 15-fold increase in the hsp90 mRNA level, which remained elevated during the entire cold treatment. It decreased again when the temperature increased to 20 °C. This change was observed between the 4th and 5th hours after the transfer to 20 °C [26]. Those results illustrate that HSP90 could be significant in the adaptation of plants to low-temperature stress and in their tolerance to low temperatures. Therefore, the questions of whether the deacclimation process causes the changes in the HSP accumulation or if it correlates with a decrease in frost tolerance of plants arises.

The aim of the current work was to characterize the disturbances in the hormonal balance and changes in the accumulation of protective heat shock proteins as a result of deacclimation in an economically important crop plant—oilseed rape. The results are discussed relative to a deacclimation-induced decrease in the frost tolerance of plants.

2. Materials and Methods

2.1. Plant Material

The experiment was conducted on four cultivars of oilseed rape (*Brassica napus* L. var. *napus* L.): Bojan, President, Rokas (winter cultivars) and Feliks (spring cultivar). These cultivars are available to cultivate in Poland. President is a hybrid cultivar (F1) while Bojan, Rokas and Feliks are population cultivars. The cultivars were selected based on a previous experiment in which their frost tolerance was characterized in controlled conditions [3]. After cold acclimation, the plants were characterized by a higher tolerance (in comparison to non-acclimated plants), while after a period of deacclimation, the frost tolerance decreased, although not to the level that was recorded in the non-acclimated control. The control plants after frost (−5 °C) had one point on a seven-point scale of injuries. The cold acclimated plants were able to survive −15 °C (three to four points on the scale of injuries), while the deacclimated plants were already severely injured by a temperature of −12 °C (one to two points on the scale of injuries). There were differences between cultivars. Rokas and Bojan had the highest basal frost tolerance (noted for non-acclimated plants), President was moderately frost tolerant, while Feliks had the lowest basal frost tolerance [3]. After cold acclimation, Rokas and Bojan were highly frost tolerant, while President and Feliks were characterized by a lower frost tolerance. After deacclimation, Rokas remained the most frost tolerant. Bojan exhibited a moderate tolerance to frost, while President and Feliks had the lowest frost tolerance. Among the tested cultivars, Rokas is a semi-dwarf cultivar. The average height of fully-developed Rokas plants, according to COBORU (Development of Polish Official Variety Testing), is 131 cm. The seeds of cultivars Bojan and Feliks were obtained from The Plant Breeding and Acclimatization Institute (IHAR) the National Research Institute in Strzelce (Radzików, Poland). The seeds of the

President cultivars were obtained from Saatbau (Środa Śląska, Poland), and the seeds of the Rokas cultivar were obtained from Syngenta (Warszawa, Poland).

2.2. Experimental Design and Sampling

The experimental model was similar to an earlier model that was described in detail by [3]. Briefly, the seeds of the oilseed rape were germinated in darkness at 24 °C (two days). Then, the seedlings were transferred into pots (18 plants per pot; details regarding soil are given in [3]). The plants were cultured in a growth chamber (20 °C day/night, 12 h photoperiod, four days; then 17 °C d/n, 12 h photoperiod, three weeks). After that, a group of 15 uniform plants was retained in each pot and the plants were pre-hardened at 14 °C d/n (12 h photoperiod, two days); 12 °C d/n (8 h photoperiod, three days) and 10 °C d/n (8 h photoperiod, two days). Then, for the cold acclimation, the temperature was set at 4 °C (8 h photoperiod, three weeks). Next, for deacclimation, the temperature was set at 16/9 °C d/n (8 h photoperiod, one week). The intensity of light was constant during the experiment (300 µmol m^{-1} s^{-1}; modified LED lamps HORTI A provided by PERFAND LED, Trzebnica, Poland; for details see [3]). The experiment was conducted in the autumn/winter seasons. The scheme of the experiment is visualized in Figure S1 (Supplementary Materials).

For all of the hormonal and HSP analyses, samples of leaves were collected from the non-acclimated plants (NA, control group), cold-acclimated plants (CA) and deacclimated plants (DA). During sampling, all tested plants were in vegetative stage-rosette, but there were some architectural differences between younger (non-acclimated) and older (cold-acclimated and deacclimated); older plants were more compact (pictures available in [3]). The best developed rosette leaves (not too young or senescing) were always selected.

2.3. Measurements

2.3.1. Analysis of the Plant Hormones and Related Metabolites

The collected leaf samples were frozen in liquid N_2 and then stored at −80 °C. Analyses were performed according to [27] with modifications. For the analyses of the hormones and related substances, the material was lyophilized and ground (MM 400, Retsch, Kroll, Germany). Weighed samples (10 mg) were spiked with a stable isotope-labelled internal standard solution (ISTD) in acetonitrile (ACN) and then extracted in 1 mL methanol/water/formic acid ($MeOH/H_2O/HCOOH$, 15/4/1, *v*/*v*/*v*). The samples were shaken for 10 min, sonicated (5 min) and centrifuged (5 min, 22,000× *g*, 15 °C, Universal R32, Hettich, Haan, Germany). The supernatant was collected and evaporated to dryness under an N_2 stream at 45 °C. The residues were dissolved in 1 mL of 3% MeOH in a 1 M aqueous solution of formic acid, sonicated (5 min), centrifuged (5 min, 22,000× *g*, 15 °C) and purified on cartridges (BondElutPlexa PCX, 30 mg, 1 mL, Agilent Technologies, Santa Clara, CA, USA). The cartridges were activated with 1 mL of methanol and 1 mL of 1 M formic acid. The samples were applied to the cartridges, aspirated slowly and washed with 1 mL of 1 M formic acid. The substances of interest were washed out with 0.5 mL of ACN/MeOH (1/1 *v*/*v*), 0.5 mL of 5% NH_3aq in ACN/MeOH (1/1 *v*/*v*) and 0.5 mL of ACN/MeOH (1/1 *v*/*v*) in succession. The collected eluate was evaporated to dryness under nitrogen and dissolved in 70 µL of ACN prior to the UHPLC analyses. Four or five replicates were analyzed using an UHPLC apparatus (Agilent Infinity 1260, Agilent, Germany) that was coupled to a triple quadruple mass spectrometer MS/MS (6410 Triple Quad LC/MS, Agilent, Savage, MD, USA) with electrospray ionization (ESI). The samples were separated on an Ascentis Express RP-Amide analytical column (2.7 µm, 2.1 mm × 150 mm; Supelco, Bellefonte, PA, USA). Further technical details are given in Table 1 and [27–30]. The following phytohormones and related metabolites were detected: cytokinins: cis-zeatin (cis-ZEA) and cis-zeatin riboside (cis-ZEA-rib); auxins: indole-3-acetic acid (IAA), oxoindole-3-acetic acid (OxIAA), indole-3-acetyl-aspartic acid (IAAsp), indole-3-carboxylic acid (I3CA), indole-3-acetonitril (IAN), indole-3-acetyl-glutamic acid (IAGlu), indole-3-acetamid (IAM); gibberellins: gibberellic acid (GA_3), gibberellin A_6 (GA_6), gibberellin A_{20} (GA_{20}), gibberellin A_{19} (GA_{19}), gibberellin A_{53} (GA_{53}), gibberellin A_7 (GA_7), gibberellin A_4 (GA_4), gibberellin A_{15} (GA_{15})

and gibberellin A_9 (GA_9); stress hormones: benzoic acid (BA), salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA) and 12-oxo-phytodenoic acid (12-oxo-PDA). The following ratios of hormones were calculated: GA_3/ABA, ratio GA_3 + GA_4 + GA_6 + GA_7/ABA and ratio IAA + cis-ZEA + GA_3 + GA_4 + GA_6 + GA_7/ABA + JA.

Table 1. The optimized mass spectrometry parameters that were used to quantify the phytohormones. The following conditions were optimal for the analyses: capillary voltage 4 kV, gas temperature 350 °C, gas flow 12 L/min and a nebulizer pressure of 35 psi. The measurements were performed using multiple reaction monitoring (MRM) in a positive polarity. MassHunter software was used to control the LC-MS/MS system and data analysis. For the MRM parameters, a MassHunter Optimizer was used. The quantities of the internal standards (ISTD) are given in parenthesis. DHZ-N15 and t-Z-R-D5–internal standards for the cytokinins; GA1-D2 and GA5-D2–internal standard for gibberellins; for the other abbreviations, see Material and Methods Section 2.3.1.

Compound		Type of Ion	Quantifier Transition (Precursor/Product Ions)	Fragmentor Voltage (V)	Collision Energy (V)	MRM Start Time (min.)
DHZ-N15	ISTD (10 pmol)	$[M + H]^+$	226.2/152	124	18	1.5
cis-ZEA		$[M + H]^+$	220.2/136.3	85	9	
oxIAA		$[M + H]^+$	192.2/146.1	54	9	4.0
IAM		$[M + H]^+$	175.1/130	66	17	
t-Z-R-D5	ISTD (10 pmol)	$[M + H]^+$	357.3/225.2	116	17	5.12
cis-ZEA-rib		$[M + H]^+$	352.2/220.3	120	9	
IAAsp		$[M + H]^+$	291.2/130.1	54	25	6.4
BA-D4	ISTD (500 pmol)	$[M + H]^+$	128.1/84.1	61	13	
BA		$[M + H]^+$	123.1/79.1	56	13	
IAGlu		$[M + H]^+$	305.2/130.1	58	29	
GA_3		$[M\text{-}H_2O + H]^+$	329.3/311.3	100	14	8.15
GA1-D2	ISTD (10 pmol)	$[M\text{-}H_2O + H]^+$	333.3/287.2	58	9	
I3CA		$[M + H]^+$	162.2/118.1	58	9	
IAA-D5	ISTD (100 pmol)	$[M + H]^+$	181.1/135.1	38	14	
IAA		$[M + H]^+$	176.1/130.3	51	9	
SA-D4	ISTD (500 pmol)	$[M + H]^+$	143.2/125.2	80	14	
SA		$[M + H]^+$	139.2/121.2	80	14	
GA6-D2	ISTD (10 pmol)	$[M\text{-}H_2O + H]^+$	331.3/115.1	96	5	10.4
GA_6		$[M\text{-}H_2O + H]^+$	329.3/283.3	104	14	
IAN-D4	ISTD (100 pmol)	$[M + H]^+$	161.1/134.1	66	13	12.0
IAN		$[M + H]^+$	157.1/130.1	71	13	
ABA-D6	ISTD (30 pmol)	$[M\text{-}H_2O + H]+$	253.4/191.3	80	14	14.6
ABA		$[M\text{-}H_2O + H]^+$	247.4/187.2	80	14	

Table 1. *Cont.*

Compound		Type of Ion	Quantifier Transition (Precursor/Product Ions)	Fragmentor Voltage (V)	Collision Energy (V)	MRM Start Time (min.)
GA5-D2	ISTD (10 pmol)	$[M\text{-}H_2O + H]^+$	287.3/115.0	96	5	15.45
GA_{20}		$[M\text{-}H_2O + H]^+$	287.3/115.0	96	5	
GA_{19}			345.2/299.1	80	9	16.8
JA-D5	ISTD (100 pmol)	$[M + H]^+$	216.3/153.2	80	5	19.5
JA		$[M + H]^+$	211.3/151.2	80	14	
GA_7		$[M\text{-}H_2O + H]^+$	313.2/223.1	104	14	18.5
GA4-D2	ISTD (10 pmol)	$[M\text{-}H_2O + H]^+$	317.3/271.2	88	9	
GA_4		$[M\text{-}H_2O + H]^+$	315.3/269.3	100	14	
GA_{53}			303.2/285.1	100	9	
GA_9		$[M\text{-}H_2O + H]^+$	271.3/225.2	136	13	22.25
GA_{15}			331.2/285.1	115	9	
dinor-12-oxo-OPDA-D5	ISTD (10 pmol)	$[M + H]^+$	270.3/252.2	84	5	
12-oxo-PDA		$[M + H]^+$	293.3/275.2	68	9	24.54

2.3.2. HSP Analysis

Measurements of the Protein Concentration in the Leaf Extracts. The samples that were obtained from leaves (1 g) were homogenized in liquid N_2 and immediately extracted using a Tricine buffer (100 mM Tricine, 3 mM $MgSO_4$, 1 mM DTT, 3 mM EGTA, pH = 8.0 and a protease inhibitor (Protease Inhibitor Cocktail Tablets, Roche, Germany)). The samples were centrifuged for five minutes at 38,030× *g* (MIKRO R, Hettich Centrifugen, Tuttingen, Germany). After the centrifugation, the supernatant was collected and the protein concentration in the obtained extracts was measured according to Bradford [31] using a SynergyTM2 Multi-Detection Microplate Reader (BioTek, Winooski, VT, USA). Bovine serum albumin (BSA) (Sigma-Aldrich, Poznań, Poland) was used as the calibration standard. The analysis was performed in three replications.

Analysis of the Accumulation of HSP90 and HSP70 (Cytosolic and Chloroplastic) in the Leaf Samples Using Immunoblotting. The same amount of protein extracts (selected after being optimized in a range of 2.5 µg to 20 µg for HSP70 cytoplasmic; 5 µg to 30 µg for HSP70 chloroplastic and 5 µg to 30 µg for HSP90), which were isolated from tested samples, were loaded and separated on 12% SDS-PAGE (1 mm polyacrylamide gel) according to the procedure of Laemmli [32]. Based on the testing, we decided to use 3 µg of the protein for the HSP70 cytoplasmic, 5 µg for the HSP70 chloroplastic and 10 µg for the HSP90. The samples were diluted with an SDS loading buffer (0.125 mM TRIS pH 6.8, 4% SDS, 20% glycerol, 5% 2-mercaptoetanol, 0.004% bromophenol blue). The molecular weight standard was Thermo Scientific PageRuler Prestained Protein Ladder (Thermo Scientific, Vilnius, Lithuania). After the proteins were separated, they were blotted to nitrocellulose membranes (0.2 µm, Trans-Blot Turbo Transfer Pack, Bio-Rad Laboratories, Inc., Hercules, CA, USA) using a BioRad Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Then, the membranes were blocked with 4% low-fat milk diluted in a Tris-buffered saline/Tween (TSB-T) buffer (containing 0.9% NaCl, 10 mM Tris) overnight. Next, the membranes were washed four times for five minutes with a TBS-T buffer and probed in the appropriate antibodies for 1.5 h (Anti-HSP70 cytoplasmic (AS08 371), 1:3000; Anti-HSP70 chloroplastic (AS08 348), 1:2000; Anti-HSP90-1 (AS08 346), 1:3000 (Agrisera, Vännäs, Sweden)). The membranes were washed four times for five minutes with a

TBS-T buffer and then incubated with the appropriate secondary antibody (Alkaline-Phosphate Conjugated Anti-rabbit, for HSP70 cytoplasmic 1:5000; for HSP70 chloroplastic 1:10,000; for HSP90 1:5000 (Sigma-Aldrich, Poznan, Poland)) for 1.5 h. Dilutions of the antibodies were selected based on the previous optimalization process and the protocol of the manufacturer. Three independent repetitions (both biological and technical) were performed. Densitometric analyses were performed to measure the protein content using ImageJ software (NIH, Bethesda, MD, USA). The averages are expressed as arbitrary units (A.U.) correlated with the area under the densitometric curves. Exemplary blots are presented in Figure S2 (Supplementary Materials).

2.4. Statistical Analyses

All of the statistical analyses (ANOVA, post-hoc Duncan's tests) were conducted using Statistica 13.1 software (StatSoft, Tulsa, OK, USA). For a specific hormone or protein, the non-acclimated, cold-acclimated and deacclimated plants (within each cultivar separately) were compared. The average data are presented as ±SD. Values that are marked with the same letters did not significantly differ according to the Duncan's test ($p < 0.05$).

3. Results

3.1. Hormonal Analyses

Twenty-three hormonal compounds were identified in the group of growth-promoting hormones and stress hormones (active forms, precursors, metabolites, conjugates) in all of the tested cultivars.

Five hormones (more characteristic for the plant stress response) and their precursors were identified: abscisic acid, 12-oxo-phytodenoic acid, jasmonic acid, benzoic acid and salicylic acid (Figure 1A–U). The concentration of abscisic acid (ABA) increased (from 485% in cultivar Bojan to 963% in cultivar Rokas) in all of the cold-acclimated plants (compared to the non-acclimated plants) and then decreased once again in the deacclimated plants. However, the ABA level in the DA plants was still significantly higher than in the NA plants (Figure 1A–D). The abscisic acid (ABA) content had the same, statistically significant pattern of changes in all of the tested cultivars.

Changes in the content of 12-oxo-phytodenoic acid (a precursor of jasmonic acid) were also observed. Lower amounts of this phytohormone were detected after cold acclimation of all of the cultivars from 51% to 77% (Figure 1E–H). After deacclimation, the 12-oxo-PDA content once again increased in three cultivars, the exception was Rokas, in which there were no changes between the CA and DA plants (Figure 1H). As for the active form (jasmonic acid), in the CA plants (Bojan, Feliks), its content was visibly higher by an average of 327% (Figure 1I,J). In President, there was a slight tendency of JA to increase, while in Rokas, no changes were observed between the NA and CA plants. Deacclimation reduced content of JA in Bojan and Feliks to the level that was observed in the NA plants. There was an opposite effect for the deacclimated plants of President and especially Rokas, in which an higher content of JA was observed (Figure 1K,L).

A lower amount of benzoic acid was detected in the cold-acclimated Bojan, Feliks and Rokas plants by an average of 11% compared to the non-acclimated plants. In President, the tendency was similar but statistically insignificant. After the deacclimation process, the effects of cold was generally reversed (Figure 1M–P). There were no differences in the level of salicylic acid in the cold-acclimated cultivars of Bojan, Feliks and President (Figure 1R–T). A decrease in the level of salicylic acid in the cold-acclimated plants was detected only in Rokas (Figure 1U). In all of the cultivars, there were no statistically significant differences in the SA level between the CA and DA plants.

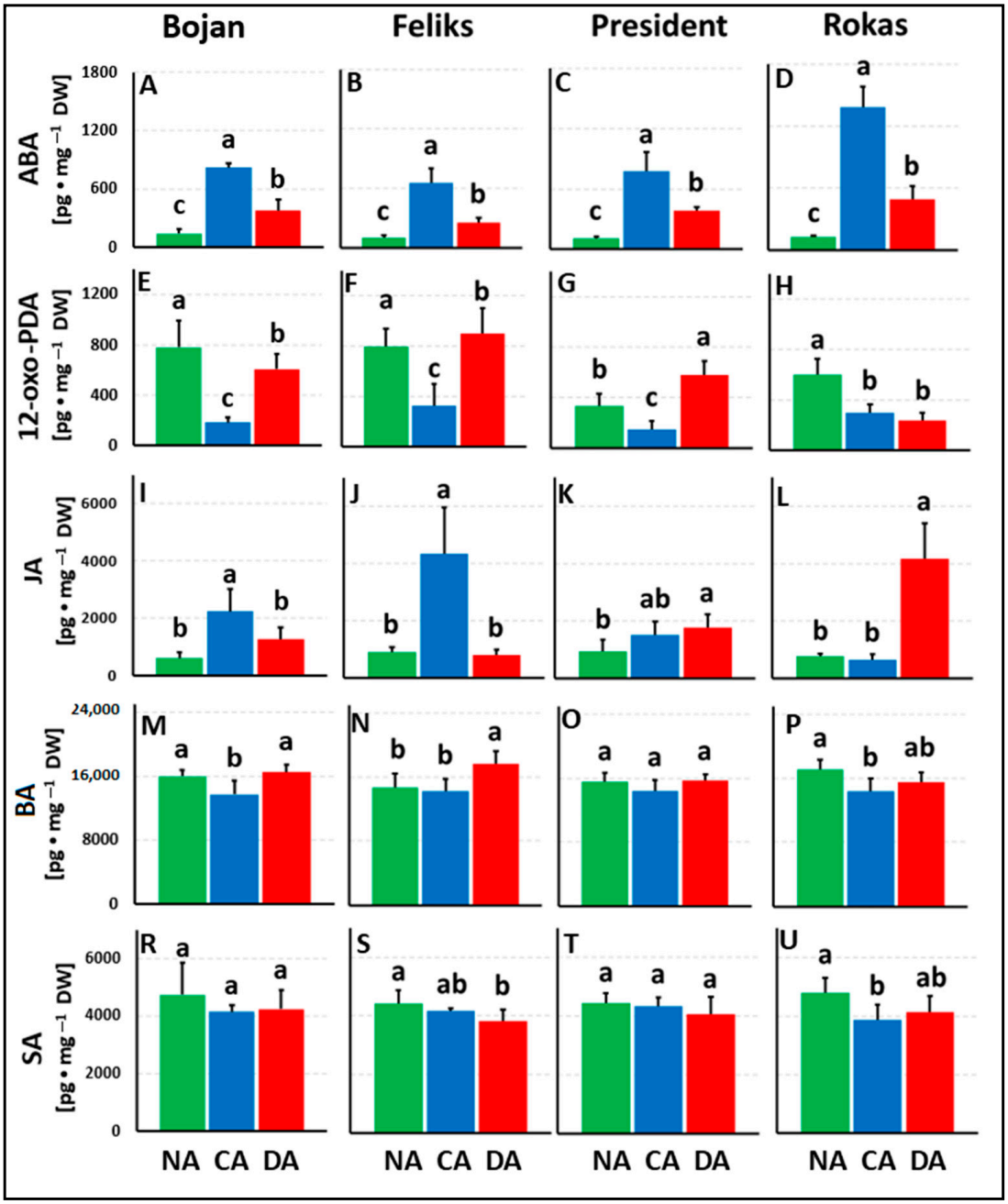


Figure 1. Content of the stress hormones: abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and selected precursors (12-oxo-phytodenoic acid (12-oxo-PDA) is a precursor of JA; benzoic acid (BA) is a precursor of SA) in four cultivars of the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. (**A**–**D**)—ABA; (**E**–**H**)—12-oxo-PDA; (**I**–**L**)—JA; (**M**–**P**)—BA; (**R**–**U**)—SA. Values marked with the same letters (within each cultivar separately) were not significantly different according to the Duncan test ($p \leq 0.05$).

The following gibberellins were identified in our studies: GA_3, GA_4, GA_6, GA_7, GA_9, GA_{15}, GA_{19}, GA_{20} and GA_{53}. In the presentation of the results, the gibberellins were divided into two groups according to their biosynthetic pathways [33]. GA_{15}, GA_9, GA_4 and GA_7 are presented in Figure 2A–P, while GA_{53}, GA_{19}, GA_{20}, GA_3 and GA_6 are presented in Figure 3A–U.

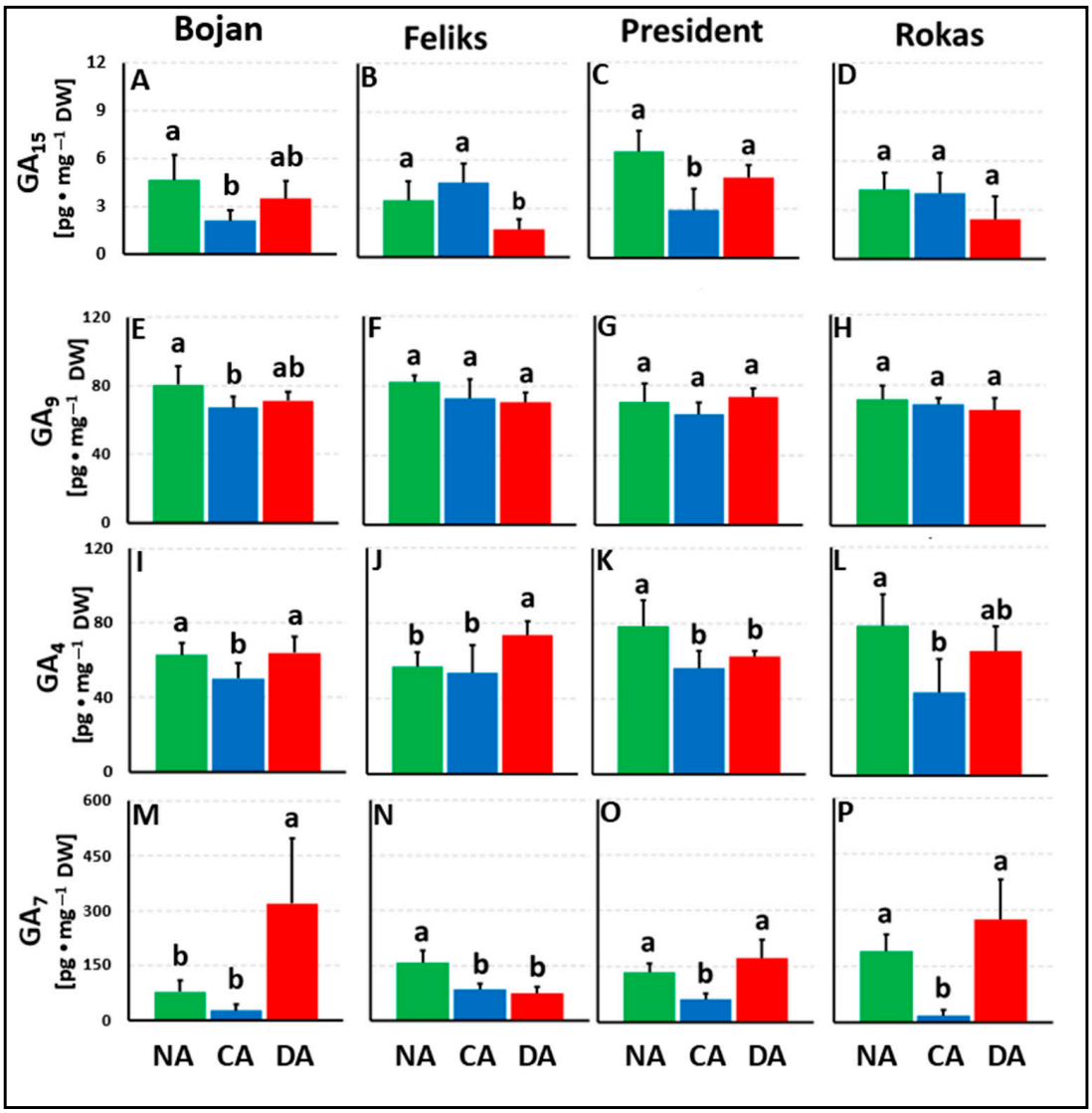


Figure 2. Content of the gibberellins GA_{15}, GA_9, GA_4 and GA_7 in four cultivars of the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. The gibberellins are presented in order based on the biosynthetic pathway from the precursor GA_{15} via GA_9 to their active forms GA_4 and GA_7 (according to [33]). (**A**–**D**)—GA_{15}; (**E**–**H**)—GA_9; (**I**–**L**)—GA_4; (**M**–**P**)—GA_7. Values marked with the same letters (within each cultivar separately) were not significantly different according to the Duncan test ($p \leq 0.05$).

In Bojan and President plants, the level of GA_{15} decreased after cold acclimation (54% and 55%, respectively), but after deacclimation, it increased to a level that was similar to the non-acclimated plants (Figure 2A,C). In the Feliks and Rokas plants, there were no differences in GA_{15} between the NA and CA plants; however, after deacclimation, the GA_{15} content had a tendency to decrease (but statistically significantly only in Feliks) (Figure 2B,D). Generally, we did not detect any significant changes in the amounts of GA_9 in the tested cultivars between the NA, CA and DA plants (Figure 2F–H); the exception was a slightly, although statistically significant, lower level of this compound in the CA Bojan plants (Figure 2E).

Cold acclimation generally reduced the concentration of GA_4 and GA_7 (Figure 2I–P); deacclimation more or less reversed this effect in almost all of the studied cultivars, which was particularly visible in the case of GA_7 (Bojan, President and Rokas; Figure 2M,O,P).

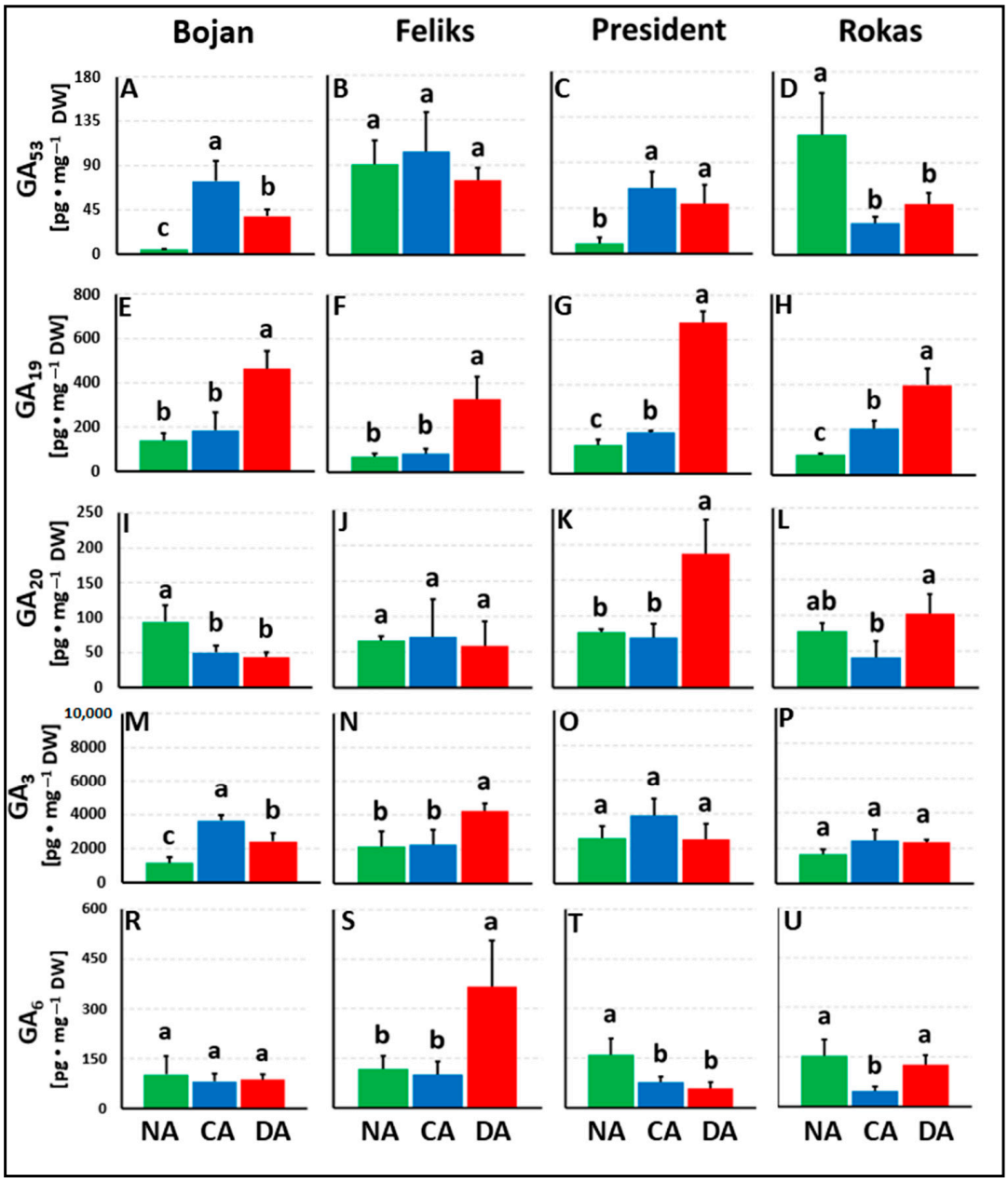


Figure 3. Content of the gibberellins GA_{53}, GA_{19}, GA_{20}, GA_3 and GA_6 in four cultivars of the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. The gibberellins are presented in order based on the biosynthetic pathway from precursor GA_{53} via GA_{19} and GA_{20} to their active forms GA_3 and GA_6 (according to [33]). (**A**–**D**)—GA_{53}; (**E**–**H**)—GA_{19}; (**I**–**L**)—GA_{20}; (**M**–**P**)—GA_3 and (**R**–**U**)—GA_6. Values marked with the same letters (within each cultivar separately) were not significantly different according to the Duncan test ($p \leq 0.05$).

The tendencies of changes in the concentration of GA_{53} were similar in the Bojan and President cultivars, which accumulated more GA_{53} after cold treatment (compared to the NA plants) by approximately 1461 and 534%, respectively (Figure 3A,C). In the Feliks plants, there was no difference in the GA_{53} level between the NA and CA plants, while in the Rokas plants, GA_{53} decreased after cold (Figure 3B,D). After deacclimation, the level of this phytohormone was similar to the level that was observed in the cold-acclimated plants in three of the four tested cultivars (Figure 3B–D).

The GA_{19} rather showed a tendency to accumulate more in cold acclimated plants (than in NA plants); interestingly, deacclimation further strengthened this effect (Figure 3E–H). For

example, the DA Rokas plants were characterized by a level of GA_{19} that was 4.5-fold higher than in the NA plants and 2-fold higher than in the CA plants (Figure 3H). The deacclimated President plants accumulated as much as a four-fold higher content of GA_{19} compared to the CA plants (Figure 3G).

In the cold-acclimated plants, the level of GA_{20} was generally similar (Feliks, President, Rokas) or lower (Bojan) than in the non-acclimated plants (Figure 3I–L). The deacclimation increased it only in the case of the President and Rokas plants (Figure 3K,L).

The level of GA_3 increased in the cold-acclimated Bojan plants and decreased in deacclimated plants (Figure 3M). In the other cultivars, cold had no effect on GA_3 (Figure 3N–P). In the deacclimated plants, the GA_3 level was clearly higher only in the Feliks plants compared to both the non-acclimated and cold-acclimated plants by approximately 1.9-fold (Figure 3N). In the case of this cultivar, a similar trend was also observed for GA_6 in which the DA plants were characterized by an average 235% higher level of this gibberellin than in the NA and CA plants (Figure 3S). The level of GA_6 after cold acclimation was similar to that after deacclimation in Bojan and President plants (Figure 3R,T). In the Rokas plants, cold decreased the content of GA_6 while deacclimation increased it once again (the accumulation of GA_6 in the non-acclimated and deacclimated plants was at a similar level; Figure 3U).

The following auxins, auxin precursors, metabolites and conjugates were identified in all of the tested cultivars: indole-3-acetamid, indole-3-acetonitril, indole-3-acetic acid, indole-3-acetyl-aspartic acid, oxoindole-3-acetic acid, indole-3-acetyl-glutamic acid and indole-3-carboxylic acid. The auxin precursors (IAM, IAN) and active auxin IAA are presented in Figure 4A–L, while the auxin conjugates (IAAsp, IAGlu), oxIAA and an indole-derivative metabolite-indole-3-carboxylic acid (I3CA) are presented in Figure 5A–P.

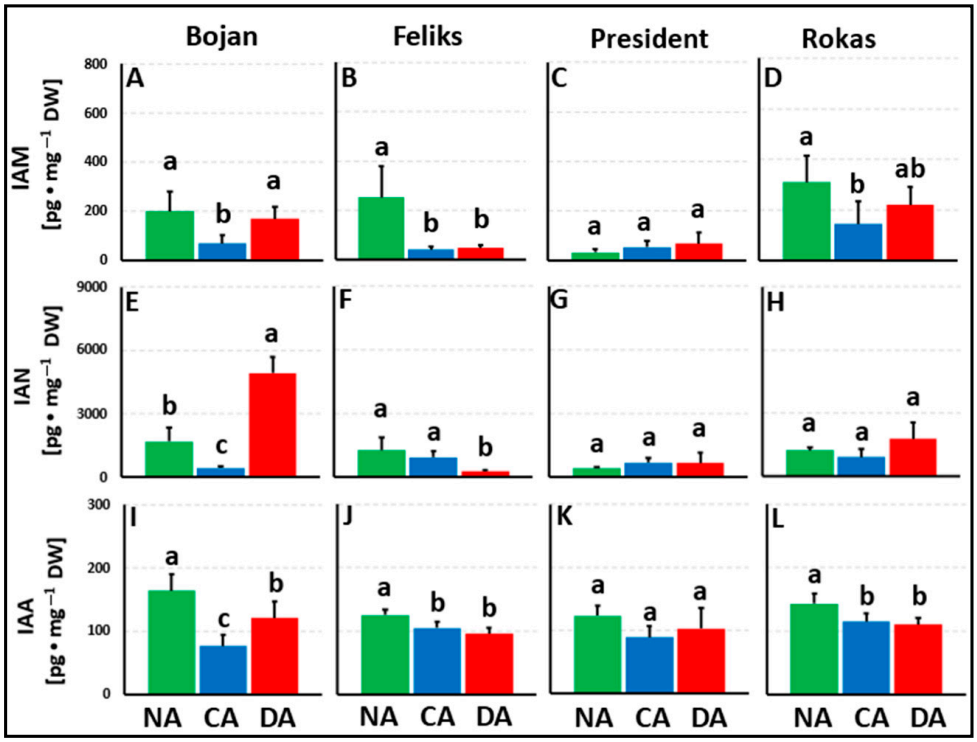


Figure 4. Content of the auxin precursors (indole-3-acetamid (IAM), indole-3-acetonitril (IAN)) and active auxin indole-3-acetic acid (IAA) in four cultivars of the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. (**A**–**D**)—IAM; (**E**–**H**)—IAN; (**I**–**L**)—IAA. Values marked with the same letters (within each cultivar separately) were not significantly different according to the Duncan test ($p \leq 0.05$).

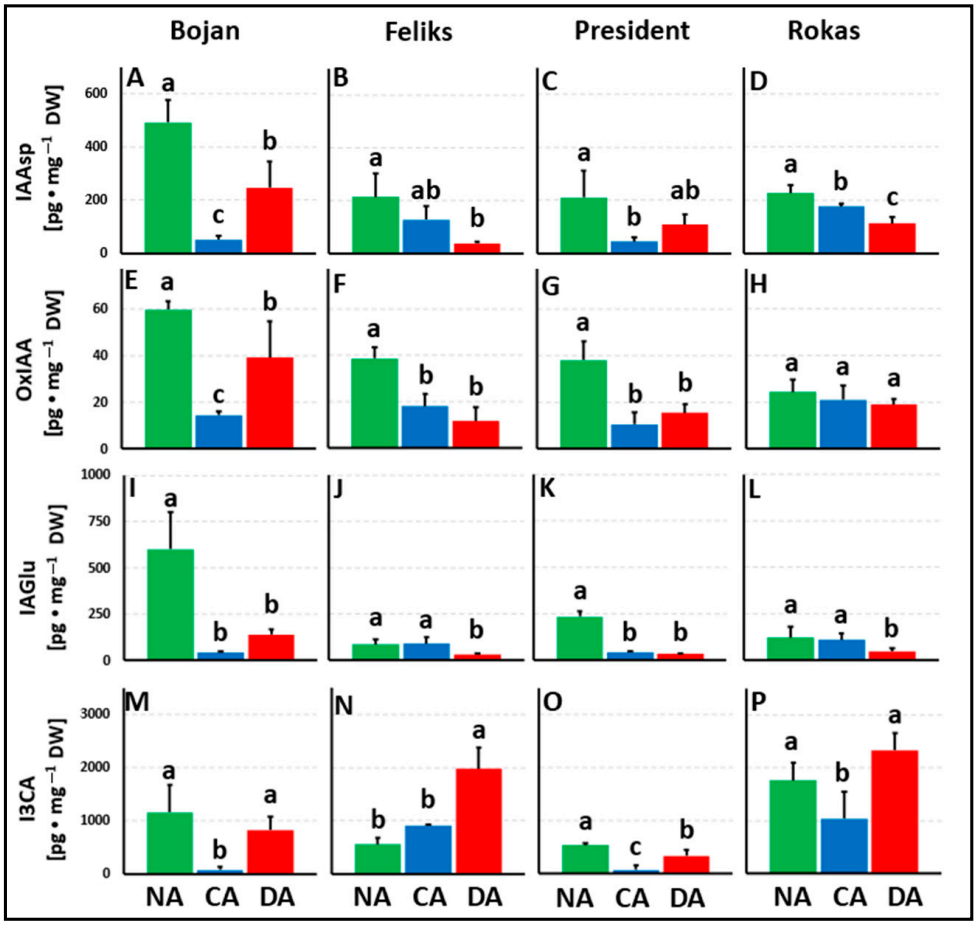


Figure 5. Content of the auxin conjugates (indole-3-acetyl-aspartic acid (IAAsp) and indole-3-acetyl-glutamic acid (IAGlu)), the oxidized auxin form (OxIAA) and the indole-derivative metabolite: indole-3-carboxylic acid (I3CA) in four cultivars of the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. (**A**–**D**)—IAAsp; (**E**–**H**)—OxIAA; (**I**–**L**)—IAGlu. (**M**–**P**)—I3CA. Values marked with the same letters (within each cultivar separately) were not significantly different according to the Duncan test ($p \leq 0.05$).

Cold acclimation reduced the level of IAM in three of the four tested cultivars, while in one cultivar (President), IAM remained at a similar level as in the non-acclimated plants (Figure 4A–D). During the deacclimation, there was a significant increase in the level of IAM in the Bojan plants—the accumulation of IAM reached the same level as that in the non-acclimated plants (Figure 4A). There was a similar tendency (statistically insignificant) in the Rokas cultivar (Figure 4D). Deacclimation did not change the content of this hormone in the Feliks and President plants (Figure 4B,C).

The content of the second precursor (IAN) decreased after cold acclimation only in the Bojan plants (there were no changes between the NA and CA plants in the other three cultivars) (Figure 4E–H). After the Bojan plants were deacclimated, IAN was higher once again and reached a level that was 190% higher than in the NA plants and 103% higher than in the CA plants. In the Feliks cultivar (Figure 4F), IAN decreased after deacclimation, while in two of the other cultivars, it remained at the same level as after cold acclimation (Figure 4G,H).

The cold-acclimated Bojan plants were characterized by a decreased level of IAA (by approximately 53% compared to the NA plants) (Figure 4I). The hormone level in this cultivar increased again after deacclimation (although it did not reach the same level as in the NA plants). In the Feliks and Rokas plants, cold acclimation lowered the concentration of IAA slightly, and this level was also maintained after deacclimation (Figure 4J,L). There

were no statistically significant differences in the accumulation of IAA (similar to IAM and IAN) between NA, CA and DA plants in the President cultivar (Figure 4K).

The leaf accumulation of the auxin conjugates IAAsp, IAGlu and the oxidized form OxIAA had the same pattern of changes in the Bojan plants. Their concentrations decreased after cold and increased again after deacclimation, although not to the level that was characteristic for the NA plants (Figure 5A,E,I). In the three other genotypes, cold also decreased (or did not change) the content of these compounds (Figure 5B–D,F–H,J–L), but the accumulation of IAAsp and IAGlu in the leaves of deacclimated Feliks, President and Rokas plants, in most of the cases, reached the lowest values compared to both the NA and CA plants (Figure 5B–D,J–L). The deacclimation of these three cultivars did not change the level of OxIAA (compared to the CA plants) (Figure 5F–H).

The amount of I3CA decreased significantly (from 41% to as much 94%) in the cold-acclimated plants (with the exception of the Feliks plants; Figure 5N). After deacclimation, the content of this hormone returned to a similar level as was detected in the NA plants (Figure 5M,O,P).

Among the cytokinins, cis-zeatin and cis-zeatin riboside were identified (Figure 6A–H). The leaf accumulation of cis-zeatin decreased in the cold-acclimated plants in all of the tested cultivars (from 75% in the Rokas plants to 81% in the President plants) (Figure 6A–D). Deacclimation increased the concentration of cis-zeatin in three of the four cultivars. The exception was the DA Bojan plants in which the cis-zeatin remained unchanged (compared to the CA plants). As for cis-ZEA-rib, generally, there were no differences between the NA, CA and DA plants in the cultivars (one exception was a slight increase in this compound in the CA Feliks plants) (Figure 6E–H).

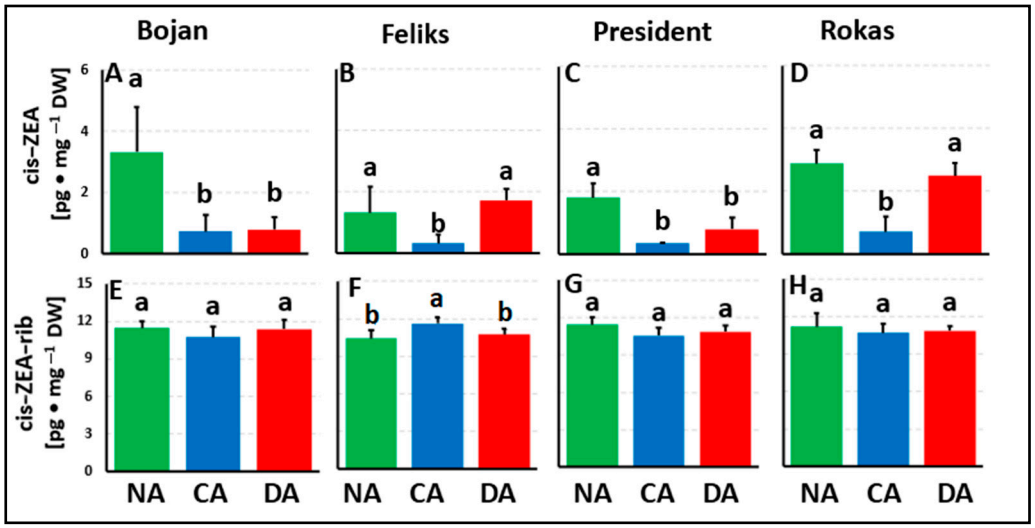


Figure 6. Content of the cytokinins–cis-zeatin (cis-ZEA) and cis-zeatin-riboside (cis-ZEA-rib) in four cultivars of the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. (**A**–**D**)—cis-ZEA; (**E**–**H**)—cis-ZEA-rib. Values marked with the same letters (within each cultivar separately) were not significantly different according to the Duncan test ($p \leq 0.05$).

3.2. HSP Analyses

The presence of cytoplasmic HSP70, chloroplastic HSP70 and HSP90 was detected in the leaves of the four cultivars of oilseed rape (Figure 7A–L). The accumulation of cytoplasmic HSP70 increased after the cold acclimation of the Bojan, Feliks, and President cultivars (Figure 7A–C). After deacclimation, the amount of this protein returned to a similar level as was observed in the non-acclimated plants of cv. Feliks and President (Figure 7B,C). In the Bojan plants, the amount of HSP70 cytoplasmic was decreased after deacclimation and finally was lower than in the non-acclimated plants (Figure 7A). The exception from this patterns was changes in cytoplasmic HSP70 in the Rokas plants. This

cultivar was characterized by a decrease in the level of the HSP70 cytoplasmic protein after cold acclimation, and a further decrease was also observed after deacclimation (Figure 7D).

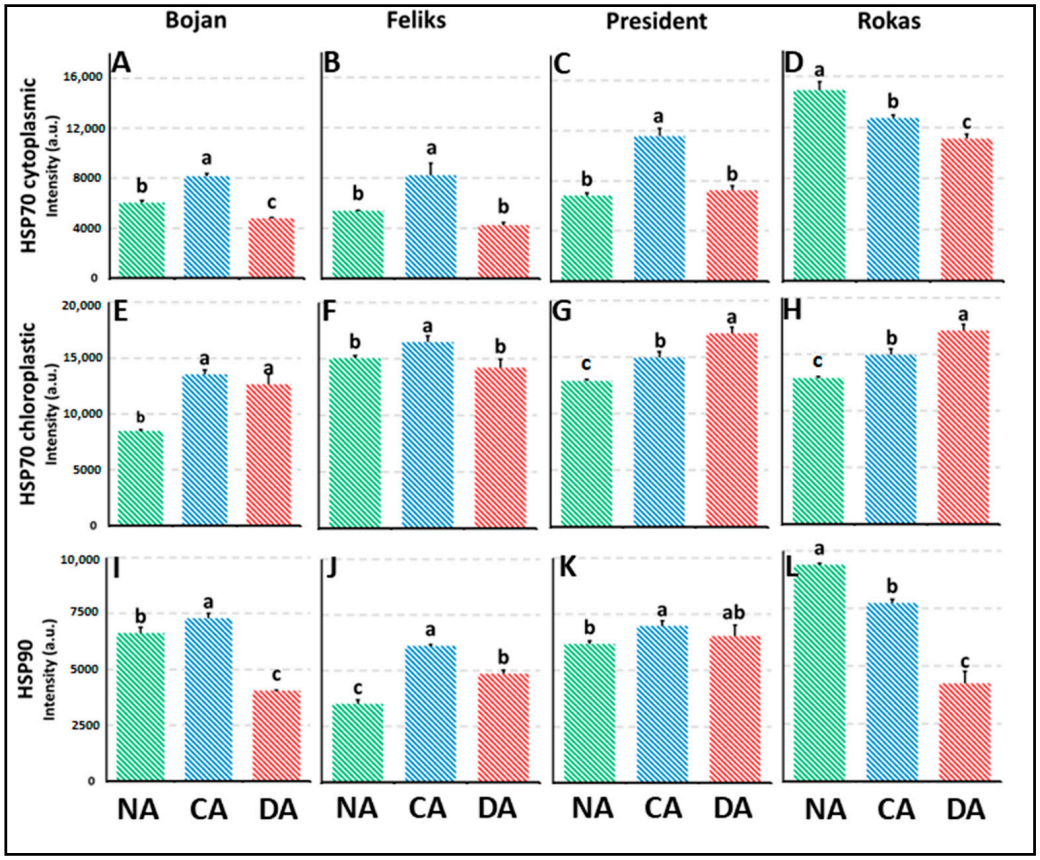


Figure 7. Changes in the accumulation of the HSP proteins in the leaves of four cultivars of the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. (**A**–**D**)—changes in the accumulation of cytoplasmic HSP70; 3 µg of the protein was loaded onto the gel. (**E**–**H**)—changes in the accumulation of chloroplastic HSP70; 5 µg of the protein was loaded onto the gel. (**I**–**L**)—changes in the accumulation of HSP90; 10 µg of the protein was loaded onto the gel. Values marked with the same letters (for each cultivar separately) were not significantly different according to the Duncan test ($p \leq 0.05$).

As for chloroplastic HSP70, in the Bojan plants, there was an increase in the amount of this protein after cold acclimation. After deacclimation, the level of chloroplastic HSP70 remained similar to what was noted after cold acclimation (Figure 7E). In cultivar Feliks, there was a similar tendency to accumulate more HSP70 chloroplastic in the cold-acclimated plants. The deacclimated Feliks plants were characterized by a lower level of this protein than the cold-acclimated plants. This level was also similar to what was detected in the non-acclimated plants (Figure 7F). Both cultivars President and Rokas had a similar tendency to accumulate HSP70 chloroplastic—the smallest amount was detected in the non-acclimated plants, while it was significantly higher in the cold-acclimated plants; the accumulation was the highest in the deacclimated plants (Figure 7G,H).

In the case of the accumulation of HSP90, a similar pattern of changes was noted for the Bojan, Feliks and President plants—cold increased the content of the protein while deacclimation more or less decreased it (Figure 7I–K). In contrast to these three cultivars, the Rokas cultivar accumulated the highest amount of HSP90 in the non-acclimated plants, and this amount was surprisingly lower in the cold-acclimated plants and decreased further in the deacclimated plants (Figure 7L).

The common tendency for all of the tested cultivars was a lower accumulation of HSP90 (so as cytoplasmic HSP70) in the DA plants (compared to the CA plants).

4. Discussion

4.1. The Impact of Deacclimation on Plant Hormone Management

Generally, the profile of the hormones detected in the oilseed rape was in agreement with the data that are available in the literature. The presence of auxins (for example IAN, IAM, IAA, oxIAA, IAAsp, IAGlu) in oilseed rape was reported by [34], gibberellins (for example GA_1, GA_3, GA_{19}, GA_{20}) by [35] and cytokinins (for example cis-ZEA and cis-ZEA-rib) by [36]. Additionally, stress hormones such as ABA, JA, SA (and its precursor BA) were reported by [37–39]. According to our best knowledge, only 12-oxo-PDA and I3CA were not previously detected in oilseed rape plants; thus, we report it for the first time here.

It is well known that the temperature in which a plant grows modifies the content of hormones in plant tissue. Regarding the cold acclimation (hardening), the changes in ABA are well known. This is related to the fact that the role of cold hardening in the case of winter plants is to increase their frost tolerance, and therefore, the increase in the level of ABA is important for the development of frost tolerance [40]. This is also confirmed by studies in which this hormone was used exogenously and an increase in frost tolerance of winter plants was noted [41,42]. In our experiment, in winter oilseed rape, after three weeks of cold acclimation, the ABA content increased unequivocally in all four of the tested cultivars (Bojan, Feliks, President, Rokas). An increase in the ABA content (from 300 to almost 600 pg/mg D.W.) in the cold was also observed in the fifth cultivar of winter oilseed rape—Pantheon, which was tested in another experiment (data not shown). These results were expected and are consistent with previous reports on oilseed rape [40]. The rapid increase in the ABA content in the leaf discs was noted within the first three days of cold acclimation at 2 °C. The ABA level remained high during the next 18 days. According to [37], increased levels of ABA were already observed in oilseed rape during early seedling growth in prehardened plants (plants growing at temperature 12 °C vs. plants growing at 20 °C).

Generally, the cold-induced increase in the ABA content in the plants of the cultivars that were studied here corresponded with a significant increase in the frost tolerance of these plants [3]. It was also observed that the most frost-tolerant cultivar, Rokas, accumulated more than 1200 pg of this hormone per mg of D.W. in the leaves after cold acclimation, while the other tested cultivars accumulated approximately two-fold less. This corresponds somewhat to the results of experiments with the exogenous use of ABA, where it was proven that while the supplementation of ABA increased the frost tolerance, the hormone was more effective in cultivars that had a naturally lower content of ABA [43].

In an earlier work [3], deacclimation (7 days 16/9 °C d/n) caused a significant decrease in frost tolerance of the ten tested cultivars of oilseed rape. Considering the significant relationship between ABA and the level of frost tolerance, a decrease in the level of this hormone as a result of deacclimation could be expected. The results obtained in this paper fully support this assumption. In all of the cultivars (Bojan, Feliks, President, Rokas), as well as in the additionally tested cultivar Pantheon (data not shown), this deacclimation caused a decrease in ABA content, although not to the level that was recorded in the control plants (those without cold hardening). This corresponds to the fact that the frost tolerance of the plants after deacclimation was lower, although it remained higher than that of the control plants [3]. Surprisingly, according to [37],the deacclimation of oilseed rape caused a slight increase in the ABA content in both of the tested cultivars of oilseed rape that were tested by the authors.

Although the role of ABA in the cold hardening and in frost tolerance of plants is the most well-known, other typical stress hormones can also contribute to improving the tolerance to temperature stress. Among them, we also studied jasmonic acid and salicylic acid in our experiment. According to the literature, the exogenous application of jasmonic acid improves the freezing tolerance of *A. thaliana* L. with or without the cold acclimation process. In contrast, blocking the endogenous JA biosynthesis resulted in plants that were hypersensitive to freezing [14]. Simultaneously, the content of JA increased also in cold-treated wheat [11]. It is in agreement with our studies, where the leaf content of JA

generally increased after cold acclimation (although an interesting exception was the most frost tolerant Rokas in which there were no changes in NA vs. CA plants). In addition, in some of the cultivars (Bojan and Feliks, partly also President) the relationship between JA and its precursor (12-oxo-PDA) was particularly clear. A higher precursor content in the control plants or deacclimated plants was usually reflected in a lower JA content. A lower content of the precursor in cold-acclimated plants was associated with a higher JA content.

It is also worth emphasizing that, unlike the other three cultivars, after deacclimation, the plants of the Rokas cultivar were characterized by a several times higher content of JA compared to the level of JA in the other cultivars. Because it is believed that JA is important for improving low temperature tolerance ([14,44] and the literature cited there), it is possible that the high levels of this hormone in this cultivar are among the factors that contribute to maintaining a high frost tolerance despite deacclimation.

In the case of the third hormone, which was salicylic acid, the differences between the NA, CA, and DA plants were not too large. Although the exogenous treatment of plants with salicylic acid may induce cold or frost tolerance, i.e., in wheat (not acclimated and cold acclimated plants [45–47]), in our opinion, the significance/importance of SA in the frost tolerance of oilseed rape remains an open question.

Because plant growth is limited during the cold acclimation period, a reduction in the level of the growth-stimulating hormones such as gibberellins, auxins and cytokinins can be expected. In the plants of the same family as oilseed rape-*Arabidopsis*, *GSF (Gibberellin Suppressing Factor)* participates in the response to abiotic stresses such as cold by suppressing the biosynthesis of gibberellins [48]. An increased deactivation of gibberellins (in response to a short period of cold) was reported in winter wheat [11]. According to [37], during the cold acclimation of oilseed rape seedlings (cultivar Górczański), the level of GA_3 decreased slightly. On the other hand, exogenous GA_3 disturbed cold acclimation, thus, increasing the susceptibility of the photosynthetic apparatus to cold-induced photoinactivation [12]. In our studies, the dominating, active gibberellin GA_3 did not change during cold acclimation (or in one cultivar—Bojan—even increased). On the other hand, a cold-induced decrease was observed for the other active gibberellins, mainly for GA_7 and GA_4 (usually accompanied by decrease in the precursor GA_{15}). In two of the four tested cultivars, the content of GA_6 also decreased. Those differences in the contents/changes of specific gibberellins between the cultivars indicates that the effect of temperature on gibberellin biosynthesis in oilseed rape may be (at least partly) cultivar-dependent. This could justify some of the differences between our results and the results in oilseed rape that were obtained by [37]. It is worth mentioning here that the level of gibberellins may dynamically change during the cooling time, which should be taken into account when performing only single/point analyses. For example, in winter wheat, the concentration of GA_3 and GA_6 (gibberellins from the group that is synthetized in the pathway via GA_{53} [33]) began to increase significantly on the 9th day of cold up to the 15th day of cold [49]. On the other hand, GA_4 and GA_7 (gibberellins from group synthetized in pathway via GA_{15}) had only one peak on the 12th day of cold and then their content decreased once again. In barley, there was a tendency to accumulate fewer GA_3 and GA_4 in the cold acclimated plants, while the amount of GA_6 tended to increase [50].

In oilseed rape, however, the gibberellin levels can already begin to increase at the end of the cold period, especially when long (i.e., ten weeks) cooling periods are used [51]. This is connected with the fact that in oilseed rape, gibberellins are engaged in development and in this aspect, cold exposure is crucial to the vernalization process. Winter cultivars require cold for the induction of stem elongation and flowering, and according to [51], vernalization influences GA content and metabolism with GAs serving as probable regulatory intermediaries between the cold treatment and subsequent stem growth. The authors observed a further increase in the level of gibberellins (especially GA_{20}, GA_1 and GA_3) eight days after the plants were moved from cold to 23 °C. In the experiment performed by [37] and in our experiment, the cold period was much shorter (three to four weeks) and there was an increase in the level of gibberellins in the plants, but during the period of deacclimation.

It should be emphasized that in the oilseed rape that we studied, the increase in the level of gibberellins as a result of deacclimation was varied (i.e., depending on the cultivar) but it concerned especially GA_{19} (four cultivars), GA_7 and GA_4 (three cultivars), GA_{20} and GA_6 (two cultivars), GA_3 and GA_{15} (one cultivar). However, unlike the experiment of [51] in which the importance of gibberellins in the context of development was examined, in our experiment, the increase in the level of gibberellins as a result of deacclimation should be interpreted as being unfavorable. Deacclimation in winter could mean a resumption of growth and could be associated with a decrease in the frost tolerance of a plant. It is worth mentioning here that exogenous gibberellins accelerate development ([52] and the literature cited there) and especially at higher concentrations, GAs can decrease the tolerance to low temperature [53,54].

Cytokinins are also engaged in the transition of winter oilseed rape to the generative phase due to vernalization [52]. In our experiment, cold decreased the cis-ZEA levels in oilseed rape (in all four cultivars), while deacclimation caused a statistically significant increase in the level of this hormone in two cultivars; in the third cultivar, a similar trend was observed, but it was not statistically significant. These trends are consistent with those that were observed in previous studies on barley, where it was also shown that cis-ZEA decreased after three weeks of cold, while after deacclimation, the level of cytokinins increased again [16]. A decrease in cis-ZEA was also observed in winter wheat after 2 weeks of cold [49]. However, for oilseed rape [36], reported that in the 21st day of cold, the concentration of cis-ZEA started to increase slightly, while maximal value was reached on the 42nd day of cold. Thus, as in the case of gibberellins, shorter periods of cooling may be associated with a lower level of cytokinins, but after a longer period of cooling, the accumulation of cytokinins can increase due to the progress of the processes of the induction of generative development. However, when it comes to deacclimation (as in the case of gibberellins), a sudden increase in the level of cytokinins as a result of this process, which may occur, e.g., in the middle of the winter, should, in our opinion, be interpreted as unfavorable as it could indicate the resumption of growth related to the lowering of frost-tolerance.

As for the hormones from the third group of growth-promoting substances—auxins, in our studies, the amount of IAA (the main active auxin) generally had a tendency to decrease in the cold acclimated plants, which is in agreement with earlier findings for wheat or barley [11,16,49]. However, the deacclimated plants of barley were characterized by an increased level of hormones such as IAA or IAA methyl ester [16]. In our work, a similar phenomenon was observed for only one of the four tested cultivars—Bojan. In this cultivar, deacclimation very clearly reversed the effect of cold by not only increasing the concentrations of IAA, but it was simultaneously connected to an increase in the production of the precursors in IAA biosynthesis (IAM and IAN). The significant increase was also recorded for IAAsp and oxIAA (and a tendency in IAGlu). OxIAA is characterized by a weak biological activity and is usually irreversibly formed in response to increases in the auxin levels [55]. Both conjugates IAAsp and IAGlu are also a form of deactivation of IAA, and their level usually increases with an increase in the IAA content [56]. The results suggest that generally the entire metabolism of auxins from biosynthesis to conjugation/deactivation in this cultivar was enhanced. In contrast to Bojan, there was a different picture for auxin metabolism in the CA and DA plants for Feliks, President and Rokas. The IAA content generally dropped slightly in these cultivars after cold and this level was maintained after deacclimation. The lower content of the main active auxin was accompanied by a relatively low content of the precursors (mainly IAM in Feliks and Rokas) as well as IAAsp or oxIAA, which reflects a less intense metabolism of auxins than in Bojan, especially after deacclimation.

In the end of this part of the discussion, it is worth emphasizing that the work of [15] provided important genetic support for our studies that were devoted to changes in the content of gibberellins and auxins in the deacclimated plants. According to the authors, during the deacclimation of *A. thaliana* (a plant from the same family as oilseed rape),

there was an overexpression of the genes associated with the metabolism of auxins and gibberellins (but also brassinosteroids, jasmonate and ethylene).

The data regarding the accumulation of I3CA in the aspect of cold acclimation and deacclimation must be taken into account for these very interesting and novel results. I3CA (indole-3-carboxylic acid) is an indolic compound that is mainly recognized as a player in plant pathogen resistance [57,58]. In our studies, its content decreased in cold (in three of the four tested cultivars), while in all four of the tested cultivars, it significantly increased after deacclimation. A simpler, although still theoretical, explanation is that this is connected to the susceptibility of plants to some pathogen infection. Plants growing in higher temperatures (deacclimated and also non-acclimated) are more susceptible to a pathogen infection [59], thus, the involvement of the I3CA accumulation as protective agent is higher (than in cold). There might also be another explanation based on the fact that I3CA is one of the factors that regulate the callose accumulation [58]. Callose is a plant polysaccharide that was mainly studied in the context of plant defense reactions, although it is also engaged in the process of growth and development [60]. During the process of cell division, the cell plate determines the correct composition of the cell wall layer—callose forms a coat-like structure that covers the surface of the cell plates [60]. Limited growth (and cell division) processes in cold might be connected to a lower requirement for callose (thus I3CA as callose synthesis regulator may be lower). After the deacclimation processes, the resumption of plant growth is linked to the higher activity of the growth regulation factors, perhaps also including a higher callose synthesis that is regulated, among others, by I3CA. The temperature-dependent changes in I3CA are a reason for further studies of this matter and the verification presented hypothesis.

To conclude this part of the discussion, a few words need to be devoted to the relationship between ABA and GA, which are hormones with an antagonistic activity [61]. To visualize general changes in the hormonal balance between the active forms of the growth-promoting and stress hormones in non-acclimated, cold-acclimated and deacclimated oilseed rape, the following ratios were calculated: GA_3/ABA, ratio GA_3 + GA_4 + GA_6 + GA_7/ABA and ratio IAA + cis-ZEA+ GA_3 + GA_4 + GA_6 + GA_7/ABA + JA (Figure 8A–F).

As for the ratio of gibberellins to ABA, the model of changes that is presented in Figure 8A–D shows that the value of the ratio decreased in cold but increased again after deacclimation, although (especially in winter cultivars) it did not reach the level that was recorded for the control plants (before acclimation). In the case of the spring cultivar—Feliks (Figure 8B,D), the effect of deacclimation was more pronounced than in the case of the winter cultivars (Figure 8A,C), which (as expected) could indicate a lower tolerance to the deacclimation of the spring cultivar. This lower tolerance to the deacclimation in a spring cultivar is even better expressed in the relationship between all of the studied active forms of the growth-promoting hormones (IAA + cis-ZEA+ GA_3 + GA_4 + GA_6 + GA_7) and stress hormones (ABA + JA) (Figure 8E,F).

Generally, the ratio of gibberellins to ABA corresponded well to the earlier results concerning cold acclimation- and deacclimation-induced changes in frost tolerance [3]. After cold acclimation, the plants were characterized by a higher tolerance, while after a period of deacclimation, the frost tolerance decreased, although not to the level that was recorded in the control plants (basal tolerance). The control plants after frost (−5 °C) had one point on a seven-point scale of injuries. The cold acclimated plants were able to survive −15 °C (three to four points on the scale of injuries), while the deacclimated plants were already severely injured by a temperature of −12 °C (one to two points on the scale of injuries).

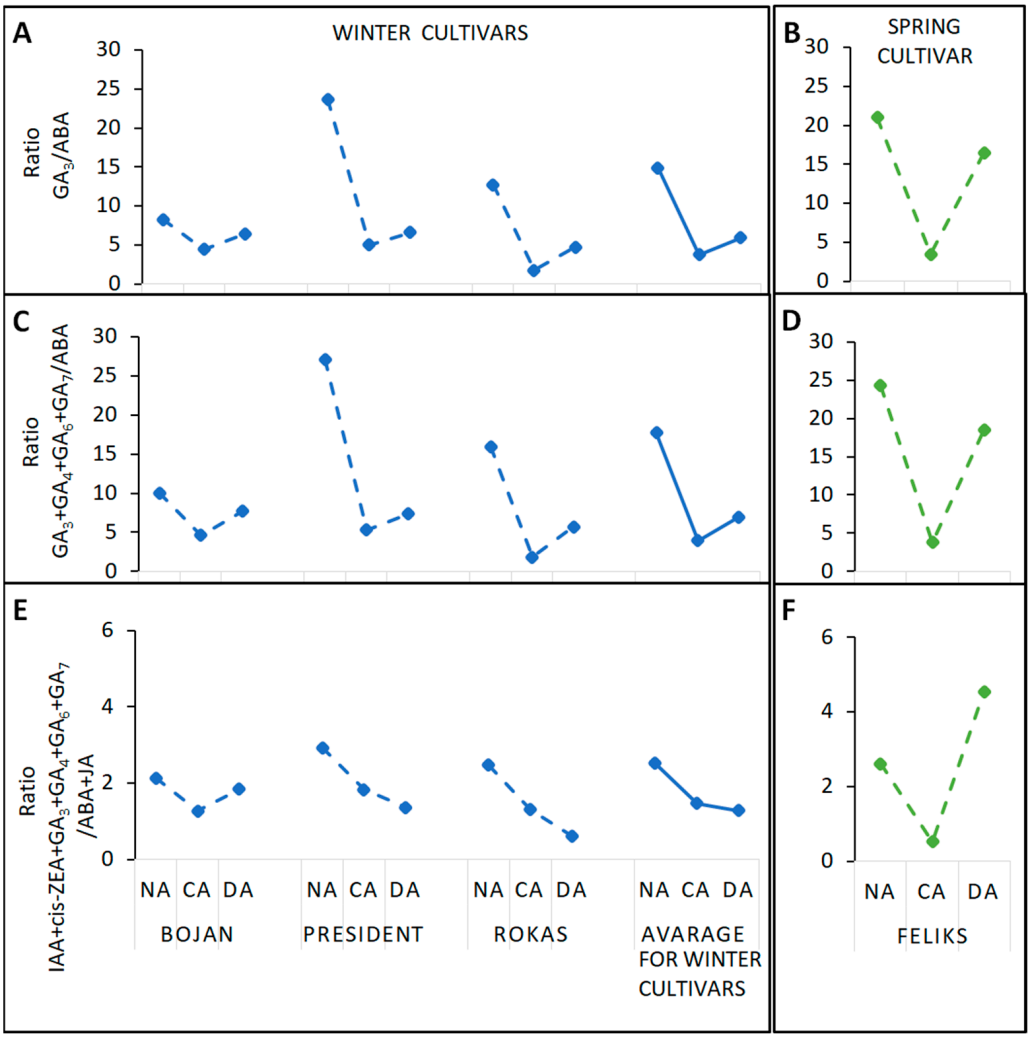


Figure 8. Visualization of the changes in the hormonal balance between the active forms of the growth promoting and stress hormones in the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) plants of winter and spring cultivars of oilseed rape. Model is based on calculations of ratio GA_3/ABA (**A**,**B**), ratio GA_3 + GA_4 + GA_6 + GA_7/ABA (**C**,**D**) and ratio IAA + cis-ZEA + GA_3 + GA_4 + GA_6 + GA_7/ABA + JA (**E**,**F**).

4.2. The Impact of Deacclimation on Heat Shock Protein Accumulation

As was mentioned in the Introduction, the heat shock proteins play an important role as chaperones and assist other proteins in folding, maintaining and stabilizing [22]. Moreover, HSP90 is an important player that mediates the stress signal transduction [62]. A similar function was also proposed for HSP70 [63]. As for chloroplastic HSP70, this protein contributes to the photoprotection and repair of photosystem II [24]. HSPs are accumulated in higher amounts (by various plants including oilseed rape), particularly during the heat stress [17,26,64], but various classes of HSPs have often also been found elevated in cold treated plants of grapevine [18], winter wheat [65] or barley [19]. That is why the elevated accumulation of HSPs in cold-acclimated oilseed rape, which was observed in our experiment, was an expected phenomenon. On the other hand, in our studies, deacclimation most often resulted in a lower accumulation of HSPs. A higher level of hsp90 mRNA under low temperature was previously described in oilseed rape, after which the accumulation of hsp90 mRNA decreased once again to the level of the control when the cold-treated plants were transferred back to 20 °C [26]. In some way, exposing plants to 20 °C here could reflect deacclimation conditions. Moreover, in *Rhododendron anthopogon* D.Don (an evergreen shrub), the expression of, among others, stromal HSP70 was up-

regulated during the cold-acclimation phases and down-regulated during the transition to the deacclimation phase [66]. Although a decrease (or increase) in gene expression does not always have to correlate with a decrease (or increase) in protein accumulation, in our oilseed rape, the directions of the changes in protein accumulation (an increase after cold acclimation and then a decrease after deacclimation) were generally consistent with the directions of the changes in the HSP gene expression that were previously found in genetic studies on oilseed rape [26] and even in another family plant—*R. anthopogon* [66].

It is worth noting that changes in level of the protective HSP proteins (an increase in the CA plants and usually a decrease in the DA plants) were accompanied by changes in their frost tolerance (at least for three cultivars). As we previously described, deacclimation significantly lowered the cold-induced frost tolerance of the tested cultivars [3]. The exception, however, was a different pattern of changes in the HSP accumulation that was recorded in the most frost tolerant cultivar—Rokas. This cultivar was generally characterized by high basal level of HSP90 (measured in non-acclimated plants) and cytoplasmic HSP70, but accumulation of these HSPs in the CA plants was surprisingly decreased. While deacclimation caused a further decrease in the level of these proteins, the deacclimated Rokas plants maintained the highest frost tolerance compared to the other cultivars [3]. This is difficult to explain, and we can only offer a theory that it had something to do with the complex dependency of HSP with many other factors, such as hormones, various signaling proteins, transcription factors, etc. [62]. We also have to remember that in our experiment, we only tracked the final accumulation of HSP and we did not know anything about the intensity of the complex processes of the synthesis or degradation of the proteins or the balance between them [67].

In the light of all the results presented in this work, it is worth making a brief comment about a potential link between the HSPs, hormone ABA and cytokinins. According to the review of [68], HSP90 may play a role in the signal transduction of ABA and this presumption was made, among others, because of the evidence that a genetic or pharmacological interference with HSP90 could result in disturbances of the ABA-induced stomata closure [69]. HSP90 clients and their potential involvement in the signaling pathways of cytokinins (such as PAS1) were also described [68]). On the other hand, an exogenous ABA treatment of *A. thaliana* and *Festuca arundinacea* Schreb. resulted in a higher level of the expression of various HSPs, together with an improved tolerance to heat stress [70]. The picture from our studies is somewhat similar to the results of [70]. In our work, an increase in the ABA content (not by exogenous treatment but as a natural effect of a low temperature) was usually accompanied by an increase in the HSPs, which was in agreement with an increase in the cold-induced frost tolerance (as was previously described for the tested cultivars [3]). Deacclimation significantly reversed these effects. Against this background, it can be seen that the level of cytokinins decreased in cold and usually increased again after deacclimation, thus, in contrast to the changes in ABA and HSPs. It is commonly known that ABA and cytokinins have antagonistic effects on many physiological processes, however, the dependency of cytokinin level on the HSP expression is more interesting. For example, as was reported by [71], transgenic *A. thaliana* overexpressing *ZmsHSP* was characterized by a lower level of cytokinins (although it was more sensitive to cytokinins). However, on the other hand, according to [67,72], cytokinins up-regulated most of the HSP70s and stimulated protein accumulation. Simultaneously, ABA (and jasmonates) have a negative effect on the HSP70 expression/HSP70 protein accumulation, which only shows that the relationship/interaction between these groups of players (ABA, cytokinin, HSP) is a result of a complex crosstalk on multiple levels that certainly (in the aspect of cold acclimation and deacclimation) requires further studies, probably using plant mutants or inhibitors as well as genetic research. To summarize, a model of the changes in the frost tolerance of cold acclimated and deacclimated oilseed rape against the background of the changes in the concentrations of ABA, cis-ZEA, HSP70 cytoplasmic, HSP70 chloroplastic and HSP90 is visualized in Figure 9.

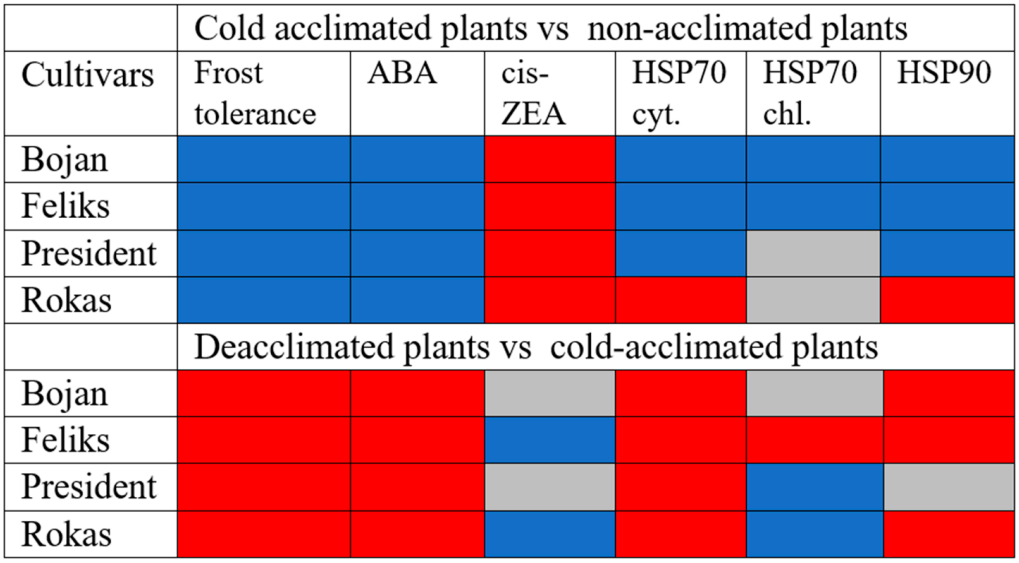


Figure 9. Directions of the changes in the frost tolerance of cold acclimated and deacclimated oilseed rape plants against the background of changes in the concentrations of ABA, cytokinin cis-ZEA, HSP70 cytoplasmic (cyt.), HSP70 chloroplastic (chl.) and HSP90. The visualization is based on the data from [3] (frost tolerance) and on Figure 1A–D, Figure 6A–D and Figure 7A–L. Blue—increase, red—decrease, grey—no changes.

5. Conclusions

In conclusion, in the winter oilseed rape, deacclimation-induced changes in the hormonal balance in the direction of the increased participation of hormones accompanied by a stimulation in plant growth and development (cytokinin, GAs (cultivar-dependent)) while there was a decrease in the concentration of the stress hormone (ABA (cultivar independent)). This is probably one of the factors that is responsible for the growth resumption of deacclimated plants and the lowering of their frost tolerance. The measurements of ABA or the ratio of gibberellins/ABA can be a tool for monitoring the process of deacclimation (and potential changes in frost tolerance) in oilseed rape. In most cases, deacclimation reversed the effect of cold acclimation on the protective heat shock proteins, which could also be responsible for lowering plant frost tolerance. An interrelation between HSP and hormones such as ABA or cytokinins in cold-acclimated and deacclimated plants seems to be an interesting matter for more detailed studies. Similarly, finding an explanation for the reason for changes in the concentration of indolic compound I3CA, which is lower in cold but higher after deacclimation, requires further research. I3CA is an indolic compound that, to date, has mainly been linked with the reaction of plants to pathogens.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13030641/s1, Figure S1: The scheme of the experiment and sampling of non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. Figure S2: The accumulation of the HSP proteins in the leaves of four cultivars of the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. The visualized bands correspond to the HSP70 cytoplasmic, HSP70 chloroplastic and HSP90 protein identified as described in Section 2.3.2.

Author Contributions: J.S. and I.S.—HSP analysis (including method optimization); M.D., J.S. and M.R.—hormonal extraction and analysis; J.S. and M.R.—plant cultivation and sampling; J.S. and A.J.—writing article; J.S.—calculation of results, figure preparation and statistical analysis under supervision of A.J.; A.J.—author of the idea for the research and funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Science Center (Poland), grant number: 2019/35/B/NZ9/02868.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Thomashow, M.F. Role of cold-responsive genes in plant freezing tolerance. *Plant Physiol.* **1998**, *118*, 1–7. [CrossRef]
2. Rapacz, M.; Jurczyk, B.; Sasal, M. Deacclimation may be crucial for winter survival of cereals under warming climate. *Plant Sci.* **2017**, *256*, 5–15. [CrossRef] [PubMed]
3. Stachurska, J.; Rys, M.; Pociecha, E.; Kalaji, H.M.; Dąbrowski, P.; Oklestkova, J.; Jurczyk, B.; Janeczko, A. Deacclimation-Induced Changes of Photosynthetic Efficiency, Brassinosteroid Homeostasis and BRI1 Expression in Winter Oilseed Rape (*Brassica napus* L.)—Relation to Frost Tolerance. *Int. J. Mol. Sci.* **2022**, *23*, 5224. [CrossRef] [PubMed]
4. Vyse, K.; Pagter, M.; Zuther, E.; Hincha, D.K. Deacclimation after cold acclimation—A crucial, but widely neglected part of plant winter survival. *J. Exp. Bot.* **2019**, *70*, 4595–4604. [CrossRef]
5. Popov, V.N.; Antipina, O.V.; Pchelkin, V.P.; Tsydendambaev, V.D. Changes in fatty acid composition of lipids in chloroplast membranes of tobacco plants during cold hardening. *Russ. J. Plant Physiol.* **2017**, *64*, 156–161. [CrossRef]
6. Miki, Y.; Takahashi, D.; Kawamura, Y.; Uemura, M. Temporal proteomics of *Arabidopsis* plasma membrane during cold- and de-acclimation. *J. Proteom.* **2019**, *197*, 71–81. [CrossRef]
7. Rys, M.; Pociecha, E.; Oliwa, J.; Ostrowska, A.; Jurczyk, B.; Saja, D.; Janeczko, A. Deacclimation of winter oilseed rape-insight into physiological changes. *Agronomy* **2020**, *10*, 1565. [CrossRef]
8. Bari, R.; Jones, J.D.G. Role of plant hormones in plant defence responses. *Plant Mol. Biol.* **2009**, *69*, 473–488. [CrossRef]
9. Tognetti, V.B.; van Aken, O.; Morreel, K.; Vandenbroucke, K.; van de Cotte, B.; de Clercq, I.; Chiwocha, S.; Fenske, R.; Prinsen, E.; Boerjan, W.; et al. Perturbation of indole-3-butyric acid homeostasis by the UDP-glucosyltransferase *UGT74E2* modulates *Arabidopsis* architecture and water stress tolerance. *Plant Cell* **2010**, *22*, 2660–2679. [CrossRef]
10. Cortleven, A.; Leuendorf, J.E.; Frank, M.; Pezzetta, D.; Bolt, S.; Schmülling, T. Cytokinin action in response to abiotic and biotic stresses in plants. *Plant Cell Environ.* **2019**, *42*, 998–1018. [CrossRef]
11. Kosová, K.; Prášil, I.T.; Vítámvás, P.; Dobrev, P.; Motyka, V.; Floková, K.; Novák, O.; Turečková, V.; Rolčik, J.; Pešek, B.; et al. Complex phytohormone responses during the cold acclimation of two wheat cultivars differing in cold tolerance, winter Samanta and spring Sandra. *J. Plant Physiol.* **2012**, *169*, 567–576. [CrossRef] [PubMed]
12. Rapacz, M. The effects of ABA and GA3 treatments on resistance to frost and high-light treatment in oilseed rape leaf discs. *Acta Physiol. Plant.* **2002**, *24*, 447–457. [CrossRef]
13. Gavelienė, V.; Novickienė, L.; Kazlauskienė, D. Effect of auxin physiological analogues on rape growth and reproductive development. *Bot. Lith.* **2007**, *13*, 101–107.
14. Hu, Y.; Jiang, L.; Wang, F.; Yu, D. Jasmonate regulates the INDUCER OF CBF expression-C-repeat binding factor/dre binding factor1 Cascade and freezing tolerance in *Arabidopsis*. *Plant Cell* **2013**, *25*, 2907–2924. [CrossRef]
15. Pagter, M.; Alpers, J.; Erban, A.; Kopka, J.; Zuther, E.; Hincha, D.K. Rapid transcriptional and metabolic regulation of the deacclimation process in cold acclimated *Arabidopsis thaliana*. *BMC Genom.* **2017**, *18*, 731. [CrossRef]
16. Pociecha, E.; Janeczko, A.; Dziurka, M.; Gruszka, D. Disturbances in the Biosynthesis or Signalling of Brassinosteroids That Are Caused by Mutations in the HvDWARF, HvCPD and HvBRI1 Genes Increase the Tolerance of Barley to the Deacclimation Process. *J. Plant Growth Regul.* **2020**, *39*, 1625–1637. [CrossRef]
17. Lindquist, S.; Craig, E.A. The heat-shock proteins. *Annu. Rev. Genet.* **1988**, *22*, 631–677. [CrossRef]
18. Zhang, J.H.; Wang, L.J.; Pan, Q.H.; Wang, Y.Z.; Zhan, J.C.; Huang, W.D. Accumulation and subcellular localization of heat shock proteins in young grape leaves during cross-adaptation to temperature stresses. *Sci. Hortic.* **2008**, *117*, 231–240. [CrossRef]
19. Sadura, I.; Libik-Konieczny, M.; Jurczyk, B.; Gruszka, D.; Janeczko, A. HSP transcript and protein accumulation in brassinosteroid barley mutants acclimated to low and high temperatures. *Int. J. Mol. Sci.* **2020**, *21*, 1889. [CrossRef]
20. Schöffl, F.; Prandl, R.; Reindl, A. Update on Signal Transduction Regulation of the Heat-Shock Response. *Plant Physiol.* **1998**, *117*, 1135–1141. [CrossRef]
21. Gupta, S.C.; Sharma, A.; Mishra, M.; Mishra, R.K.; Chowdhuri, D.K. Heat shock proteins in toxicology: How close and how far? *Life Sci.* **2010**, *86*, 377–384. [CrossRef] [PubMed]
22. Picard, D. Heat-shock protein 90, a chaperone for folding and regulation. *Cell Mol. Life Sci.* **2002**, *59*, 1640–1648. [CrossRef] [PubMed]
23. Marshall, J.S.; DeRocher, A.E.; Keegstra, K.; Vierling, E. Identification of heat shock protein hsp70 homologues in chloroplasts. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 374–378. [CrossRef] [PubMed]
24. Schroda, M.; Vallon, O.; Wollman, F.A.; Beck, C.F. A chloroplast-targeted heat shock protein 70 (HSP70) contributes to the photoprotection and repair of photosystem II during and after photoinhibition. *Plant Cell* **1999**, *11*, 1165–1178. [CrossRef] [PubMed]

25. Su, P.H.; Li, H.M. Arabidopsis stromal 70-kD heat shock proteins are essential for plant development and important for thermotolerance of germinating seeds. *Plant Physiol.* **2008**, *146*, 1231–1241. [CrossRef] [PubMed]
26. Krishna, P.; Sacco, M.; Cherutti, J.F.; Hill, S. Cold-induced accumulation of hsp90 transcripts in *Brassica napus*. *Plant Physiol.* **1995**, *107*, 915–923. [CrossRef] [PubMed]
27. Dziurka, M.; Janeczko, A.; Juhász, C.; Gullner, G.; Oklestková, J.; Novák, O.; Saja, D.; Skoczowski, A.; Tóbiás, I.; Barna, B. Local and systemic hormonal responses in pepper leaves during compatible and incompatible pepper-tobamovirus interactions. *Plant Physiol. Biochem.* **2016**, *109*, 355–364. [CrossRef]
28. Płażek, A.; Dubert, F.; Kopeć, P.; Dziurka, M.; Kalandyk, A.; Pastuszak, J.; Wolko, B. Seed hydropriming and smoke water significantly improve low-temperature germination of *Lupinus angustifolius* L. *Int. J. Mol. Sci.* **2018**, *19*, 992. [CrossRef]
29. Cioć, M.; Dziurka, M.; Pawłowska, B. Changes in Endogenous Phytohormones of *Gerbera jamesonii* Axillary Shoots Multiplied under Different Light Emitting Diodes Light Quality. *Molecules* **2022**, *27*, 1804. [CrossRef]
30. Dziurka, K.; Dziurka, M.; Muszyńska, E.; Czyczyło-Mysza, I.; Warchoł, M.; Juzoń, K.; Laskoś, K.; Skrzypek, E. Anatomical and hormonal factors determining the development of haploid and zygotic embryos of oat (*Avena sativa* L.). *Sci. Rep.* **2022**, *12*, 548. [CrossRef]
31. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef] [PubMed]
32. Laemmli, U. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*, 680–685. [CrossRef] [PubMed]
33. Igielski, R.; Kępczyńska, E. Gene expression and metabolite profiling of gibberellin biosynthesis during induction of somatic embryogenesis in Medicago truncatula Gaertn. *PLoS ONE* **2017**, *12*, e0182055. [CrossRef]
34. Mácová, K.; Prabhullachandran, U.; Štefková, M.; Spyroglou, I.; Pěnčík, A.; Endlová, L.; Novák, O.; Robert, H.S. Long-Term High-Temperature Stress Impacts on Embryo and Seed Development in *Brassica napus*. *Front. Plant Sci.* **2022**, *13*, 844292. [CrossRef]
35. Rood, S.B.; Pearce, D.; Pharis, R.P. Identification of Endogenous Gibberellins from Oilseed Rape. *Plant Physiol.* **1987**, *85*, 605–607. [CrossRef] [PubMed]
36. Tarkowská, D.; Filek, M.; Biesaga-Kościelniak, J.; Marcińska, I.; Macháčková, I.; Krekule, J.; Strnad, M. Cytokinins in shoot apices of *Brassica napus* plants during vernalization. *Plant Sci.* **2012**, *187*, 105–112. [CrossRef]
37. Rapacz, M.; Waligórski, P.; Janowiak, F. ABA and gibberellin-like substances during prehardening, cold acclimation, de- and reacclimation of oilseed rape. *Acta Physiol. Plant.* **2003**, *25*, 151–161. [CrossRef]
38. Wang, Z.; Tan, X.; Zhang, Z.; Gu, S.; Li, G.; Shi, H. Defense to *Sclerotinia sclerotiorum* in oilseed rape is associated with the sequential activations of salicylic acid signaling and jasmonic acid signaling. *Plant Sci.* **2012**, *184*, 75–82. [CrossRef]
39. Zheng, X.; Koopmann, B.; von Tiedemann, A. Role of salicylic acid and components of the phenylpropanoid pathway in basal and cultivar-related resistance of oilseed rape (*Brassica napus*) to *Verticillium longisporum*. *Plants* **2019**, *8*, 491. [CrossRef]
40. Smoleńska-Sym, G.; Gawrońska, H.; Kacperska, A. Modifications of abscisic acid level in winter oilseed rape leaves during acclimation of plants to freezing temperatures. *Plant Growth Regul.* **1995**, *17*, 61–65. [CrossRef]
41. Lalk, I.; Dörffling, K. Hardening, abscisic acid, proline and freezing resistance in two winter wheat varieties. *Physiol. Plant.* **1985**, *63*, 287–292. [CrossRef]
42. Churchill, G.C.; Reaney, M.J.T.; Abrams, S.R.; Gusta, L.V. Effects of abscisic acid and abscisic acid analogs on the induction of freezing tolerance of winter rye (*Secale cereale* L.) seedlings. *Plant Growth Regul.* **1998**, *25*, 35–45. [CrossRef]
43. Bravo, L.A.; Zúñiga, G.E.; Alberdi, M.; Corcuera, L.J. The role of ABA in freezing tolerance and cold acclimation in barley. *Physiol. Plant.* **1998**, *103*, 17–23. [CrossRef]
44. Kim, H.; Seomun, S.; Yoon, Y.; Jang, G. Jasmonic acid in plant abiotic stress tolerance and interaction with abscisic acid. *Agronomy* **2021**, *11*, 1886. [CrossRef]
45. Ignatenko, A.; Talanova, V.; Repkina, N.; Titov, A. Exogenous salicylic acid treatment induces cold tolerance in wheat through promotion of antioxidant enzyme activity and proline accumulation. *Acta Physiol. Plant.* **2019**, *41*, 80. [CrossRef]
46. Taşgín, E.; Atící, Ö.; Nalbantoğlu, B. Effects of salicylic acid and cold on freezing tolerance in winter wheat leaves. *Plant Growth Regul.* **2003**, *41*, 231–236. [CrossRef]
47. Wang, W.; Wang, X.; Zhang, X.; Wang, Y.; Huo, Z.; Huang, M.; Cai, J.; Zhou, Q.; Jiang, D. Involvement of salicylic acid in cold priming-induced freezing tolerance in wheat plants. *Plant Growth Regul.* **2021**, *93*, 117–130. [CrossRef]
48. Chen, H.I.; Li, P.F.; Yang, C.H. NAC-Like Gene Gibberellin Suppressing Factor Regulates the Gibberellin Metabolic Pathway in Response to Cold and Drought Stresses in *Arabidopsis*. *Sci. Rep.* **2019**, *9*, 19226. [CrossRef]
49. Janeczko, A.; Biesaga-Kościelniak, J.; Dziurka, M.; Filek, M.; Hura, K.; Jurczyk, B.; Kula, M.; Oklestkova, J.; Novak, O.; Rudolphi-Skórska, E.; et al. Biochemical and Physicochemical Background of Mammalian Androgen Activity in Winter Wheat Exposed to Low Temperature. *J. Plant Growth Regul.* **2018**, *37*, 199–219. [CrossRef]
50. Sadura, I.; Pociecha, E.; Dziurka, M.; Oklestkova, J.; Novak, O.; Gruszka, D.; Janeczko, A. Mutations in the HvDWARF, HvCPD and HvBRI1 Genes-Involved in Brassinosteroid Biosynthesis/Signalling: Altered Photosynthetic Efficiency, Hormonal Homeostasis and Tolerance to High/Low Temperatures in Barley. *J. Plant Growth Regul.* **2019**, *38*, 1062–1081. [CrossRef]
51. Zanewich, K.P.; Rood, S.B. Vernalization and gibberellin physiology of winter canola. *Plant Physiol.* **1995**, *108*, 615–621. [CrossRef] [PubMed]

52. Filek, M.; Biesaga-Koscielniak, J.; Machackova, I.; Krekule, J. Generative Development of WinterRape (*Brassica napus* L.)—The Role of Vernalization. *Int. J. Plant Dev. Biol.* **2007**, *1*, 57–63.
53. Moblidowska, I. Effects of some growth regulators on frost damage. *Cryobiology* **1968**, *5*, 175–187. [CrossRef] [PubMed]
54. Wang, X.; Xu, C.; Cang, J.; Zeng, Y.; Yu, J.; Liu, L.; Zhang, D.; Wang, J. Effects of exogenous GA3 on wheat cold tolerance. *J. Agric. Sci. Technol.* **2015**, *17*, 921–934.
55. Pěnčík, A.; Simonovik, B.; Petersson, S.V.; Henyková, E.; Simon, S.; Greenham, K.; Zhang, Y.; Kowalczyk, M.; Estelle, M.; Zažímalová, E.; et al. Regulation of auxin homeostasis and gradients in *Arabidopsis* roots through the formation of the indole-3-acetic acid catabolite 2-oxindole-3-acetic acid. *Plant Cell* **2013**, *25*, 3858–3870. [CrossRef]
56. Östin, A.; Kowalyczk, M.; Bhalerao, R.P.; Sandberg, G. Metabolism of indole-3-acetic acid in arabidopsis. *Plant Physiol.* **1998**, *118*, 285–296. [CrossRef]
57. Gamir, J.; Pastor, V.; Cerezo, M.; Flors, V. Identification of indole-3-carboxylic acid as mediator of priming against *Plectosphaerella cucumerina*. *Plant Physiol. Biochem.* **2012**, *61*, 169–179. [CrossRef]
58. Pastor-Fernández, J.; Pastor, V.; Mateu, D.; Gamir, J.; Sánchez-Bel, P.; Flors, V. Accumulating evidences of callose priming by indole- 3- carboxylic acid in response to *Plectospharella cucumerina*. *Plant Signal. Behav.* **2019**, *14*, 1608107. [CrossRef]
59. Cohen, S.P.; Leach, J.E. High temperature-induced plant disease susceptibility: More than the sum of its parts. *Curr. Opin. Plant Biol.* **2020**, *56*, 235–241. [CrossRef]
60. Wang, B.; Andargie, M.; Fang, R. The function and biosynthesis of callose in high plants. *Heliyon* **2022**, *8*, e09248. [CrossRef]
61. Liu, X.; Hou, X. Antagonistic regulation of ABA and GA in metabolism and signaling pathways. *Front. Plant Sci.* **2018**, *9*, 251. [CrossRef] [PubMed]
62. Xu, Z.S.; Li, Z.Y.; Chen, Y.; Chen, M.; Li, L.C.; Ma, Y.Z. Heat shock protein 90 in plants: Molecular mechanisms and roles in stress responses. *Int. J. Mol. Sci.* **2012**, *13*, 15706–15723. [CrossRef]
63. Aghaie, P.; Tafreshi, S.A.H. Central role of 70-kDa heat shock protein in adaptation of plants to drought stress. *Cell Stress Chaperones* **2020**, *25*, 1071–1081. [CrossRef] [PubMed]
64. Ludwig-Müller, J.; Krishna, P.; Forreiter, C. A glucosinolate mutant of *Arabidopsis* is thermosensitive and defective in cytosolic Hsp90 expression after heat stress. *Plant Physiol.* **2000**, *123*, 949–958. [CrossRef] [PubMed]
65. Vítámvás, P.; Prášil, I.T.; Kosová, K.; Planchon, S.; Renaut, J. Analysis of proteome and frost tolerance in chromosome 5A and 5B reciprocal substitution lines between two winter wheats during long-term cold acclimation. *Proteomics* **2012**, *12*, 68–85. [CrossRef]
66. Rathore, N.; Kumar, P.; Mehta, N.; Swarnkar, M.K.; Shankar, R.; Chawla, A. Time-series RNA-Seq transcriptome profiling reveals novel insights about cold acclimation and de-acclimation processes in an evergreen shrub of high altitude. *Sci. Rep.* **2022**, *12*, 15553. [CrossRef] [PubMed]
67. Berka, M.; Kopecká, R.; Berková, V.; Brzobohatý, B.; Černý, M. Regulation of heat shock proteins 70 and their role in plant immunity. *J. Exp. Bot.* **2022**, *73*, 1894–1909. [CrossRef] [PubMed]
68. Di Donato, M.; Geisler, M. HSP90 and co-chaperones: A multitaskers' view on plant hormone biology. *FEBS Lett.* **2019**, *593*, 1415–1430. [CrossRef]
69. Clément, M.; Leonhardt, N.; Droillard, M.J.; Reiter, I.; Montillet, J.L.; Genty, B.; Lauriére, C.; Nussaume, L.; Noël, L.D. The cytosolic/nuclear HSC70 and HSP90 molecular chaperones are important for stomatal closure and modulate abscisic acid-dependent physiological responses in *Arabidopsis*. *Plant Physiol.* **2011**, *156*, 1481–1492. [CrossRef]
70. Wang, X.; Zhuang, L.; Shi, Y.; Huang, B. Up-regulation of HSFA2c and HSPs by ABA contributing to improved heat tolerance in tall fescue and *Arabidopsis*. *Int. J. Mol. Sci.* **2017**, *18*, 1981. [CrossRef]
71. Cao, Z.; Jia, Z.; Liu, Y.; Wang, M.; Zhao, J.; Zheng, J.; Wang, G. Constitutive expression of ZmsHSP in *Arabidopsis* enhances their cytokinin sensitivity. *Mol. Biol. Rep.* **2010**, *37*, 1089–1097. [CrossRef] [PubMed]
72. Černý, M.; Jedelský, P.L.; Novák, J.; Schlosser, A.; Brzobohatý, B. Cytokinin modulates proteomic, transcriptomic and growth responses to temperature shocks in *Arabidopsis*. *Plant Cell Environ.* **2014**, *37*, 1641–1655. [CrossRef] [PubMed]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.