International Journal of
Plant Biology

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Root ABA Accumulation Delays Lateral Root Emergence in Osmotically Stressed Barley Plants by Decreasing Root Primordial IAA Accumulation

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Abstract: Increased auxin levels in root primordia are important in controlling root branching, while their interaction with abscisic acid (ABA) likely regulates lateral root development in water-deficient plants. The role of ABA accumulation in regulating root branching was investigated using immunolocalization to detect auxin (indoleacetic acid, IAA) and ABA (abscisic acid) in root primordia of the ABA-deficient barley mutant *Az34* and its parental genotype (cv. Steptoe) barley plants. Osmotic stress strongly inhibited lateral root branching in Steptoe plants, but hardly affected *Az34*. Root primordial cells of Steptoe plants had increased immunostaining for ABA but diminished staining for IAA. ABA did not accumulate in root primordia of the *Az34*, and IAA levels and distribution were unaltered. Treating *Az34* plants with exogenous ABA decreased root IAA concentration, while increasing root primordial ABA accumulation and decreasing root primordial IAA concentration. Although ABA treatment of *Az34* plants increased the root primordial number, it decreased the number of visible emerged lateral roots. These effects were qualitatively similar to that of osmotic stress on the number of lateral root primordia and emerged lateral roots in Steptoe. Thus ABA accumulation (and its crosstalk with auxin) in root primordia seems important in regulating lateral root branching in response to water stress.

Keywords: *Hordeum vulgare* L.; ABA-deficient mutant *Az34*; hormones; immunolocalization; primordia; osmotic stress

Citation: Akhiyarova, G.; Veselov, D.; Ivanov, R.; Sharipova, G.; Ivanov, I.; Dodd, I.C.; Kudoyarova, G. Root ABA Accumulation Delays Lateral Root Emergence in Osmotically Stressed Barley Plants by Decreasing Root Primordial IAA Accumulation. *Int. J. Plant Biol.* **2023**, *14*, 77–90. https://doi.org/10.3390/ijpb14010007

Academic Editor: Adriano Sofo

Received: 28 November 2022
Revised: 21 December 2022
Accepted: 3 January 2023
Published: 9 January 2023

1. Introduction

Lateral root development is genetically and environmentally regulated, with the inhibition of lateral root development by low soil water availability considered an important adaptive response [1,2]. Nevertheless, extensive branching in the upper soil layers may allow root exploitation of light rainfalls that fail to infiltrate the soil to great depths [3]. Roots position new lateral branches according to the spatial distribution of available water in the substrate [4] and not in dry patches [5]. This adaptive response allows continued water uptake even as the soil dries.

Various growth hormones are involved in regulating root growth through complex signaling networks, with abscisic acid (ABA)–auxin crosstalk in lateral roots providing an adaptive strategy under drought stress conditions [6,7]. ABA likely exerts its repressive effects on lateral root formation through auxin [8]. The promotive effects of auxin on root branching might be inhibited by the repressive actions of ABA during lateral root emergence and initiation [9–11]. Auxin-sensitive reporter constructs such as DR5::GUS [12] and DR5::YFP [13] or immunohistochemical localization [14] have demonstrated auxin accumulation in root primordial cells. However, to the best of our knowledge, immunolocalization was not used to study the accumulation of ABA in the cells of lateral root primordia, even

though immunohistochemistry determined ABA accumulation in the stele of *Arabidopsis* primary root tips [15] and the beginning of the root hair zone of primary barley roots [16]. While immunohistochemical approaches allow the spatial accumulation of ABA and IAA in water-stressed plants to be determined, they have not yet been applied together in studies aiming to determine the regulation of lateral root branching.

Mutants with an impaired ability to synthesize hormones under stress conditions provide a useful resource to investigate the importance of phytohormonal regulation of lateral root branching. For example, the ABA-deficient tomato mutants *flacca* and *notabilis* had more and longer lateral roots when grown in vitro compared to their wild-type (WT), even though all genotypes had the same primary root length [17]. The ABA-deficient *Arabidopsis* mutant *nced3* had more and longer lateral roots (LRs) [18], while *aba2-3*, *aba2-4* and *aba3-2* also had more LR growth [19]. Disrupting genes responsible for the control of auxin synthesis also affects lateral root development in mutants [20]. Still, most of these studies were conducted under optimal conditions that may not adequately reflect the role of ABA (or indoleacetic acid (IAA) under water deficit.

Lateral root branching is important in regulating the responses of cereal crops to water deficit [21]. Moreover, ABA maintains primary root elongation [22] and inhibits root branching [23] of maize plants grown at low soil water availability. Nevertheless, lateral root growth of ABA-deficient cereals has attracted little attention. When grown in split-pots with distinct dry and wet soil compartments, both Steptoe plants and the ABA-deficient barley mutant *Az34* showed a similar root biomass distribution between the two pots [24]. Nevertheless, *Az34* had lower root ABA concentrations than Steptoe plants under optimal conditions [16] and failed to accumulate ABA in the roots when an atmospheric water deficit was imposed [25]. However, ABA and auxin concentrations in the cells of root primordia, and lateral root branching phenotypes of these genotypes have not been studied, irrespective of soil water availability. To test the importance of stress-induced changes in root hormone concentration, and their possible relationship to root branching, a histochemical technique enabled simultaneous immunolocalization of IAA and ABA in root sections. To determine the role of water-stress induced root ABA accumulation in these phenotypic responses, the ABA-deficient barley mutant *Az34* and its Steptoe were grown hydroponically with an osmotic stress applied to simulate decreased soil water availability. Moreover, exogenous ABA was applied to the mutant in attempting to revert its phenotype. We hypothesized that root ABA accumulation was necessary to inhibit lateral root branching in response to water deficit, either independently or via its interaction with IAA.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Seeds of barley *Hordeum vulgare* L. (ABA deficient mutant *Az34* and its wild-type cv. Steptoe) were germinated for 3 d in darkness at 21–24 °C on rafts made from sealed glass tubes tied together, which were then suspended over 0.1 strength Hoagland–Arnon nutrient medium (0.5 mM KNO_3, 0.5 mM $Ca(NO_3)_2$, 0.1 mM KH_2PO_4, 0.1 mM $MgSO_4$, 0.5 mM $CaSO_4$) in 3-litre containers and grown at an irradiance of 400 µmol m^{-2} s^{-1} and a 14-h photoperiod. When seedlings were 3 days old, they were placed into containers with either polyethylene glycol (PEG 6000, 0.15 MPa) or 0.1 mg/L ABA in the nutrient solution. Three days after PEG addition, roots were fixed in a mixture of ethanol and glacial acetic acid (3:1), stained with acetocarmine and emerged lateral roots and their primordia (detected inside the primary roots) were counted under the microscope as described [26].

2.2. Exogenous ABA Treatment

Two days after adding 0.1 mg/L of exogenous ABA to plants grown under control conditions, roots of *Az34* were harvested for hormone immunoassay, as previously described [25,27]. Hormones were extracted from homogenized roots of barley plants with 80% ethanol overnight at 4 °C. ABA, and the auxin IAA were partitioned with diethyl

ether from the aqueous residue after its dilution with distilled water and acidification with HCl to pH 2.5. Then, the hormones were transferred from the organic phase into a solution of $NaHCO_3$, re-extracted from the acidified aqueous phase with diethyl ether and immunoassayed after methylation using antibodies to ABA and IAA [26]. Reducing the amount of extractant at each stage of extraction, re-extraction increased the selectivity of hormone recovery [28]. Together with the approaches mentioned below, the specificity of immunoassay for ABA was confirmed in previous experiments, with fluridone-treated plants (which inhibits ABA biosynthesis) having lower ABA concentrations [29].

2.3. Immunolocalization of Hormones

Two days after adding PEG to the nutrient medium, 5 mm long root sections were collected at different positions (located from 10 to 45 mm) from the root tip. Root segments were fixed in 4% carbodiimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, (Merck, Darmstadt, Germany) for 4 h as described earlier [16,30] to prevent the washing out of hormones from tissues during dehydration. For better structural preservation, this procedure was followed by fixation with 4 % paraformaldehyde (Riedel de Haens, Seelze, Germany) for 10 h. During this process, carboxylic groups of ABA and IAA were linked with amino groups of proteins in the tissue. After dehydration with ethanol solutions of increasing grades (up to 96%), samples were embedded in the methacrylate resin (JB-4, Hatfield, PA, USA) as recommended by manufacturers. Histological sections (1.5 μm thick) were cut with a rotation microtome (HM 325, MICROM Laborgerate GmbH, Walldorf, Germany) and placed on slides. Sections were then incubated in 0.1 M Na-phosphate buffer (pH 7.3) containing 0.05% Tween 20 and 0.2% gelatin for 30 min and called below as PGT. Specific rabbit antisera against ABA [16,25,31,32] and IAA [26,30,33] were used for their immunolocalization. Anti-ABA or anti-IAA sera (20 μL) in PGT (1:80 and 1:40, respectively), were poured over some sections and incubated in a moist chamber at room temperature for 2 h. Some sections were incubated with the non-immune serum to check specificity of immunostaining. To visualize serum binding with ABA and IAA, sections were treated for 1 h at room temperature with the goat antibodies against rabbit immunoglobulin, which were labeled with colloidal gold (Aurion, Wageiningen, Netherlands). After three washes with 0.1 M Na-phosphate buffer containing 0.05% Tween 20, sections were treated with silver enhancer (Aurion, Wageiningen, Netherlands) for 15–20 min and examined under a light microscope. Excess silver was removed with distilled water. Preparations were visualized under an Axio Imager.A1 light microscope (Carl Zeiss, Jena, Germany) supplied with an AxioCam MRc5 digital camera (Carl Zeiss, Jena, Germany).

Intensity of immunostaining for IAA and ABA was estimated as described [34]. Staining values were averaged for the areas of primordia. Images were taken from 10 independent sections per treatment. Intensity of staining was expressed in arbitrary units: maximal staining was taken as 100%, and minimal staining was 0%.

Earlier specificity and reliability of immunostaining was confirmed in experiments, where increased immunostaining was detected in the plants treated with exogenous hormones (positive control), as well as by decreased immunostaining of the sections of primary roots of ABA-deficient mutant (negative control) [16]. In the present study, immunostaining for the two hormones of the identical sections, fixed in a similar way, confirmed that differences in staining were due to specificity of the antibodies.

2.4. Staining of Nuclei by Schiff Reagent

Sections were hydrolyzed with hydrochloric acid at 24 °C (1 M HCl, 5 min; 5 M HCl, 30 min; 1 M HCl, 5 min), slides were stained with Schiff's reagent (Feulgen reaction for a period of 12 h and then washed in SO_2-water (3 × 5 min) and H_2O [35].

2.5. Statistics

Data were expressed as means ± S.E., which were calculated in all treatments using MS Excel. Significant differences between means were analyzed by a *t*-test.

3. Results

In the absence of osmotic stress, the *Az34* mutant and its parental cultivar Steptoe had similar numbers of lateral roots and primordia (Figure 1). No lateral roots had emerged before PEG was added to the nutrient medium, and later changes in the total number of lateral roots were due to the osmotic stress treatment. Three days of osmotic stress decreased the number of lateral roots of Steptoe plants by 40% compared to the control plants, but had no effect on the ABA-deficient mutant (Figure 1). Osmotic stress increased the number of primordia in the Steptoe, but this response was attenuated in the mutant. Primary root length was similar in both genotypes, and not affected by the osmotic stress, at least over 3 days (Table 1).

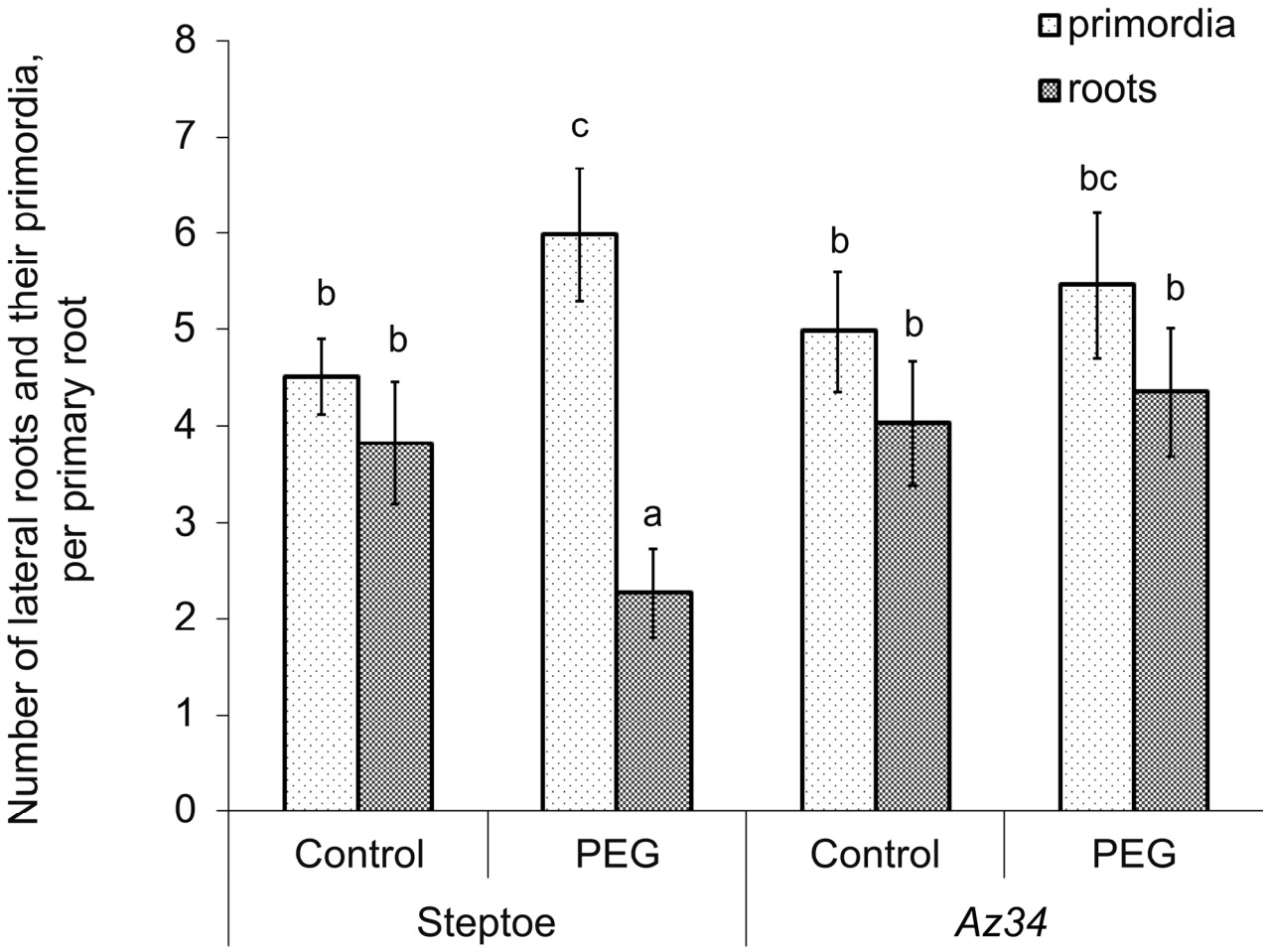

Figure 1. Number of lateral roots and their primordia in 6-day-old ABA-deficient *Az34* barley plants and their parental cultivar Steptoe grown under control conditions, or exposed for 3 days to 6 % polyethylene glycol (PEG). Significant differences are marked with different letters. Values are means ± S.E. (n = 25) ($p \leq 0.05$, *t*-test).

Table 1. The length of all seminal roots (mm) of Steptoe and *Az34* grown under normal conditions and polyethylene glycol (PEG) treatment. Data are means ± SE of 50 replicates. No statistical differences were detected in these experiments.

Genotypes	Normal Conditions	PEG Treatment
Steptoe	199 ± 6	206 ± 11
Az34	184 ± 9	201 ± 8

In consecutive slides reflecting a developmental gradient from small primordia to lateral root emergence, when plants were grown under normal conditions the two genotypes did not differ in the level and distribution of IAA (Figure 2) and ABA (Figure 3) between the root cells (Figure 2). The subcellular localization of ABA and IAA clearly differed, the latter concentrating in the nuclei (nuclear localization was confirmed with the help of the Schiff reagent—Figure S1B). Interestingly, the preferential staining of nuclei for IAA was detected in primordia with elongating cells (Figure 2B,C,E,F) and was not observed at earlier stages of primordial development (Figure 2A,D). In primordia ready to emerge from the primary root, the distribution of immunostaining for the two hormones between the central cylinder and the cortex also differed (compare Figure 2B,C,E,F with Figure 3B,C,E,F). Closer to the base of these primordia, nuclei staining for IAA was greater in the cells of the central cylinder, while that of ABA was similar in intensity either in the central cylinder or the cortex. Interestingly, staining with the Schiff reagent revealed more stained nuclei in the emerging central cylinder or the cortex of the primordia (Figure S1B), while many of the cellular nuclei remained unstained in the case of IAA immunolocalization (Figure S1A). Thus, not all the nuclei were labeled with anti-IAA serum. Despite the difference in the subcellular localization of IAA and ABA, and their distribution between root zones during development of root primordia, there were no differences between the genotypes in staining for each of the hormones in the absence of osmotic stress.

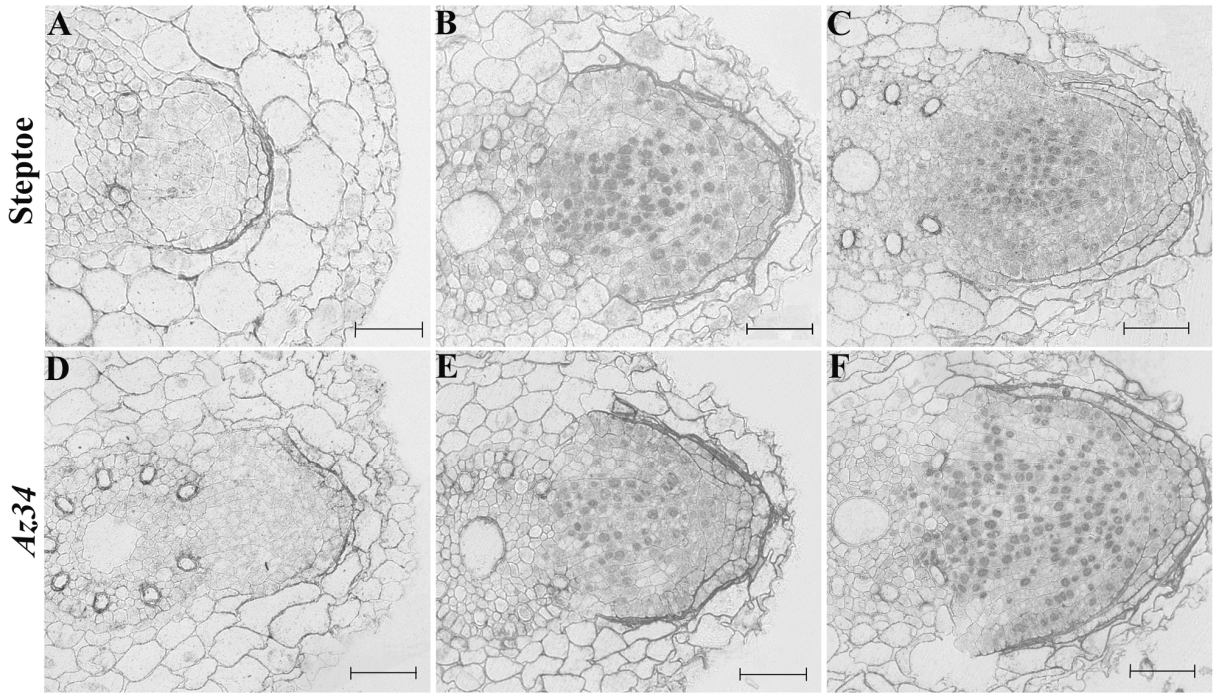

Figure 2. Immunostaining of IAA in root primordia of Steptoe (**A**–**C**) and *Az34* (**D**–**F**) at successive stages of their development. Scale bar is 50 µm.

Although there were no genotypic differences in the intensity of staining of developing primordia for either hormone when grown under optimal conditions, under osmotic stress the subcellular localization and distribution of ABA and IAA between the cells of the stele and cortex differed between genotypes (Figures 4 and 5). Osmotic stress increased staining of primordial cells for ABA in Steptoe plants, but did not change this in *Az34* (Figure 4). For IAA a different pattern was detected, with weaker immunostaining in the stressed Steptoe than the mutant (Figure 5). Increased staining of root primordial cells for ABA in the stressed Steptoe plants, and their weakened staining for IAA, was statistically significant as shown by semi-quantitative analysis of the staining with the help of the ImageJ program (Figure 6). The difference in root staining between stressed plants of the two genotypes was not only in root cell IAA levels, but also in the subcellular distribution of the hormone. Thus increased nuclei staining for IAA disappeared in the root cells of stressed Steptoe plants (Figure 5C), but remained in *Az34* (Figure 5D). Thus the capacity of Steptoe plants to accumulate ABA in primordial cells under stress conditions was accompanied by decreased IAA level in those cells, while the absence of ABA changes in the mutant may be related to the absence of changes in IAA.

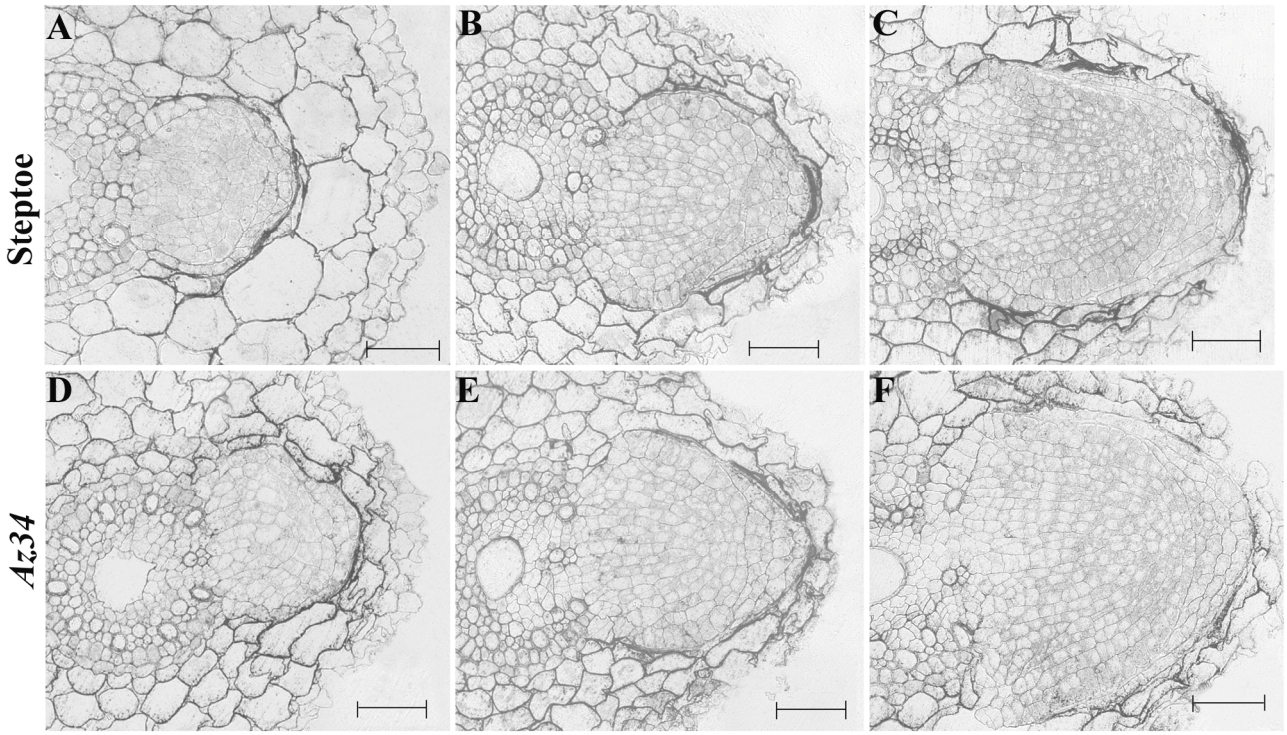

Figure 3. Immunostaining of ABA in root primordia of Steptoe (**A**–**C**) and *Az34* (**D**–**F**) at successive stages of their development. Scale bar is 50 μm.

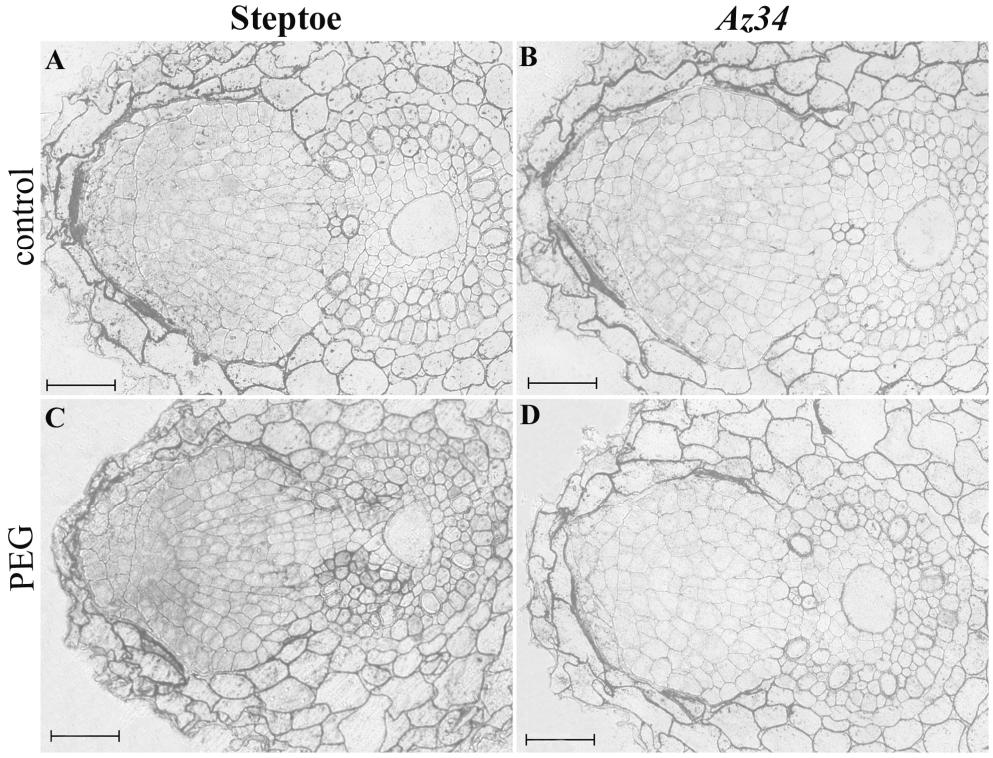

Figure 4. Effect of 6% polyethylene glycol (PEG) treatment on immunolocalization of ABA in the cells of root primordia of Steptoe (**A**,**C**) and *Az34* (**B**,**D**) plants; (**A**,**B**)—plants grown under normal conditions; (**C**,**D**)—stressed plants. Scale bar is 50 μm.

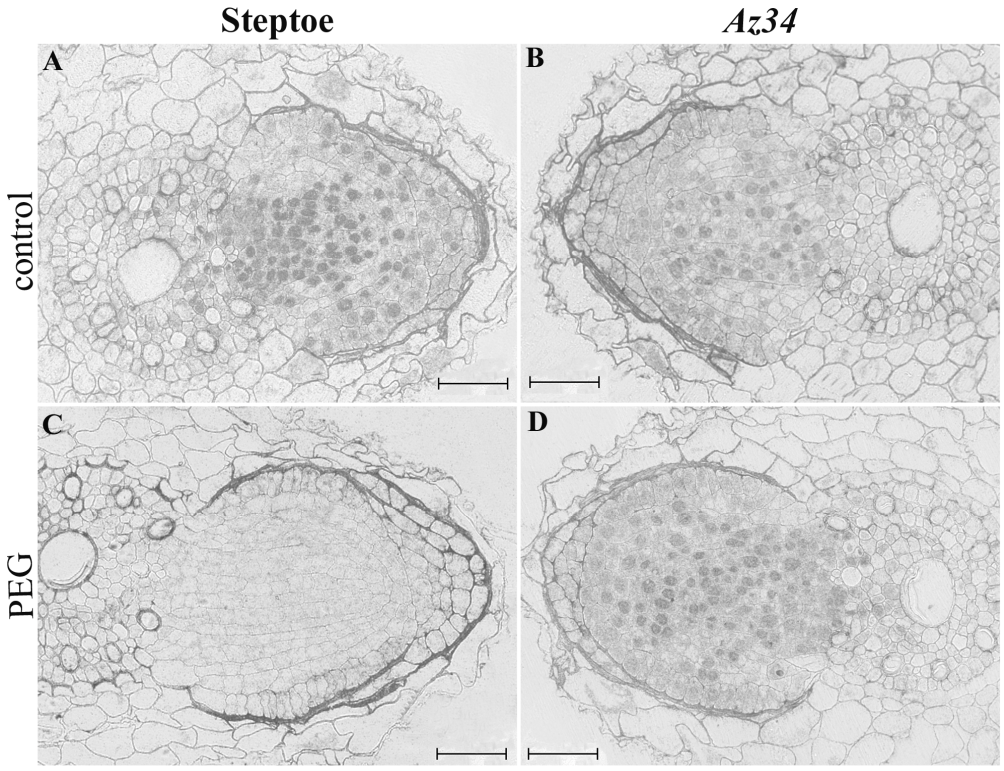

Figure 5. Effect of 6% polyethylene glycol (PEG) treatment on immunolocalization of IAA in the cells of root primordia of Steptoe (**A**,**C**) and *Az34* (**B**,**D**) plants; (**A**,**B**)—plants grown under normal conditions; (**C**,**D**)—stressed plants. Scale bar is 50 μm.

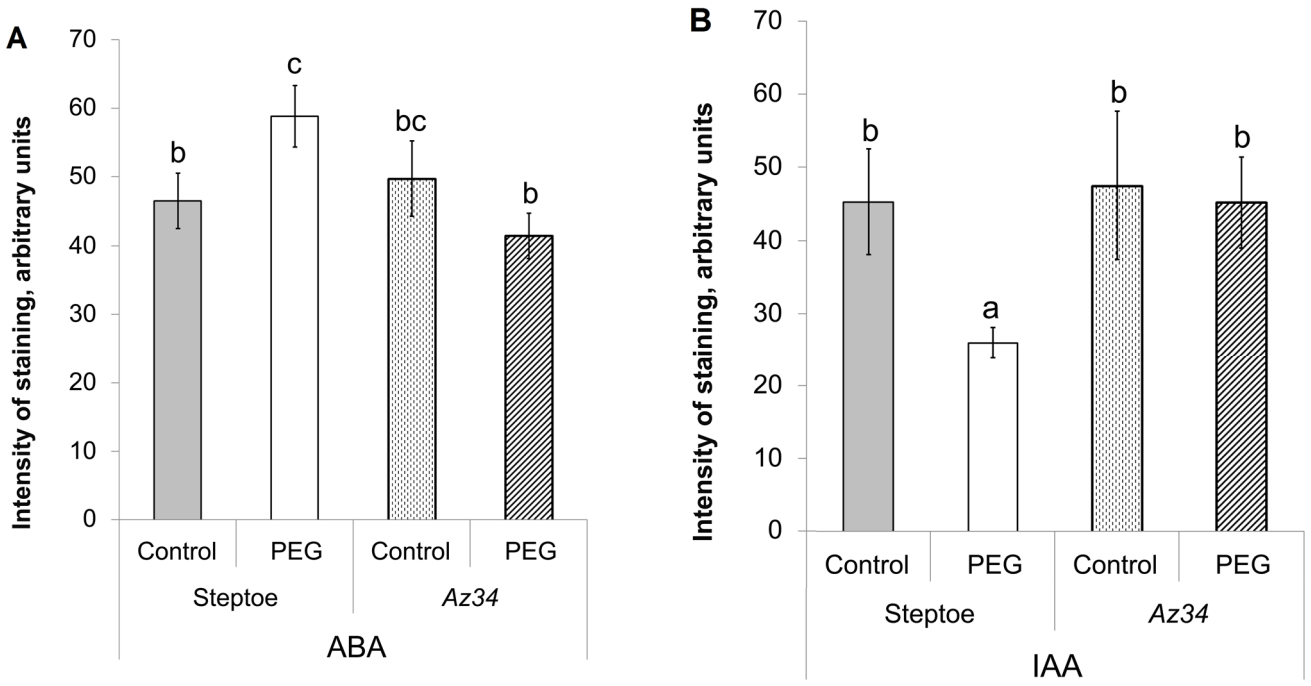

Figure 6. Intensity of immunostaining for ABA (**A**) and IAA (**B**) of root primordia of *Az34* and Steptoe plants grown under normal or stress conditions (6% polyethylene glycol (PEG)). Images were taken from the sections presented in Figures 2, 3, 5 and 6. Means (arbitrary units, maximal staining taken as 100%, minimal—as 0%). Significant differences are marked with different letters. Values are means ± S.E. (n = 25) ($p \leq 0.05$, t-test).

To confirm whether root ABA status affected the pattern of root IAA concentration and lateral branching, ABA was added to the nutrient solution of *Az34* plants grown under optimal conditions (Figure 7). This treatment decreased bulk root IAA concentration by 34% and the number of lateral roots by 56%, but increased the number of root primordia by 55% as compared to plants untreated with ABA. ABA treatment had no effect on the primary root length in this experiment, but increased immunostaining for ABA. Such ABA accumulation was accompanied by weakened immunostaining for IAA (Figures 8 and 9), while nuclear staining for IAA disappeared as in PEG-treated Steptoe plants (Figure 5). Thus exogenous ABA treatment was able to phenotypically revert lateral root branching and IAA response of the ABA-deficient mutant, making them similar to Steptoe plants under stress conditions.

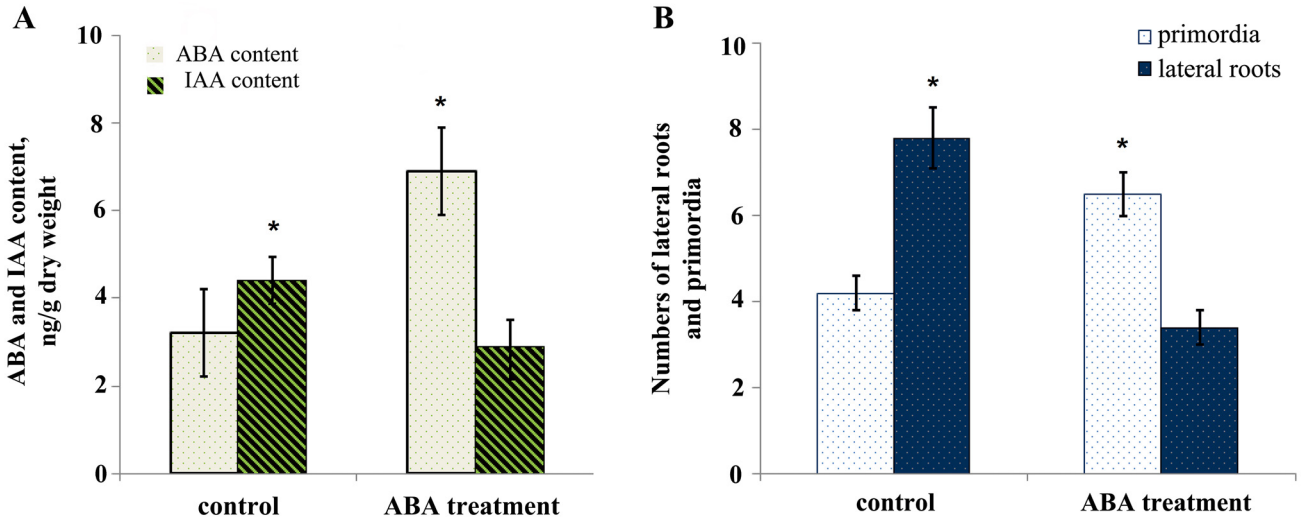

Figure 7. ABA and IAA content in roots (**A**) and numbers of primordia and lateral roots (**B**) in ABA-deficient *Az34* barley plants grown under control conditions or exposed for 3 days to 0.1 mg/L ABA. Significantly different means from control are marked with asterisk. Values are means ± S.E. ($n = 25$) ($p \leq 0.05$, *t*-test).

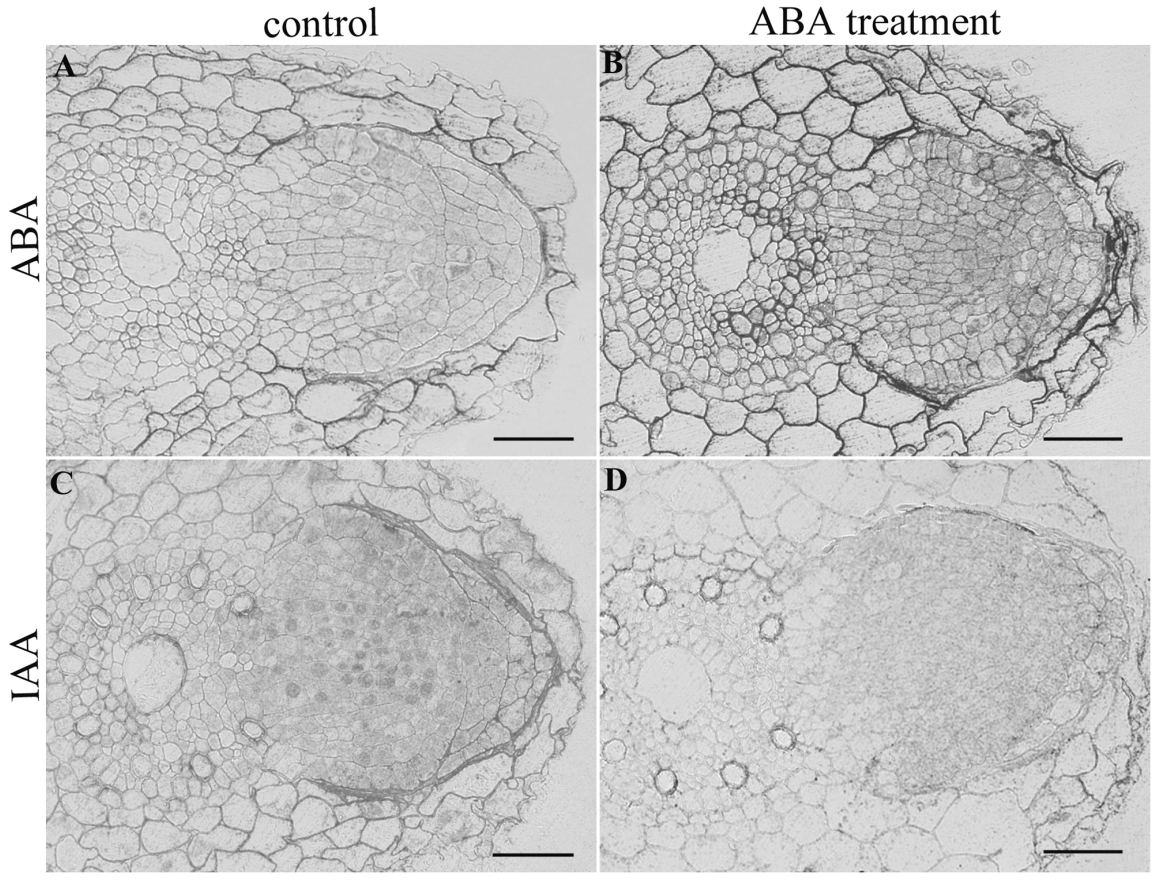

Figure 8. Effect of 0.1 mg/L ABA treatment on immunolocalization of ABA (**A**,**B**) and IAA (**C**,**D**) in the cells of root primordia of ABA–deficient *Az34* barley plants. (**A**,**C**)—plants grown under normal conditions; (**B**,**D**)—plants grown under ABA treatment for 3d. Scale bar is 50 μm.

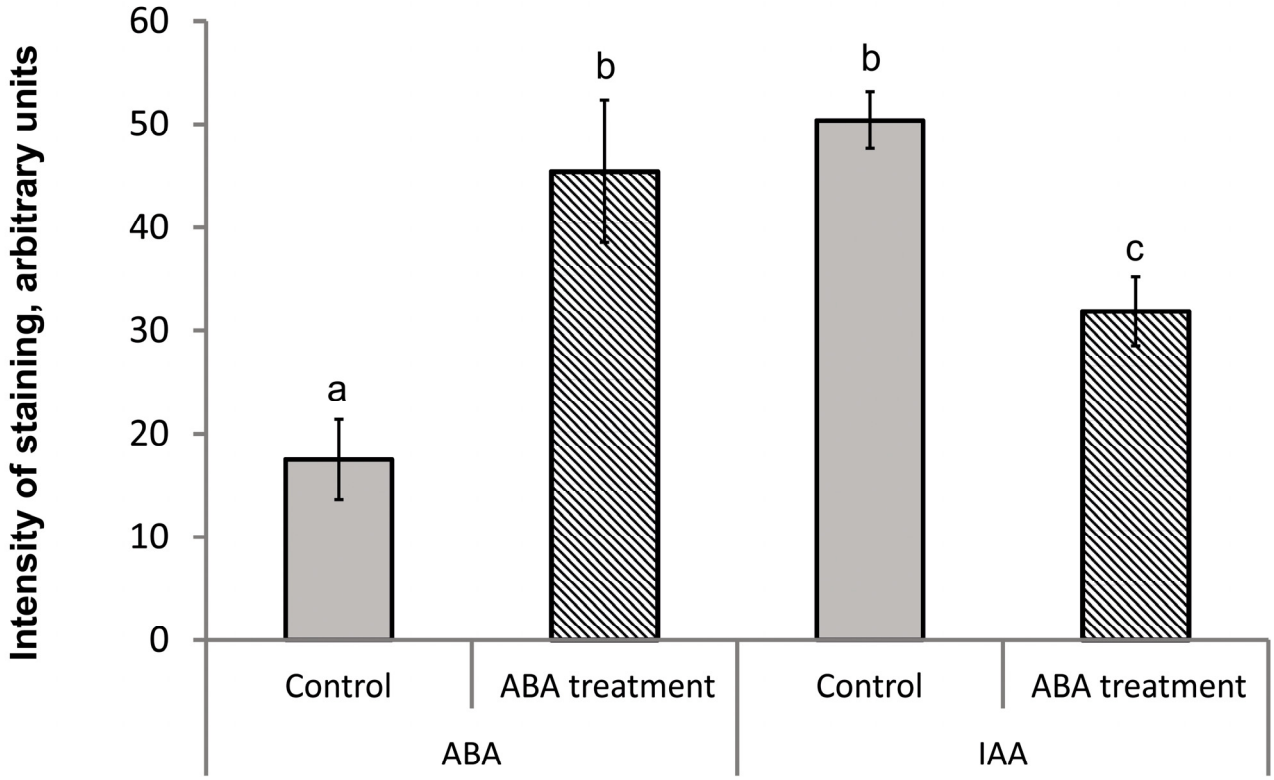

Figure 9. Intensity of immunostaining for ABA and IAA of root primordia of *Az34* grown under normal conditions or under 0.1 mg/L ABA treatment. Means (arbitrary units, maximal staining taken as 100%, minimal—as 0%). Significant differences are marked with different letters. Values are means ± S.E. (n = 25) ($p \leq 0.05$, t-test).

4. Discussion

Both auxins and ABA can influence lateral root development, which is likely important for regulating growth responses to water deficit. Since ABA's effects on root branching may be due to its cross-talk with IAA, we simultaneously determined localization of these hormones in the cells of root primordia under normal conditions and moderate PEG-induced water deficit. Differential ABA and IAA accumulation in root primordia of Steptoe plants and the ABA-deficient barley mutant *Az34* under osmotic stress was correlated with inhibition of lateral root branching in the parental cultivar plants, but no such effect in the mutant. Exogenous ABA treatment of *Az34* restored a parental cultivar response, further supporting the hypothesis that root ABA accumulation inhibits lateral root emergence at low soil water availability.

Root primordial nuclei of both genotypes were immuno-labelled for IAA, but not for ABA (Figure 2, Figure 3 and Figure S1). The presence of IAA in the nuclei is not surprising as the auxin TIR1 receptor is localized in the nuclei and auxin perception is known to be a nuclear effect [36]. Although the PYR/PYL/RCAR family of ABA receptors is also nuclear localized [37,38], the absence of nuclei immunostaining for ABA suggests that ABA signaling in root primordia uses other receptors, such as the GCR1 receptor located in the plasma membrane [39]. Previous studies indicate more intensive staining for ABA in the central cylinder of *Arabidopsis* [15] and barley [16] primary roots, with the maximum level in a single cell layer surrounding the root stele [15]. This pattern was not detected in barley root primordia, although the root central cylinder was expected to show increased staining for ABA when emerged lateral roots are studied.

Increased immunostaining for ABA of primordial cells of ABA-treated roots agrees with data obtained for primary root tips [16] and confirmed the reliability and specificity of immunostaining for this hormone (positive control). Interestingly, immunostaining for IAA followed an opposite pattern to ABA. In parental cultivar plants, more intensive staining for ABA in the primordia of stressed roots contrasted with weaker immunostaining for IAA in control (unstressed) roots. Exogenous ABA treatment also decreased staining for IAA of primordial cells of *Az34* plants. Lower immunostaining for IAA of stressed Steptoe primordia as well as ABA-treated *Az34* plants agrees with data showing that drought and ABA treatments may decrease auxin level by up-regulating *GH3* genes that encode enzymes that conjugate amino acids to IAA, particularly in the lateral root primordia [6]. Thus, ABA may modulate auxin-signaling by inducing *GH3* enzyme genes, ultimately reducing lateral root formation. Alternatively, changes in auxin transporter levels and localization may reduce root auxin concentrations in an ABA-dependent [11,40] and osmotic stress dependent manner [7].

When osmotically stressed, the ABA-deficient *Az34* barley mutant had more lateral roots than its parental cultivar Steptoe (Figure 1). Thus, cereals and *Arabidopsis* seem to regulate lateral root growth similarly, since ABA-deficient mutants (such as the biosynthetic mutant *aba2-1*) have greater lateral root development than Steptoe plants under osmotic stress [1]. Both osmotic stress and ABA-treatment abolished preferential staining for IAA of the root cell nuclei of Steptoe plants, correlating with the decreased branching. Since this differential subcellular staining was absent at early stages of primordial development, increased IAA levels in root cell nuclei are likely important for lateral root emergence. Under osmotic stress, this increased staining of the root cell nuclei was retained in *Az34* plants, and the lateral root number was maintained. These results suggest ABA accumulation prevents IAA accumulation in the nuclei of stressed plants, thereby delaying lateral emergence.

For the first time, immunohistochemistry demonstrated greater staining for ABA in lateral roots of stressed Steptoe plants but not in the ABA deficient mutant. Stressed Steptoe plants had fewer lateral roots but more root primordia than the control Steptoe plants. In both PEG-treated or control plants, the sum of primordia and emerged lateral roots remained the same, suggesting that stress-induced ABA accumulation inhibited root emergence, as previously reported ([10] and references therein). Although ABA accumulation maintains primary root elongation in stressed plants [41], an opposing effect on lateral root emergence has been detected [1,40,42]. Further experiments are needed to explain this discrepancy. Although osmotic stress increases bulk root ABA accumulation ([43] and references therein), ABA accumulation in primordial cells (Figure 4) likely inhibited lateral root emergence. Despite osmotic stress, the lack of ABA accumulation in root primordia of the ABA-deficient mutant *Az34* allowed normal lateral root emergence.

This comparative study of immunolocalization of ABA and IAA in the ABA-deficient barley mutant *Az34* and its parental cultivar Steptoe revealed root primordial hormone accumulation. Stress-induced ABA accumulation in Steptoe plants correlated with decreased IAA concentrations and changes in the intracellular distribution of IAA. The *Az34* mutant did not accumulate ABA under osmotic stress, but exogenous ABA treatment decreased root IAA concentration of this genotype (Figures 7A and 8) and decreased lateral root number while increasing the number of primordia. These effects mimicked the effects of stress-induced ABA accumulation in Steptoe primordia. Thus, stress-induced changes in hormone levels and their cross-talk in the cells of root primordia likely regulate root branching.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijpb14010007/s1, Figure S1. Immunolocalization of IAA (A) and staining with the Schiff reagent (B) of developing lateral root Steptoe primordia. Scale bar is 50 µm.

Author Contributions: Conceptualization, G.S., G.K. and I.C.D.; methodology, D.V., G.A., R.I. and G.S.; software, I.I.; formal analysis, D.V., G.A., R.I. and G.S.; investigation, D.V., G.A., R.I. and G.S.; resources, D.V.; data curation, G.S; writing—original draft preparation, G.K.; writing—review and editing, I.C.D. and G.S.; visualization, G.A. and G.S.; supervision, G.K.; project administration, G.K.; funding acquisition, G.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Ministry of Science and Higher Education of Russia, grant number AAAA-A18-118022190099-6 and Russian Foundation for Basic Research, grant number 20-04-00305.

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.

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