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Effects of Elevated Temperature and Salicylic Acid on Heat Shock Response and Growth of Potato Microplants

Jelena Rudić [1], Danijel Pantelić [1], Jasmina Oljača [2] and Ivana Momčilović [1,*]

[1] Institute for Biological Research "Siniša Stanković"-National Institute of the Republic of Serbia, University of Belgrade, Bulevar despota Stefana 142, 11060 Belgrade, Serbia; jelena.rudic@ibiss.bg.ac.rs (J.R.); danijel.pantelic@ibiss.bg.ac.rs (D.P.)

[2] Faculty of Agriculture, University of Belgrade, Nemanjina 6, 11080 Belgrade, Serbia; jasmina.oljaca@agrif.bg.ac.rs

* Correspondence: ivana.momcilovic@ibiss.bg.ac.rs

Abstract: Potato is a globally important, highly heat-susceptible crop species. We investigated the effects of prolonged exposure to elevated temperatures and exogenous salicylic acid (SA) on microplant growth and heat-shock response (HSR) in three unrelated potato genotypes/cultivars. Long-term exposure to 29 °C (mild heat stress) caused a significant reduction in the number of surviving explants and shoot morphometric parameters in heat-sensitive genotypes, while exposure to 26 °C (warming) caused only a decline in shoot growth. Interestingly, 26 °C-temperature treatment stimulated root growth in some investigated genotypes, indicating a difference between favorable temperatures for potato shoot and root growth. SA showed a protective effect regarding potato shoot growth at 26 °C. At 29 °C, this effect was genotype-dependent. SA did not affect the number of roots and inhibited root elongation at all temperature treatments, indicating the difference between shoot and root responses to applied SA concentration. Although HSR is mainly considered rapid and short-lived, elevated transcript levels of most investigated *HSF*s and *HSP*s were detected after three weeks of heat stress. Besides, two *StHSF*s and *StHSP21* showed elevated expression at 26 °C, indicating extreme potato heat-susceptibility and significance of HSR during prolonged warming. SA effects on *HSF*s and *HSP*s expression were minor and alterable.

Keywords: potato; micropropagation; heat stress; HSP; HSF; salicylic acid

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1. Introduction

Potato, *Solanum tuberosum* L., is the most important non-grain crop in the world, a source of staple food for millions of people worldwide. Compared to other crops, potato is highly susceptible to high temperatures that can cause heat stress, i.e., a multitude of biochemical, physiological, and morpho-anatomical changes that affect plant growth and development [1]. In potato, these changes include acceleration of stem growth, enhanced branching, reduced total leaf area, and reduced or inhibited root growth, but the most prominent alterations relate to the reduction of tuber initiation and enlargement, leading to a decline in potato yield [2,3]. Regarding physiological changes, high temperatures affect photosynthesis and respiration, and shift assimilate supply to the above-ground plant parts with less assimilated carbon incorporated into the tuber's starch [4,5]. At the cellular and molecular level, heat stress disrupts the integrity of plant cellular membranes, changes protein conformation (heat denaturation), degrades the PSII component of the photosynthetic apparatus, and impairs electron transport chains in chloroplasts and mitochondria [6].

To cope with high-temperature stress, plants employ heat-protective mechanisms that lead to modification of organelles and the cytoskeleton organization, modulation of membrane fluidity, and accumulation of antioxidant enzymes accompanied with the production of antioxidants and other protective molecules [7]. One of the major mechanisms is the heat-shock response (HSR) which results in the rapid synthesis of heat-shock proteins

(HSPs) upon plant exposure to high temperature. HSPs are molecular chaperones that play a central role in plant heat tolerance by promoting the refolding of heat-denatured proteins, forming complexes with denatured proteins to protect them from aggregation or mediating disaggregation of insoluble protein aggregates [8–10]. The HSPs complement comprises five main classes of proteins, designated by their approximate molecular masses (kDa), amino acid sequence homologies, and activities as molecular chaperones: HSP100, HSP90, HSP70, HSP60, and small HSP with molecular masses 15–30 kDa (sHSPs). The sHSPs are the most diversified family of HSP in plants (angiosperm), comprising seven subfamilies that are cytoplasmic/nuclear localized (CI–CVII) and five sHSP subfamilies that localize to organelles: the endoplasmic reticulum (ER), peroxisomes (PX) chloroplasts (CP), and the mitochondria (MTI and MTII) [11]. Fast expression of HSP genes is regulated by heat-shock factors (HSFs)—transcription factors that trimerize under stress conditions, translocate to the cell nucleus, and activate HSP transcription [12].

Salicylic acid (SA) is considered an endogenous growth regulator and signaling molecule involved in plant defense responses to the presence of pathogens and abiotic stresses [13]. Heat stress promotes the accumulation of endogenous SA in plants [14–16]. The study of *Arabidopsis thaliana* mutants with contrasting SA accumulation revealed that the content of endogenous SA and SA-inducible thermotolerance mechanisms are of considerable importance in plant basal thermotolerance [14]. Pan et al. [15] reported that inhibition of SA biosynthesis during heat acclimation in pea plants not only reduced the endogenous SA content but also the level of heat tolerance. Molecular mechanisms underlying SA-mediated heat tolerance are not fully understood. SA-potentiated HSR in tomato included enhancement of HSF binding to DNA [17]. One of the proposed mechanisms may involve SA interaction with non-expresser of pathogenesis-related (NPR) proteins, which stimulates the transcriptional regulation of various defense-related signaling cascades by controlling the expression of WRKY transcription factors [16,18]. However, besides NPR proteins, almost 100 candidate SA-binding proteins (SABP) have been identified in plants [19]. The outcome of signaling through these regulatory networks might be observed SA-related alterations in HSR, such as enhancement of HSP biosynthesis [17], increased proline production [20], changes in the activities of plasma membrane H^+ and Ca^{2+}-ATPase [21], and modulation of antioxidant enzymes expression and activity [22]. The significance of SA in plant heat tolerance was confirmed by the exogenous application of this growth regulator, either through spraying or adding to the nutrient solution, which promoted thermotolerance in a large number of species [23].

Besides the increasing frequency of heatwaves, global climate change will cause a gradual rise in average ambient temperatures in most potato-growing regions in the 21st century [24]. Therefore, investigations regarding potato physiological, biochemical, and molecular responses to long-term exposure to elevated temperatures, as well as effects of protective compounds such as SA, are gaining importance [5,25]. Thus far, HSR has been mainly investigated via short-term exposure of plants to severely high temperatures (heat-shock); data regarding its significance during prolonged warming or mild heat stress are scarce [26]. Moreover, investigations regarding SA involvement in potato heat tolerance were related to the effect of exogenous SA on virus-infected plants subjected to high-temperature treatments [27,28]. In the present study, we investigated the effects of SA on plant growth and development in potato cultivars Agria, Désirée, and Kennebec, after three-week exposure to 21 °C, 26 °C, and 29 °C, using an in vitro system. Treatment with 21 °C was considered a control and slightly elevated 26 °C was applied to explore the upper limit of temperature optimum for growth and development of potato microplants, while treatment with 29 °C was chosen to induce long-term mild heat stress and to avoid damage caused by excessively high temperatures. Three unrelated tetraploid potato varieties were used to assess eventual differences in HSR and growth response among genotypes. Temperature and SA effects on HSR were investigated by following the expression of three HSFs (HSFA2, HSFA3, and HSFA5) and six HSPs (HSP17.6-CI, HSP21-CP, HSP23.9-MTI, HSP70-1, HSP70-2, and HSP101) at the transcript level in microplant leaves. Our results

provide the basis for further research of potato HSFs and HSPs and can be important for a better understanding of potato plants' response to moderately elevated temperature and SA.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Three commercial *S. tuberosum* L. cultivars, Agria, Désirée, and Kennebec, were used in experiments. Virus-free tubers were obtained from Solanum Komerc, Guča, Serbia. In vitro cultures were established from surface-sterilized sprouts, which were fragmented into 1 cm-long cuttings carrying a single axillary bud and placed on a basal medium (BM) composed of Murashige and Skoog salt mixture [29], Linsmaier and Skoog vitamins [30], 0.7% agar, 3% sucrose, and 100 mg L^{-1} myo-inositol, and supplemented with 0.5 mg L^{-1} 6-benzylaminopurine (BAP; Sigma Aldrich, St. Louis, MO, USA). The shoots obtained on this medium gave rise to plantlets when transferred to BM without BAP. Microplants were grown in the culture growth room at 21 °C and 16 h of light (light flux 90 µmol m^{-2} s^{-1}), then routinely subcultured every four weeks on BM using single-node stem cuttings (SNC).

2.2. SA and Temperature Treatments

SNCs (10–15 mm) from 4-week-old potato microplants were transferred on BM (control) or BM supplemented with 1×10^{-5} M SA (10 SNCs per jar). SA (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 96% ethanol and added to BM prior to the sterilization at 114 °C for 25 min, while the equivalent volume of ethanol was added in control. The explants were grown in the growth chamber (Aralab, Rio de Mouro, Portugal) under the following conditions: photoperiod 16/8 h (light/dark), illumination 90 µmol m^{-2} s^{-1}, relative humidity 70% and three different temperatures: 21 °C, 26 °C, or 29 °C. After three weeks, the percentage of surviving explants and plant morphometric parameters (the number of leaves and nodes, shoot length, shoot fresh mass, the number of roots, root length, and root fresh mass) were measured. SNCs that gave rise to plantlets (>10 mm) were calculated as surviving explants. Necrotic explants and explants with bud developmental arrest were not calculated. Since every singular stem node of microplants was bearing a leaf, this parameter was described as the number of leaves and nodes. The length of the longest root per plant was measured and averaged for the root length parameter.

Fully developed leaves (third, fourth, and fifth leaf from the apex) were collected after SA and temperature treatments, frozen in liquid nitrogen, stored at −80 °C, and further used for RNA analysis. Three biological replicates per experiment were used for qRT-PCR analysis.

2.3. RNA Extraction and qRT-PCR Analysis

Total RNA was extracted from frozen potato leaves (0.5 g) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in combination with DNase I (Fermentas, Hanover, MD, USA) treatment. The RNA quality was evaluated by electrophoresis on a 2% agarose gel and assessed by the 28S/18S rRNA ratio. Total RNA was used for the first-strand cDNA synthesis using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA) and oligo(dT) primers according to manufacturer's instructions. The primers for qRT-PCR analysis were designed using the Primer-BLAST tool available from the NCBI web site (https://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed on 10 March 2021). The sequences of the forward and reverse primers for each gene are described in Table S1. All qRT-PCR reactions were run in the Applied Biosystems QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). Each cDNA sample was run in triplicates on a 96 well plate, using 10 µL volumes of a mixture comprising 100 ng cDNA, forward (7.5 µM) and reverse primers (7.5 µM), and Maxima SYBR Green/ROX qPCR Master Mix (2x) (Thermo Fisher Scientific, Waltham, MA, USA). The qRT-PCR amplification conditions were as follows 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. After amplification, a melt curve analysis was carried out on the amplicons to

confirm that only a specific product was generated in each reaction. The expression values of the individual genes were normalized with respect to the averaged expression level of the internal control genes *60SL36* and *CYC*. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [31].

2.4. Potato Genomic Resources and Description of HSF and HSP Genes and Proteins

Data regarding eleven potato *HSF*- and *HSP*-coding genes, including accession numbers, chromosomal location, sequences, and corresponding transcript and protein sequences, were based on the doubled monoploid *S. tuberosum* Group Phureja DM 1-3 516 R44 and retrieved from Ensembl Plants (DM v4.04, https://plants.ensembl.org/index.html, accessed on 2 March 2021). Information about *HSP70-2* was also obtained from the NCBI database (Gene ID 102600309, https://www.ncbi.nlm.nih.gov/, accessed on 2 March 2021).

The physicochemical properties of HSF and HSP proteins encoded by investigated genes, including instability index, aliphatic index, isoelectric points, and molecular weight, were calculated by the online ProtParam tool (http://web.expasy.org/protparam/, accessed on 5 April 2021) [32].

2.5. Statistical Analysis

Statistical analysis of plants' morphometric data was performed using STATISTICA 10 (StatSoft, Inc., 1984–2011, Tulsa, OK, USA). The data were analyzed using three-factor analysis of variance (ANOVA) with SA application, temperature, and cultivar as categorical predictors, followed by Newman–Keuls multiple comparison test at a confidence level $p < 0.05$. The experiments were repeated three times with four replicas per temperature treatment (10 SNCs per replica) for each cultivar. The qRT-PCR data were analyzed using statistical software SPSS 25 (IBM, Chicago, IL, USA) and tested for homogeneity of variance by Levene's test. In addition, all values were subjected to one-way ANOVA followed by either Tukey's HSD (for equal variances) or Games–Howell (for unequal variances) tests for post-hoc pairwise comparisons. A *p*-value less than 0.05 was accepted as statistically significant for both tests.

3. Results

3.1. Effects of SA and Temperature Treatments on the Survival of Explants

The survival of potato explants was significantly affected by the investigated factors of SA, temperature, and cultivar (Table S2). Significant cultivar : temperature and temperature : SA interactions indicated different effects of temperature treatments on survival rates among potato genotypes and different effects of SA application on potato explant-survival between three temperatures, respectively. All three cultivars showed a high survival rate at 21 °C and 26 °C, which was not influenced by the SA application (Figure 1). Survival of explants was significantly reduced at 29 °C in two cultivars, Agria and Kennebec, and SA application improved surviving rate in Agria. Explant necrosis was rarely observed, while the developmental arrest of explant buds was more frequent, resulting in a reduced number of developed plants at 29 °C temperature. The survival of Désirée explants was not affected by any of the temperature treatments, nor SA application.

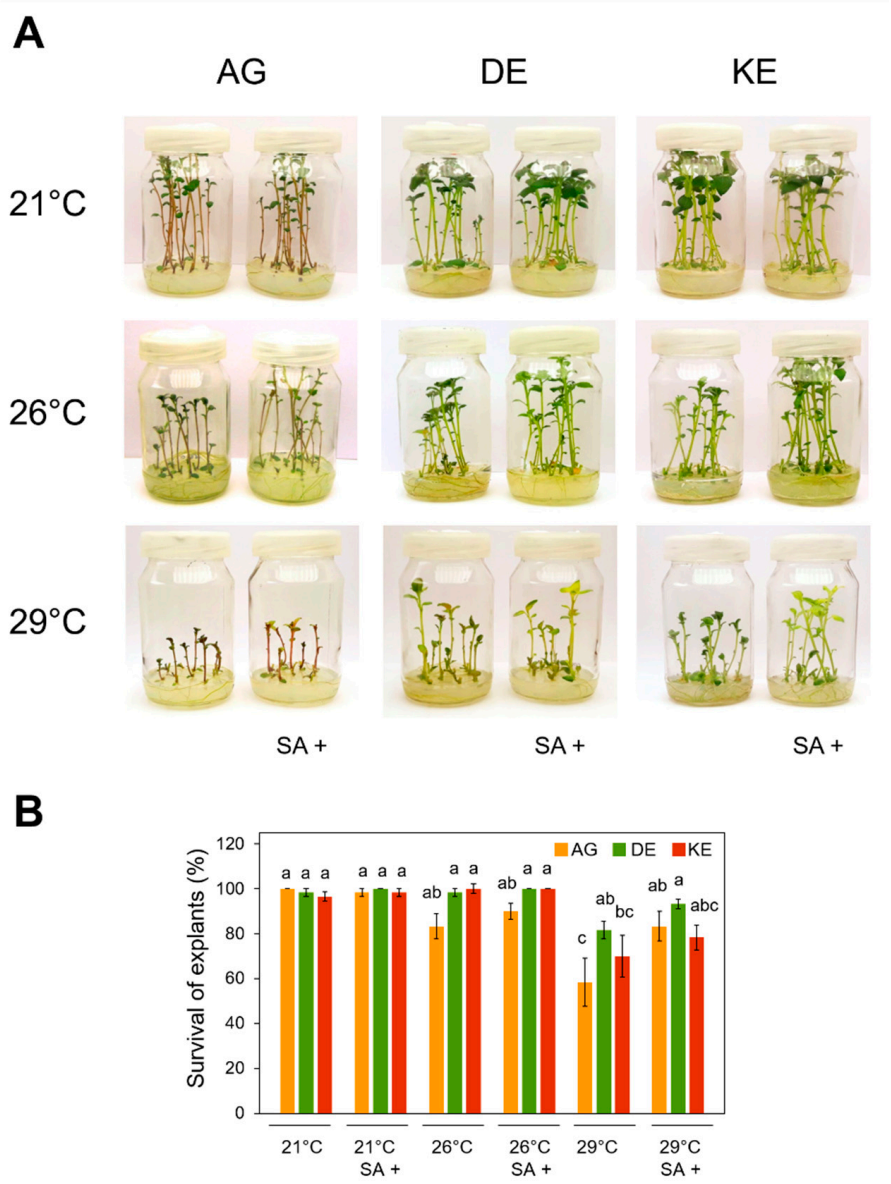

Figure 1. Effects of SA and temperature on the survival of potato explants and plant growth. (**A**) Microplants were grown from SNC explants placed on basal medium (jar on the left) or basal medium supplemented with 1×10^{-5} M SA (jar on the right) at 21 °C, 26 °C, or 29 °C. (**B**) The percentages of surviving explants, defined as SNCs that gave rise to plantlets >10 mm, were determined after three weeks. Error bars indicate standard errors of the means. Data were analyzed using three-factor ANOVA (cultivar, temperature, and SA application as factors), followed by Newman–Keuls multiple comparison test. Means labeled with different letters are significantly different at a confidence level of $p \leq 0.05$. AG—Agria, DE—Désirée, KE—Kennebec, SA+—salicylic acid treatment.

3.2. Effects of SA and Temperature on Growth and Development of Potato Plantlets

After three weeks of microplants' growth on BM or BM supplemented with 1×10^{-5} M SA at 21 °C, 26 °C or 29 °C, the following morphometric parameters were measured: the number of leaves and nodes, shoot length, fresh shoot mass, the number of roots, root length, and fresh root mass (Figure 2). Only fully developed leaves were counted; partly developed leaves of the apical bud were excluded.

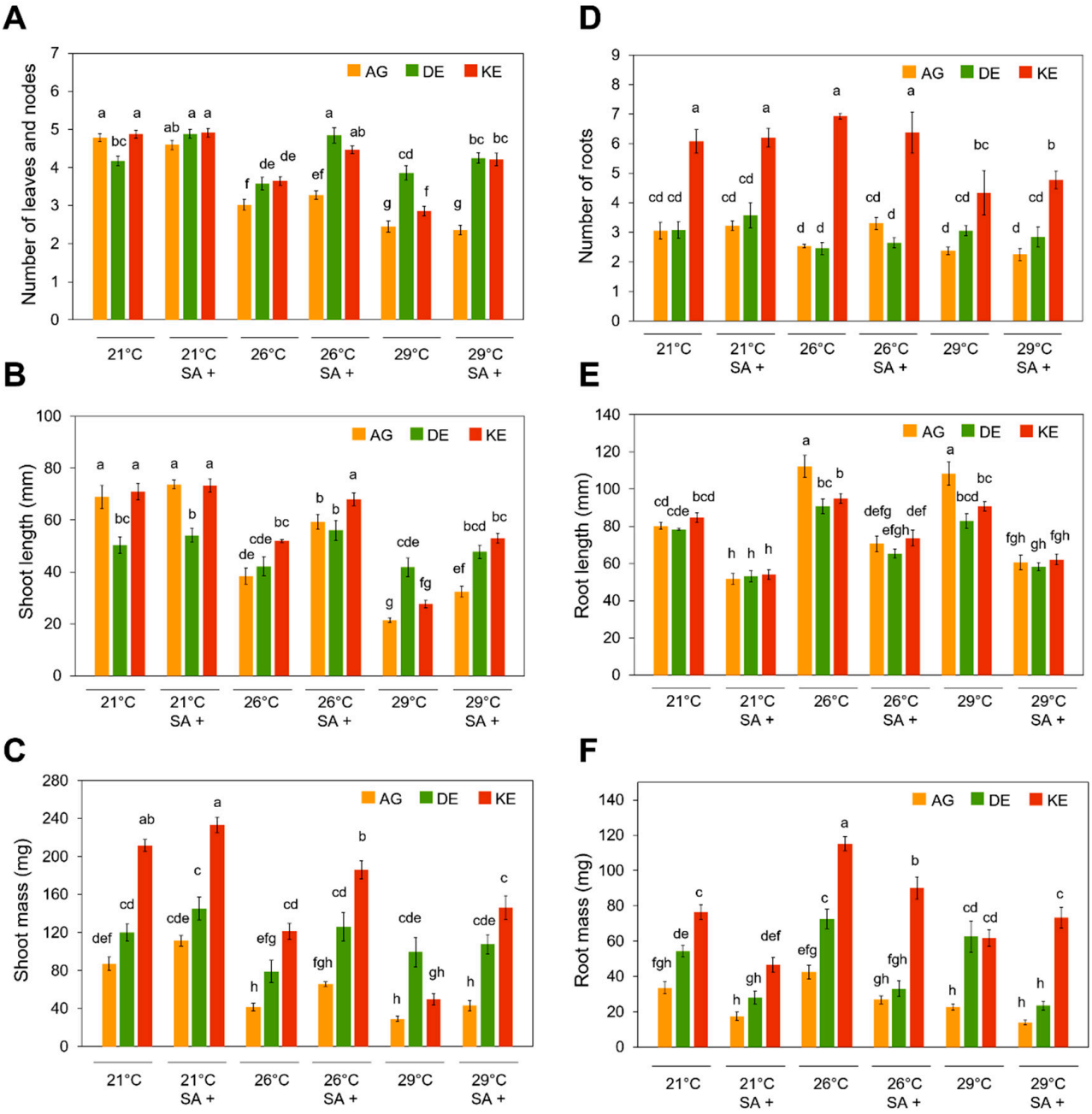

Figure 2. Effects of SA and temperature treatments on growth and development of potato microplants. The number of (**A**) leaves and nodes, (**B**) shoot length, (**C**) shoot fresh mass, (**D**) the number of roots, (**E**) root length, and (**F**) root fresh mass were measured after three weeks of plantlets' growth on basal medium or basal medium supplemented with 1×10^{-5} M SA at 21 °C, 26 °C or 29 °C. Error bars indicate standard errors of the means. Statistical analysis of data was performed using three-factor ANOVA (cultivar, temperature, and SA application as factors), followed by Newman–Keuls multiple comparison test. Means labeled with different letters are significantly different at a confidence level of $p \leq 0.05$. AG—Agria, DE—Désirée, KE—Kennebec, SA+—salicylic acid treatment.

The development of leaves was significantly affected by each investigated factor of SA, temperature, and cultivar (Table S3). Significant three-way interaction of cultivar : temperature : SA (Table S3) indicates that the effect of SA differs between cultivars at some of the temperature treatments. SA did not affect the number of leaves in Agria at all temperature treatments, while it stimulated leaf development in Désirée under 21 °C and 26 °C, and

Kennebec during exposure to 26 °C and 29 °C (Figure 2A). Compared to treatment at 21 °C, exposure to 26 °C significantly reduced the number of leaves in all tested cultivars. Temperature treatment of 29 °C reduced leaf number in Agria and Kennebec.

The growth of shoots, measured as shoot length and shoot mass, was also significantly influenced by each investigated factor, SA, temperature, and cultivar (Tables S4 and S5). Significant three-way interaction of cultivar : temperature : SA was determined, indicating that the effect of SA differs between cultivars at some of the temperature treatments. In all tested cultivars, SA did not affect shoot length at 21 °C, while it induced shoots elongation at 26 °C. Interaction between the elevated temperature of 29 °C and SA application resulted in shoot elongation only in Agria and Kennebec (Figures 1 and 2B). Compared to the shoot length at 21 °C, elevated temperatures (26 °C and 29 °C) caused a significant decrease in shoot length in Agria and Kennebec, while did not affect the length of Désirée's shoots. Regarding shoot mass, SA treatment had an effect only in Kennebec at elevated temperatures (26 °C and 29 °C). Compared to the results obtained at 21 °C, exposure to 29 °C caused a decrease in shoot mass in Agria and Kennebec, while in Kennebec, a significant decline in shoot mass was also observed during exposure to 26 °C (Figure 2C). Interestingly, 29 °C-temperature treatment did not affect shoot mass in Désirée.

SA application did not affect the number of roots in tested potato cultivars (Figure 2D, Table S6). The number of developed roots was significantly affected only by factor cultivar and temperature. Significant cultivar : temperature interaction indicated different effects of temperature treatments on the root number between potato genotypes. Indeed, Agria and Désirée plantlets formed a similar number of roots at all three temperature treatments, while in Kennebec, a temperature of 29 °C significantly reduced the root number.

The elongation and growth of roots, measured as root length and root fresh mass, were significantly influenced by each of the investigated factors of SA, temperature, and cultivar (Tables S7 and S8). SA application caused a decrease in the root length in all tested cultivars at all temperature treatments (Figure 2E). The temperature treatment did not affect root length in Désirée and Kennebec, while elevated temperatures of 26 °C and 29 °C stimulated root elongation in Agria.

Compared to the root mass at 21 °C, treatments with elevated temperatures of 26 °C and 29 °C did not affect the root mass in Agria and Désirée, while in cv. Kennebec, a temperature of 26 °C significantly increased the root mass. Three cultivars responded differently to SA treatment. SA application did not affect the root mass in cv. Agria at all investigated temperature treatments. Conversely, SA caused a decrease in the fresh root mass in cv. Désirée at all temperature treatments, as well as in cv. Kennebec at 21 °C and 26 °C (Figure 2F).

3.3. Potato HSFs and HSPs Selected for Expression Analysis

Three HSF genes, *StHSFA2*, *StHSFA3*, and *StHSFA5*, were selected for analysis due to reported heat responsiveness and high expression levels in shoots and leaves of potato plants during heat stress [33]. In addition, potato HSP genes *StHSP17.6*, *StHSP21*, *StHSP23.9*, *StHSP70-1*, *StHSP70-2*, and *StHSP101* that encode proteins that belong to different HSP classes and operate in different cell compartments (mitochondria, cytosol, chloroplasts) were selected for expression analysis. The information about analyzed *StHSF* and *StHSP* genes and their respective proteins, including chromosomal localization, transcript number and length, protein amino acids numbers, the theoretical molecular weight, physicochemical properties, and intracelular localization is presented in Table 1. The complete nucleotide sequence for *StHSP70-2* was retrieved from the NCBI database (Gene ID 102600309) since the partial nucleotide sequence was presented in Ensembl Plants (Table 1, Figure S1B).

Table 1. Characteristics of the analyzed HSP and HSF genes and proteins of potato (*Solanum tuberosum* L.).

Name	Gene ID	Chromosomal Localization	Transcript ID	Transcript Length (bp)	Protein Length (aa)	pI	Instability Index	Aliphatic Index	MW (kDa)	Protein Localization
StHSP17.6	PGSC0003DMG400030426	Chr. 6: 56,893,292–56,894,077	PGSC0003DMT400078201	786	154	5.83	44.31	65.71	17.6	cytosol
StHSP17.6	PGSC0003DMG400030339	Chr. 6: 56,900,911–56,901,677	PGSC0003DMT400078006	767	154	6.20	50.37	65.06	17.6	cytosol
StHSP17.6	PGSC0003DMG400030340	Chr. 6: 56,905,147–56,905,872	PGSC0003DMT400078007	726	154	7.91	44.85	65.71	17.6	cytosol
StHSP21 *	PGSC0003DMG400003219	Chr. 3: 47,576,728–47,579,687	PGSC0003DMT400008351	2776	233	6.98	39.66	70.21	25.8	chloroplast
StHSP23.9	PGSC0003DMG400004808	Chr. 8: 52,375,001–52,376,265	PGSC0003DMT400012249	1028	211	6.45	57.07	71.61	23.9	mitochondria
StHSP70-1	PGSC0003DMG400024887	Chr. 1: 80,158,423–80,162,913	PGSC0003DMT400064031	2644	706	5.16	27.82	85.21	75.1	chloroplast
StHSP70-2	102600309 (NCBI) PGSC0003DMG400027611	Chr. 11:12,761,196–12,763,127	XM_006361313.2 PGSC0003DMT400070986	2577 1032	696 257	5.26 -	29.14 -	84.31 -	74.8 -	chloroplast
StHSP101	PGSC0003DMG400024644	Chr. 3: 55,148,140–55,152,302	PGSC0003DMT400063352	3139	912	5.82	36.29	96.57	101.1	cytosol
StHSFA2	PGSC0003DMG400008223	Chr. 8: 35,415,712–35,418,551	PGSC0003DMT400021232	2521	254	5.12	64.12	69.84	29.3	cytosol/nucleus
StHSFA3	PGSC0003DMG401002683	Chr. 9: 3,849,902–3,856,182	PGSC0003DMT400021233	2744	226	4.58	64.32	76.77	26.0	cytosol/nucleus
			PGSC0003DMT400021234	2350	353	4.92	56.69	74.22	40.7	
			PGSC0003DMT400021235	1817	340	5.14	56.88	71.62	39.1	
			PGSC0003DMT400006917	1494	497	4.78	54.25	68.07	54.7	
			PGSC0003DMT400006919	3530	380	4.98	52.01	66.97	41.7	
			PGSC0003DMT400006920	3276	357	4.97	56.23	60.39	39.2	
			PGSC0003DMT400006921	2645	480	4.81	55.73	64.60	52.9	
			PGSC0003DMT400006922	3013	480	4.81	55.73	64.60	52.9	
StHSFA5	PGSC0003DMG400004662	Chr. 12:59,294,378–59,298,391	PGSC0003DMT400011871	1700	478	5.48	57.97	70.98	53.4	cytosol/nucleus

Note: * indicates frequently used name based on the molecular weight of mature protein without chloroplast targeting sequence; bp, base pair; aa, amino acid; pI, theoretical isoelectric point; MW, molecular weight; kDa, kilodalton.

3.4. Effects of SA and Temperature on HSPs and HSFs Expression

HSFs and *HSPs* expression was analyzed in fully developed leaves of potato microplants after three weeks of growth at 21 °C, 26 °C or 29 °C and SA treatment.

Elevated temperatures induced expression of the *StHSFA2* gene; depending on the cultivar, a 4.8–8.0-fold increase was detected at 26 °C, and a 3.2–5.4-fold increase at 29 °C (Figure 3A). SA treatment caused slight induction of *StHSFA2* expression only at 29 °C in Kennebec. *StHSFA3* was also up-regulated by moderately elevated temperatures (Figure 3B). Depending on the cultivar, a 2.7–4.6-fold increase was detected at 26 °C, and a 1.8–3.7–fold increase at 29 °C in leaves of microplants grown on BM. SA treatment caused slight induction of *StHSFA3* expression at 21 °C, and more prominent induction at 26 °C. Interestingly, *StHSFA5* expression levels were similar at all temperature and SA treatments, except for a slight increase at 29 °C in cultivar Desiree (Figure 3C).

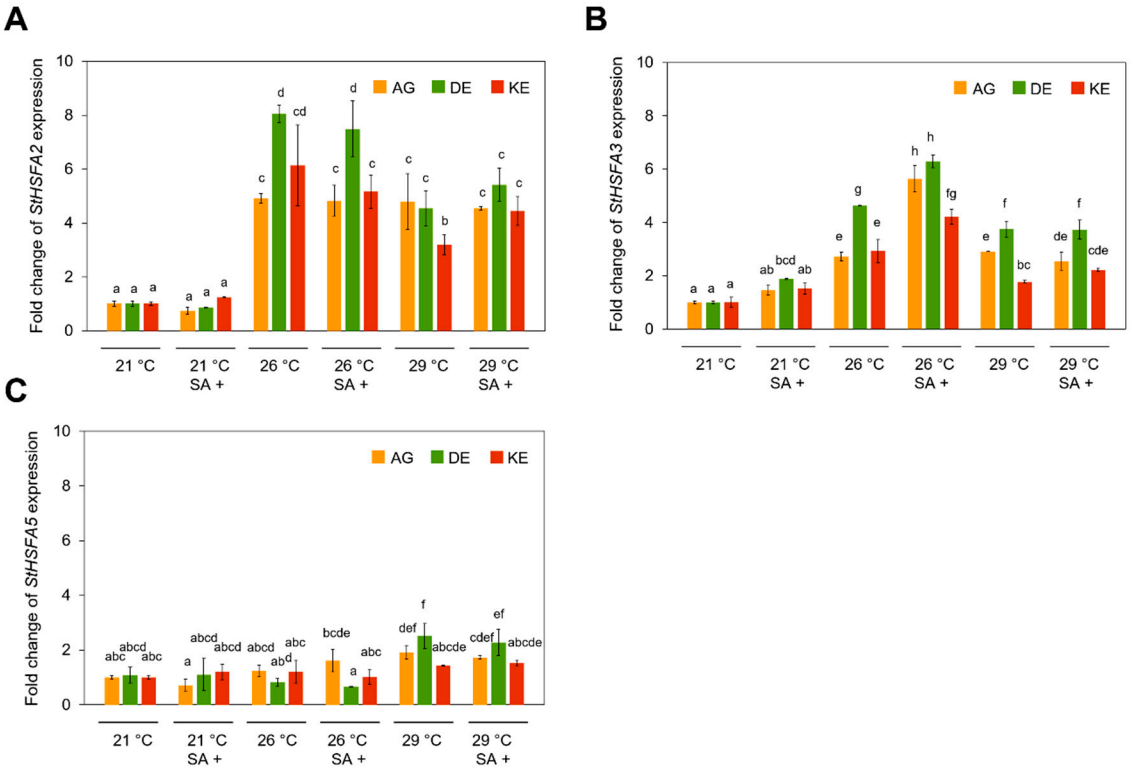

Figure 3. Effects of SA and temperature treatments on HSF expression in leaves of potato microplants. Fold change in gene expression of (**A**) *StHSFA2*, (**B**) *StHSFA3*, and (**C**) *StHSFA5* was calculated by the $2^{-\Delta\Delta Ct}$ method with respect to untreated control (21 °C, without SA). The mRNA levels were normalized with two constitutive genes (*60SL36* and *CYC*). Error bars represent the standard error of the mean fold change calculated from three independent biological replicates. Statistical analysis of data was performed using one-way ANOVA followed by either Tukey's HSD (for equal variances) or Games–Howell (for unequal variances) multiple comparison tests. Means labeled with different letters are significantly different at a confidence level of $p \leq 0.05$. AG—Agria, DE—Désirée, KE—Kennebec, SA+—salicylic acid treatment.

Transcripts of three *StHSP17.6* genes (Table 1) are characterized by highly similar nucleotide sequences (Figure S1A), encoding proteins with almost identical amino acid sequences, and the joint set of primers was used to detect their expression. The level of *HSP17.6* transcripts increased significantly by the elevated temperature of 29 °C in three potato cultivars: 4-fold in Agria, 3.2-fold in Désirée and 2.3-fold in Kennebec (Figure 4A). SA application caused mainly a slight reduction in the transcript abundance at 21 °C and 26 °C. The expression of the *StHSP21* gene was strongly induced by 29 °C temperature treatment;

depending on the cultivar, the increase in transcript level was in the 11.0–28.0-fold range (Figure 4B). A temperature of 26 °C also induced *StHSP21*, while SA caused a reduction in transcript level. Expression of the *sHSP* gene encoding mitochondrial HSP23.9-MTI was significantly induced only by the elevated temperature of 29 °C, and SA enhanced *StHSP23.9* expression in cultivar Kennebec at this temperature treatment (Figure 4C). Depending on the cultivar, a 4.8–8.0-fold increase in transcript level was detected at 29 °C compared to 21 °C.

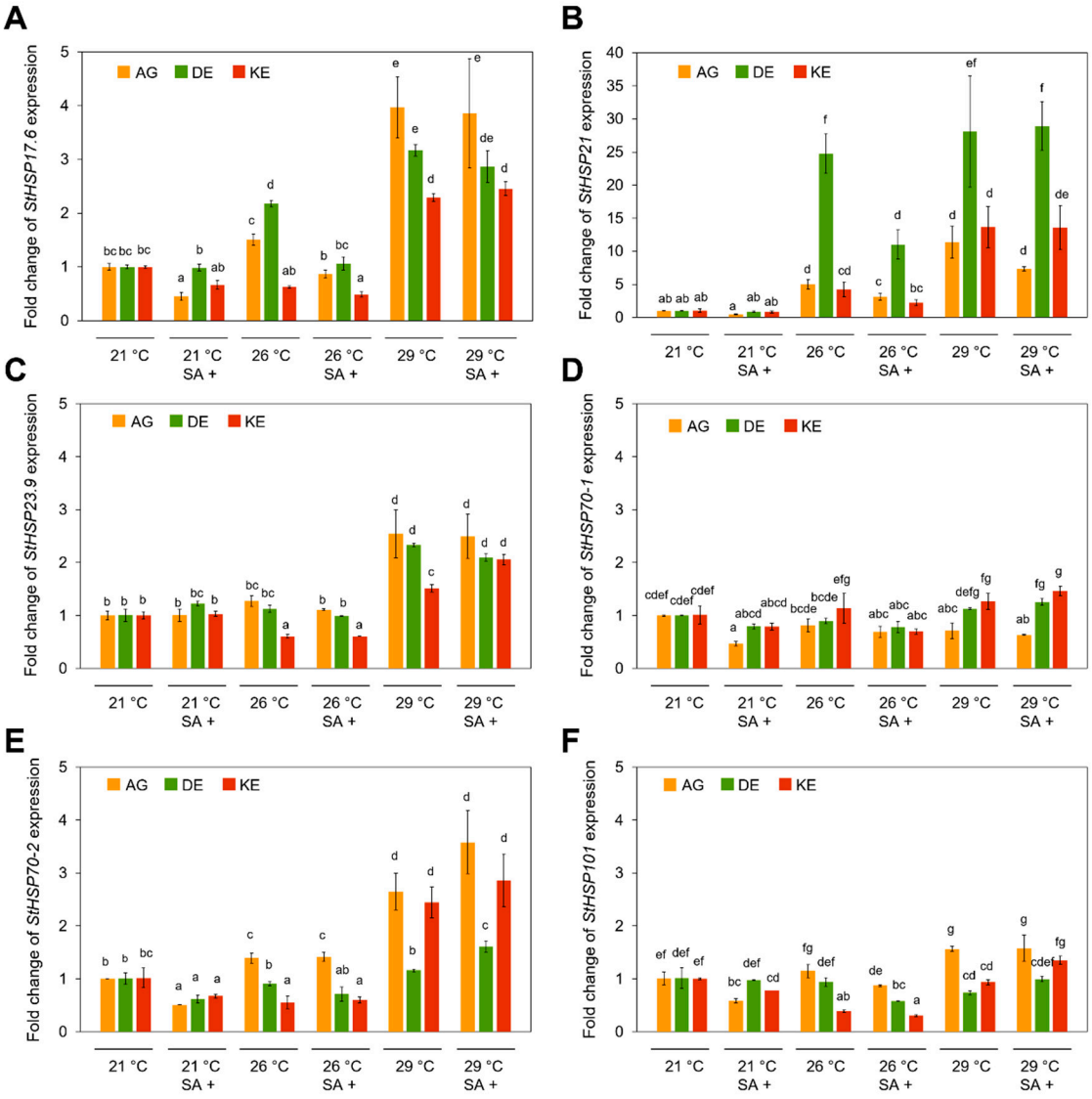

Figure 4. The expression profile of *HSP* genes in leaves of potato microplants. The expression of (**A**) *StHSP17.6*, (**B**) *StHSP21*, (**C**) *StHSP23.9*, (**D**) *StHSP70-1*, (**E**) *StHSP70-2*, and (**F**) *StHSP101* in response to SA and temperature treatments was calculated by the $2^{-\Delta\Delta Ct}$ method as folds relative to the untreated control (21 °C, without SA). The mRNA levels were normalized with two constitutive genes (*60SL36* and *CYC*). Error bars represent the standard error of the mean fold change which were calculated from three independent biological replicates. Statistical analysis of data was performed using one-way ANOVA followed by either Tukey's HSD (for equal variances) or Games–Howell (for unequal variances) multiple comparison tests. Means labeled with different letters are significantly different at a confidence level of $p \leq 0.05$. AG—Agria, DE—Désirée, KE—Kennebec, SA+—salicylic acid treatment.

Expression analysis of two genes encoding chloroplast HSP70 was also conducted. *StHSP70-1* was slightly upregulated at 29 °C in cultivars Désirée and Kennebec (Figure 4D). The temperature of 29 °C caused stronger induction of *StHSP70-2* expression (Figure 4E) in Agria (2.7-fold increase) and Kennebec (2.5-fold increase). *StHSP101* has shown an irregular expression pattern in some investigated genotypes (Figure 4F); in brief, it was downregulated in Kennebec at 26 °C, slightly upregulated at 29 °C in Agria, and SA promoted its expression in Kennebec at 29 °C-temperature treatment.

4. Discussion

In the present study, we investigated effects of SA and temperature treatments (21 °C, 26 °C or 29 °C) on microplant growth and HSR in three unrelated potato genotypes/cultivars: Agria, Désirée, and Kennebec. The growth and development of potato plants are substantially affected by ambient temperature. The literature data suggest that the optimal temperature range for the most developmental processes in potato is 20–25 °C [2,3]. However, some processes, such as shoot growth, stem elongation, leaf primordial development, and leaf appearance, have higher temperature optima (≥28 °C) [2]. Interestingly, after initial findings that the favorable temperatures for plantlets' node formation in vitro are 20 °C or 25 °C [34], the effects of mildly elevated temperatures on growth of potato microplants were not investigated. Some authors used temperatures above 25 °C for a routine subculturing of potato microplants [35–37]. Since the current production of high-quality seed potato is based on virus-free microplant material [38], optimizing the temperature conditions for the growth and development of potato plantlets in vitro is of utmost importance. Treatment with slightly elevated temperature of 26 °C was applied in our study to explore the upper limit of temperature optimum for the growth and development of potato microplants, while 29 °C was chosen as a temperature that might induce mild heat stress. According to several reports, temperatures in the 28–30 °C range can cause heat stress in some potato cultivars/genotypes [5,28].

Long-term mild heat stress (29 °C, three weeks) caused reduction in the number of surviving explants, leaf number, shoot length, and shoot fresh mass in Agria and Kennebec, and did not affect shoot growth in cv. Désirée (Figures 1 and 2A–C). These results indicated that Désirée was the most heat-tolerant cultivar of the three investigated. Long-term exposure to slightly elevated temperature of 26 °C was not severe enough to significantly affect the survival of explants in three investigated cultivars. However, this temperature treatment caused a decline in the leaf number, shoot length, and shoot fresh mass in cvs. Agria and Kennebec microplants during three weeks of cultivation on BM which suggested that temperature of 26 °C is above optimal temperature range for potato shoot growth in vitro. Heat stress (29 °C, three weeks) did not affect the number of formed roots and root fresh mass, except for the reduction in the root number in cv. Kennebec (Figure 2D). Long-term exposure to slightly elevated 26 °C did not affect the root number in three cultivars, but did stimulate root elongation in cv. Agria (Figure 2E) and also caused an increase in the fresh mass of roots in cv. Kennebec (Figure 2F), indicating a difference between optimal temperature range for potato root and shoot growth in vitro.

Previous findings have shown that exogenous SA could alleviate the adverse effects of abiotic stresses on potato. Beneficial effects of SA application on potato growth and tuberization under salt stress have been observed in cultivar Proventa and line "97-980" using an in vitro system [37]. Furthermore, López-Delgado et al. [39] reported that SA induced freezing tolerance in potato cv. Granate plants raised in a greenhouse, while Mostafa et al. [40] outlined the positive effects of SA on the yield of cv. Spunta grown in the field under water deficiency conditions. However, investigations regarding SA effects on potato heat tolerance have so far mainly focused on excessive high-temperature treatments (35–42 °C) important for in vitro thermotherapy of virus-infected plants and related to the survival of explants [27,41]. In our study, SA application promoted survival of cv. Agria explants, stimulated shoot elongation of all three cultivars, and yielded an increase in a leaf number and shoot mass in cv. Kennebec at 29 °C. SA also promoted

shoot elongation and mass increase at 26 °C, suggesting an SA protective role regarding potato shoot growth during prolonged warming. Conversely, exogenous SA did not affect the number of roots in tested cultivars at any temperature treatments but inhibited root elongation in all tested cultivars and temperature treatments and caused a decline in the fresh root mass in cv. Désirée (21 °C, 26 °C and 29 °C), and cv. Kennebec (21 °C and 26 °C). SA concentration of 10^{-5} M used in present work, which was selected by previous testing of SA in concentration range 10^{-4}–10^{-6} on several potato genotypes (unpublished results) and literature data [27], was probably supra-optimal for elongation and thickening of microplant roots in particular potato genotypes/cultivars. Pasternak et al. [42] reported that low concentrations of exogenous SA (<5 × 10^{-5} M) enhanced adventitious roots and altered architecture of the apical root meristem in *A. thaliana*, whereas a high concentration of SA (>5 × 10^{-5} M) inhibited root growth. According to the authors, auxin accumulation or depletion in the root tip followed low- or high-concentration SA treatments, respectively, and exogenous SA probably affected root growth by altering auxin biosynthesis and transport. Several groups of authors investigated the mechanisms underlying SA-auxin crosstalk in the regulation of root growth [43,44]. Tan et al. [43] reported that *A. thaliana* seedlings grown on 2–4 × 10^{-5} M SA exhibited shorter, partially agravitropic primary roots, as well as fewer lateral roots, and identified the PIN2 auxin transporter as a SA target. This auxin transporter is hyperphosphorylated in response to SA, leading to inhibition of auxin transport and auxin-mediated root development, including root growth, gravitropic response, and lateral root organogenesis. Armengot et al. [44] showed that exogenous SA treatment (2.5 × 10^{-4} M) of Arabidopsis wild-type seedlings and constitutive high SA levels in Arabidopsis mutants caused a decrease in root length and the number of lateral roots and proposed a model in which the protein kinase CK2 acts as a link between SA homeostasis and transcriptional regulation of auxin-efflux transporters. The SA concentrations used in the Arabidopsis studies mentioned above were in the same range or higher than the one used in our study, and similar mechanisms that include negative regulation of auxin transport may be responsible for the detected inhibition of root growth in three potato cultivars.

In the present study, effects of SA and temperature treatments on potato HSR were investigated by following the expression of three HSFs, StHSFA2, StHSFA3, and StHSFA5 in potato cultivars Agria, Désirée, and Kennebec. Twenty-seven members of the HSF family were identified in potato by bioinformatics analysis; the expression pattern analysis using the potato RNAseq data revealed that *StHSF* genes have distinct spatial expression patterns, while the promoter analysis indicated that all *StHSF*s should be responsive to various abiotic stresses [33]. According to qRT-PCR analysis conducted by Tang et al. [33], *StHSFA2* (*StHSF004*), *StHSFA3* (*StHSF005*), and *StHSFA5* (*StHSF009*) transcripts were predominant in leaves of potato plants after 2 h, 6 h, and 24 h exposure to high temperature of 35 °C, with up to 100-fold increase compared to expression level before stress treatment. These findings suggested that, compared to other HSFs, transcription factors encoded by *StHSFA2*, *StHSFA3*, and *StHSFA5* play a major role in potato response to heat stress. However, our study has shown that only *StHSFA2* and *StHSFA3* were up-regulated after three weeks of exposure to 29 °C, while *StHSFA5* expression levels did not change. Besides, the increase in the transcript level was in the 3.2–5.4-fold range for *StHSFA2* and 1.8–3.7-fold range for *StHSFA3*. We speculate that a lower increase in transcript level of *StHSFA2* and *StHSFA3* and lack of *StHSFA5* response to heat stress is probably due to the moderately elevated temperature of 29 °C used in our study and duration of stress treatment. Interestingly, a similar expression pattern was observed at 26 °C compared to control (21 °C). SA only slightly promoted expression of *StHSF*s in some of potato genotypes at elevated temperatures.

Besides HSFs, our study encompassed investigation of effects of SA and temperature on expression of genes encoding proteins of HSP70, HSP100, and sHSP classes. Among sHSPs, expression of HSP17.6-CI, HSP21-CP, and HSP23.9-MTI was followed. The sHSPs are molecular chaperones which play important roles in plant heat tolerance by binding heat-denatured proteins in an ATP-independent manner during heat stress, preventing

their aggregation and maintaining them in a state competent for later ATP-dependent refolding by other chaperones [45]. Genes encoding HSP17.6-CI, HSP21-CP, and HSP23.9-MTI were induced by 29 °C temperature treatment with the highest, 28-fold increase in cv. Desiree *StHSP21* transcripts. Interestingly, temperature of 26 °C also induced expression of *StHSP17.6* and *StHSP21*. In both cases, Désirée was the genotype that showed the highest level of induction, which is in concordance with observed higher level of heat-tolerance regarding plantlets growth. SA-promoted enhancement was detected only for *StHSP23.9* in Kennebec at 29 °C, while application of this compound caused decline in the expression of *StHSP17.6* and *StHSP21* at 26 °C.

Expression of genes encoding two chloroplast HSP70s, HSP70-1, and HSP70-2, was also investigated. HSP70s are a family of conserved and ubiquitously distributed heat-shock proteins. Members of the family play important roles in protein folding and transport through cellular membranes under non-stress conditions and/or promote refolding of denatured proteins under stress conditions [46]. They are present in different cellular compartments, including cytosol, endoplasmic reticulum, mitochondria, and chloroplasts. Expression analysis of two chloroplast *StHSP70* genes revealed a slight increase in *StHSP70-1* transcript abundance and stronger induction in *StHSP70-2* expression (2.5–2.7-fold increase) after long-term heat stress in some of the investigated potato genotypes. Chloroplasts are one of the major targets of heat damage in plant cells. Considering the thermolability of several important proteins of photosynthetic apparatus [6], it is not surprising that high expression levels of both chloroplast HSP70-2 and HSP21 chaperones were maintained during prolonged heat stress. The sHSP cooperate with HSP70 in the reparation of heat-damaged proteins. The sHSPs chaperone role, including HSP21, comprises binding of heat-denatured proteins to prevent their aggregation during heat stress, but denatured proteins are refolded and reactivated by HSP70 [8]. A strong upregulation of *StHSP70-2* (PGSC0003DMG400027611) and *StHSP21* (PGSC0003DMG400003219) gene expression was also reported for cv. Russet Burbank pot-grown plants after 3-day exposure to 35 °C day/28 °C night temperature regime [47].

The expression at transcript level of potato HSP101, a member of the HSP100 class, was also investigated. HSP100 are chaperones involved in the disaggregation of insoluble protein aggregates and/or degradation of irreversibly damaged polypeptides [10]. HSP101/ClpB (DMT400063352) is a cytosolic, constitutively expressed protein in potato, accumulation of which was confirmed in leaves of potato plants under short-term heat stress [48]. Our results, however, indicate minor upregulation of *StHSP101* transcripts (1.6-fold increase) during prolonged mild heat stress in microplants of just one investigated genotype, Agria.

SA is a phytohormone and signaling molecule whose significance in promoting heat tolerance was confirmed in a large number of plant species, mainly after short-term exposure to severely high temperatures (10–15 °C above optimal growth temperature) [23]. The present study revealed that, with few exceptions, SA (1×10^{-5} M) does not play a significant role in regulating *StHSF*s and *StHSP*s expression, and therefore HSR, during prolonged exposure of potato microplants to mildly elevated temperatures (26 °C and 29 °C). Due to observed protective SA effects regarding potato shoot growth, we speculate that SA acts through mechanisms other than HSR to preserve shoot morphogenesis during prolonged warming and mild heat stress.

5. Conclusions

In the present study, we reported the effects of SA and prolonged exposure to elevated temperature on microplant growth and HSR in three unrelated potato genotypes/cultivars, Agria, Désirée, and Kennebec. A slightly elevated temperature of 26 °C was found to be above the optimal temperature range for potato shoot growth, while stimulated root elongation and mass increase in some investigated genotypes, indicating a difference between favorable temperatures for potato root and shoot growth in vitro. In Désirée, long-term mild heat stress did not significantly affect the number of surviving explants or

shoot and root morphometric parameters, implying that it can be considered a relatively heat-tolerant potato cultivar.

Exogenous SA showed a protective effect regarding potato shoot growth during prolonged warming at 26 °C, but this effect was genotype-dependent during mild heat stress. Conversely, SA did not affect the number of roots and inhibited root elongation in all tested cultivars and temperature treatments, indicating the difference between shoot and root response to applied SA concentration.

Although HSR is mainly considered rapid and short-lived, the results of our study revealed that elevated levels of two *HSF* transcripts, *StHSFA2* and *StHSFA3*, and most investigated *HSP*s are present after three weeks of mild heat stress. Furthermore, increased levels of *StHSFA2*, *StHSFA3*, and *StHSP21* transcripts were also detected at 26 °C, implying extreme heat susceptibility in potato and significance of HSR even during prolonged warming. Effects of exogenous SA on HSFs and HSPs expression were minor and variable.

Our results provide the basis for further research of potato plant response to prolonged warming and mild heat stress, as well as SA, and can be important for a better understanding of potato HSR.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae8050372/s1. Figure S1: the alignments of *StHSP17.6* and *StHSP70-2* nucleotide sequences; Table S1: primers used for amplification of *HSP*, *HSF*, *60SL36*, and *CYC*; Tables S2–S8: statistical analysis of surviving explants and plant morphometric parameters.

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