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RESEARCH ARTICLE

Expression of the aldehyde oxidase 3, ent-copalyl diphosphate synthase, and *VIVIPAROUS 1* genes in wheat cultivars differing in their susceptibility to pre-harvest sprouting

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Abstract

The quality of wheat grains is often negatively affected by pre-harvest sprouting (PHS), a complex trait with a poorly understood genetic background. In this study two wheat cultivars differing in their susceptibility to PHS were used to investigate expression of three genes: *AAO3*, *CPS3* and *VP1*. *AAO3* is coding for aldehyde oxidase 3, an enzyme involved in the synthesis of abscisic acid. *CPS3* codes for ent-copalyl diphosphate synthase which belongs to the pathway of gibberellic acid synthesis. The product of *VP1 (VIVIPAROUS 1*) is a transcription factor which controls expression of the former two genes. The study was carried out using both developing and sprouting-induced grains. In Piko, a wheat cultivar susceptible to PHS, accumulation of the *AAO3* transcript was significantly decreased, during the last stages of grain development, in comparison to Sława, a cultivar tolerant to PHS. In case of the *CPS3* and *VP1* transcripts, the differences between cultivars were especially evident from 17th to 31st day after pollination. In turn, after induction of sprouting within spikes, accumulation of the *AAO3* and *VP1* mRNA in the Sława grains was lower in comparison to that observed in the Piko grains. Moreover, accumulation of the *CPS3* transcript was significantly higher for Piko than for Sława, both in sprouting and non-sprouting grains. According to our knowledge this report provides the first description of the *AAO3* and *CPS3* expression in the context of PHS, and in the future it would be valuable to correlate this information with the data on the accumulation of ABA and GA_3.

Additional key words: ABA; GA; germination; grain development; *Triticum aestivum* L.; quantitative PCR.

Abbreviations used: *AAO3* (gene coding for aldehyde oxidase 3); ABA (abscisic acid); *CPS3* (gene coding for ent-copalyl diphosphate synthase); DAP (days after pollination); GA (gibberellic acid); GI (germination index); PCR (polymerase chain reaction); PHS (pre-harvest sprouting); qPCR (quantitative PCR); RQ (relative quantity); *VP1* (gene coding for transcription factor)

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Introduction

Pre-harvest sprouting (PHS) – germination of the cereal grains in ears before harvest, occurs usually due to rainfalls and high humidity. During this process, an increase of the α-amylase activity in the endosperm, results in degradation of starch to reduced sugars. Moreover, sprouted grains have significant changes in the protein content. In sprouted samples Simsek *et al*. (2014) detected higher levels of free asparagine, which is a precursor for acrylamide formation in baked products. PHS-associated deterioration of grain quality causes great economic losses both to farmers and food processing industry. The major factor that inhibits PHS even under favorable conditions is grain dormancy. Grain dormancy depends on temperature (Walker-Simmons & Sesing, 1990) and is controlled by many developmental processes as well as relative amounts of abscisic acid (ABA) and gibberellic acid (GA) (Cadman *et al.,* 2006). Studies have shown that the *de novo* synthesis of ABA in the embryo or

endosperm and not the maternal ABA is required to induce dormancy (Karssen *et al.,* 1983; Nambara & Marion-Poll, 2003). Prior to an increase in the embryo GA content or sensitivity to the hormone, a decline in the ABA content is usually required (Jacobsen *et al.,* 2002; Ali-Rachedi *et al.,* 2004). It appears that the ABA:GA ratio, and not the absolute hormone contents, is critical for grain dormancy and germination. In some species, including wheat, two peaks of ABA concentration occur during grain development (King, 1976; Hsu, 1979; Karssen *et al.,* 1983; Finkelstein *et al.,* 1985; Suzuki *et al.,* 2000; Finkelstein & Rock, 2002). However, Walker-Simmons (1987) reported that in wheat, the embryonic ABA peaked near 40 days after pollination (DAP). Although the process of ABA biosynthesis in seeds is well known, there is little information about the expression of genes involved in this pathway in cereals. The final step of ABA biosynthesis is oxidation of abscisic aldehyde to ABA by aldehyde oxidase, the enzyme encoded by the *AAO3* gene (Seo *et al.,* 2000). The experiments performed by González-Guzmán *et al.* (2004) and Seo *et al*. (2004) showed that among four aldehyde oxidase genes in *Arabidopsis, AAO3* played a major role in ABA biosynthesis in seeds. The biosynthetic pathway of gibberellins also has been thoroughly studied in *Arabidopsis*. Gibberellins are involved in many processes, including seed development and germination. In wheat, some genes involved in the synthesis of gibberellins (GAs) have been identified. The ent-copalyl diphosphate synthase (*CPS*) gene product is involved in the first step of this pathway – the conversion of geranylgeranyl pyrophosphate (GGPP) to copalyl pyrophosphate (CPP). For the wheat gene three cDNAs were indentified: *Ta*CPS1, *Ta*CPS2 and *Ta*CPS3, but only the last one encoded enzyme responsible for gibberelin biosynthesis (Toyomasu *et al.,* 2009). In inactivation of ABA-regulated genes and repression of GA-activated genes, such as α-amylase, the *VIVIPAROUS 1* (*VP1*) gene has been described. The role of *VP1* in dormancy of cereals has been analyzed in many studies. This gene encodes a transcription factor essential for grain maturation and embryo dormancy. In the *viviparous 1* maize mutant, the inactivation of the *VP1* locus during the late phase of embryogenesis results in embryo disruption and germination without any other abnormalities (McCarty *et al.,* 1991; McCarty, 1995). The *VP1* gene may also be important for dormancy maintenance in wheat; in dormant cultivars, the expression of *VP1* is much stronger than in non-dormant ones (Nakamura & Toyama, 2001). In turn, McKibbin *et al*. (2002) and Wilkinson *et al*. (2005) reported that most of the *VP1* transcripts were incorrectly spliced and consequently they did not encode the full-length protein. These alternatively spliced transcripts of the *VP1* gene were identified in PHS-sensitive genotypes of wheat. The *ABI3/VP1* gene was also alternatively spliced in tomato, particularly in seeds at different developmental stages and in response to phytohormonal and abiotic stresses (Gao *et al*., 2013). Also in *Arabidopsis* alternative splicing of the the *ABI3/VP1* gene was developmentally regulated (Sugliani *et al*., 2010).

The aim of this study was to compare two wheat (*Triticum aestivum* L.) cultivars, differing in their susceptibility to PHS, on the basis of expression of two genes involved in ABA and GA biosynthesis (*AAO3* and *CPS3*, respectively) and the *VP1* gene during grain development and sprouting.

Material and methods

Plant material

Two winter wheat cultivars, Piko and Sława, were used in our study. Both cultivars were registered in The Polish National List, Piko in 2002, Sława in 2001. Piko was derived from the cross (Semidwarf × Kraka) × (Kranich × Caribo) (Nordsaat Saatzucht GmbH, Germany), whilst Sława was obtained from the cross Lanca × Juwell 15 (Poznańska Hodowla Roślin, PHR Sp.z o.o, Poland). Both cultivars develop red grains which, when reacting with phenol, change color to brown. Both cultivars were used for baking.

These cultivars were chosen on the basis of our previous (2005-2007) field and laboratory experiments in which their pre-harvest sprouting capabilities were evaluated. According to our data, Piko grains exhibited higher susceptibility to PHS than Sława grains. For our purposes both cultivars were cultivated at the Experimental Station of the Department of Plant Breeding and Seed Science, located in Prusy near Krakow (N 50°07'03" and E 20°05'13"), and the trials were prepared each year by sowing the seeds collected from isolated ears. For this study we used the plant material from the 2009 field experiment. The average temperatures in May, June, and July were 13.1, 17.6 and 20.8°C, respectively, the rainfall was 302.5, 135.1 and 105.2 mm, respectively (the average weather conditions in the years 2005-2015, recorded at the experimental station in May, June and July were as follows: temperatures - 13.9, 17.5 and 19.9°C, respectively; rainfalls - 92.5, 95.1 and 96.7 mm, respectively). Forty-five grams of seeds of each cultivar were sown manually on three plots. Each plot consisted of 10 rows 1.5 m long - the rows were 25

cm apart. The plants were evaluated in respect of the date when the pollen was released from the florets in the middle part of the spike. We harvested ten spikes from the middle part of each plot, 10, 14, 17, 24, 31, 38 and 45 days after pollination and the grains from middle parts of the spikes were used for preparing the biological samples for germination tests and expression analyses. The grains from each plot were used as one biological replication. The germination tests (see below) were prepared immediately after harvesting the grains, while for RNA isolation the grains were stored at -80°C. At the last developmental stage (45 DAP, grain humidity was 113 g/kg and 107 g/kg for Piko and Sława, respectively) also spikes without any signs of external sprouting were collected and used for the induction of sprouting (see below). The grains harvested at that time were also stored (for 5 weeks) at room temperature before using them in an ABA responsiveness assay. The picked spikes and grains from each plot were used as individual biological replicates.

Germination experiments

Grains harvested at different developmental stages (from 24 to 45 DAP), as well as the physiologically mature grains stored for 5 weeks at room temperature after harvest, were germinated. Grains were placed in 14-cm Petri dishes with three layers of filter paper and 10 mL of distilled water. Each dish had 25 grains of each cultivar. Incubation was performed at 20°C in the darkness. Grains after 5 weeks of storage were also incubated in 10, 50, 100 and 150 µmol/L of ABA (Sigma-Aldrich) solutions, at 20 and 25°C. Three replications for each cultivar were performed. Each replication consisted of the grains collected from one field plot. Germinating grains were counted daily and removed. Germination was considered when the grain coat over the embryo was at least broken or ruptured, resulting in partial exposure of the embryo. Germination capability was assessed after a 7-d induction using the germination index (GI) value (Walker-Simmons, 1987), which is calculated as follows: $GI = [(7\times n1) + (6\times n2) + (5\times n3) + (4\times n4) + (3\times n5) + (2\times n6) + (1\times n7)]/(\text{total days} \times \text{total number of grains})$, where 7, 6,....1 are weights given to the grains germinated on the first ($n1$), second ($n2$), and subsequent days until the seventh day ($n7$), respectively. The maximum value of GI is 1.0.

Evaluation of sprouting sensitivity

Spikes collected at grain physiological maturity (45 DAP) without visible sprouting damage were placed in a moisture chamber at 20°C ± 1 for maximum 6 days. Three replications for each cultivar, with each sample consisting of 10 spikes, were performed. Each replication consisted of the spikes collected from one field plot. The spikes were placed in a vertical position on a special device (a frame with a stretched 0.5 cm mesh net). The spacing between the spikes was 2 cm in a row and 3 cm between rows. High humidity was maintained during the experiment: the spikes were sprayed using a fine nozzle (1 mm), with 2 L of water four times a day at: 7.00-8.00 a.m., 11.00 a.m.-12.00 noon, 3.00-4.00 p.m., and 6.00-7.00 p.m. After the last spraying, the spikes were covered with a plastic foil for overnight. Estimation of grain sprouting was conducted on the third day of incubation for five randomly chosen spikes from each replication when first germination signs were visible. The percentage of sprouting grains was determined. Among the sprouting grains, three stages of germination were distinguished: 1 - broken or ruptured grain coat over the embryo resulting in a partial embryo exposure, 2 - grains with the emerging radicle up to 2 mm in length, and 3 - grains with the coleoptile and radicle longer than 2 mm. The remaining spikes were left in the same conditions until the sixth day. For molecular analysis, sprouting grains (stage 1) collected on the third day and non-sprouting ones collected on the sixth day of incubation were used. For RNA isolation, each sample was performed in three biological replicates and stored at -80°C.

RNA isolation

For total RNA isolation, 1 g of grain samples (corresponding to 12-20 grains depending to the developmental stage of the grains) were homogenized in liquid nitrogen using a mortar and pestle, and 100 mg of the resulting powder was transferred to 2 mL tubes. The RNA pellet, obtained according to the protocol described by Singh *et al.* (2003) was resuspended in 100 µL of sterile Milli-Q water and incubated for 4 h on ice. After incubation, the mixture was centrifuged (15000 rpm, 10 min, 4°C) (Biofuge Stratos, Kendro Laboratory Products, Hanau, Germany). The supernatant was transferred into new Eppendorf tubes, and RNA precipitation using LiCl was performed as described by Simlat *et al.* (2013). The obtained RNA pellet was resuspended in 20 µL of sterile Milli-Q water and stored at -80°C until further use. The quantity of RNA was determined by measuring absorbance at 260 nm and by standard agarose electrophoresis.

Quantitative PCR (qPCR)

To synthesize the first strand of cDNA, we used 1 µg of RNA. Prior to reverse transcription, RNA was treated

Table 1. Susceptibility to pre-harvest sprouting of wheat cultivars Piko and Sława, harvested at 45 DAP

	Piko			Sława		
Total sprouting grains [%][a]	50.40 ±6.84			14.87 ±8.15		
$LSD_{0.05\ (0.01)}$	17.058 (28.287)					
Stages of sprouting[b]	**1**	**2**	**3**	**1**	**2**	**3**
Contribution of sprouting grains [%][a]	18.15±6.03	28.38±5.77	53.47±7.94	33.33±6.03	60.00±8.89	6.67±1.15
$LSD_{0.05\ (0.01)}$	11.585 (16.348)					

[a] The presented results are the mean values from independent replicates (n = 3) ± SE. [b] Stages of sprouting are defined as follows: 1 – broken or ruptured grain coat over embryo resulting in partial embryo exposure, 2 – grains with emerging radicle maximally 2 mm long, 3 – grains with coleoptile and radicle longer than 2 mm

with DNase I without RNase activation (Fermentas Int Inc) (2 U DNase I / 1 µg RNA) and incubated for 30 min at 37°C. Reverse transcription was performed using the First Strand cDNA Synthesis Kit (Fermentas Int Inc) with random hexamer primers in 20 µL reactions according to the manufacturer's instructions. Quantitative PCR was performed using the 7500 Fast Real Time PCR System (Applied Biosystems, USA) with SYBR Green to monitor double-stranded DNA synthesis. Three technical replicates of each cDNA sample were performed for qPCR analysis. For amplification, cDNA was diluted 10-fold, and 1 µL was used in 20 µL qPCR reaction mixture with 2X Fast SYBR Master Mix (Applied Biosystems, USA) and 0.5 µmol/L of each primer. The following thermal profile was used for all reactions: 95°C for 20 s, 40 cycles of 95°C for 3 s, and 60°C for 1 min. Melting curve analysis was performed to evaluate the presence of non-specific PCR products and primer dimers. Relative gene expression was calculated in different samples using the ΔCt method. *Actin* gene was used as an internal control. The relative mRNA level was normalized for each sample to that of 10 DAP for the respective cultivar. For all genes, standard curves were generated using a dilution series of cDNA. The obtained data were analyzed using 7500 Software v2.0 (Applied Biosystems, USA). The primers used for detection of transcript accumulation were designed on the basis of the sequence records: AK332105 (*AAO3*), AB439590 (*CPS3*), AB047554 (*VP1*), and AY663392 (*actin*). The *VP1* cDNA was amplified with primers anchored in the region coding for the B3-domain and therefore both the correctly and alternatively spliced mRNAs were targeted in the assay. The primers were designed and tested for possibility of homo- and hetero-dimers formation using PrimerQuest and Oligo Analyzer programs available online (http://eu.idtdna.com/Home/Home.aspx). The gene specific primers were as follows: *AAO3*-fwd: GGC GTT GTG ATT GCT GAA AC, *AAO3*-rev: TAG CTG TCA TGT TGG ATG GC, *CPS3*-fwd: GCG GTC TCC ATG AAG GAA AT, *CPS3*-rev: TCC ACC AAC AGG TAG TGT CT, *VP1*-fwd**:** CAC TGG TGA CTT TGT TCG GT**,** *VP1*-rev: GTG CTT GGC TAG ATC CTG TT, *actin*-fwd: AGG TGC CCT GAG GTC CTC TT, and *actin*-rev: GTA GGT CGT CTC GTG GAT TCC A.

Statistical analysis

Differences among mean values of germination index, percentage of sprouted grains, and relative quantity of gene expression were analyzed using a factorial-design analysis of variance (*ANOVA*). In these analyses, the least significant difference (*LSD*) was calculated using the Student's t-test at 0.05 and 0.01 probability. The statistical analyses of data were performed using Statistica, v 10 (www.statsoft.com).

Results

Germination during grain development

The grains of both cultivars harvested at 24, 31, 38, and 45 DAP were induced to germinate in Petri dishes. Each day, the grains with visible signs of external germination were removed. For both cultivars, germination capabilities increased progressively during grain development. For Piko, the values of the germination index ranged from 0.05 at 24 DAP to 0.82 at 45 DAP. For Sława, the GI values ranged from 0.04 to 0.6, but they did not differ significantly at 24 and 31 DAP. In all analyzed developmental stages, except 24 DAP, the germination index of Sława was significantly lower than that of Piko (Fig. 1).

Sensitivity of mature grains to sprouting in spikes

With respect to sprouting in spikes harvested at 45 DAP, both cultivars showed different tendencies. The grains of Piko sprouted faster and better than those of Sława. The mean percentage of sprouted grains observed for Piko reached 50.4%, whereas in Sława it was 14.9% (Table 1). The cultivars also differed in the progress of grain sprouting. In Piko, more than 50% of

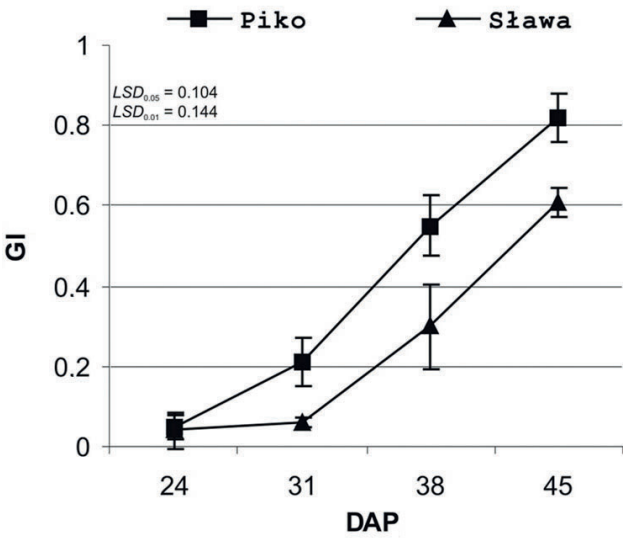

Figure 1. Germination index (GI) for wheat cultivars Piko and Sława. Grains were harvested at different days after pollination (DAP), and incubated for 7 days in water at 20ºC. Each value represents the mean of three independent replicates. Bars represent standard deviation

the germinated grains were those with well-developed radicles and coleoptiles (stage 3), whilst in Sława the most numerous were grains with the radicles no longer than 2 mm (stage 2).

Sensitivity of mature grains to ABA

Table 2 shows that the cultivars differed in the GI, also when germination was tested in the presence of ABA (the experiment was performed for the physiologically mature grains stored at room temperature for 5 weeks after harvest). Exogenous ABA inhibited germination of grains in both cultivars. However, the inhibitory effect of ABA was more pronounced for PHS-tolerant Sława than for PHS-susceptible Piko. A greater inhibitory effect was observed when grains were germinated at 25°C, and only for Sława the concentration of exogenous ABA had significant effect on grain germination.

Transcript accumulation during grain development

Figure 2 shows that relative quantitation (RQ) of the *AAO3* mRNA in Piko and Sława grains harvested at successive stages of development clearly indicates greater accumulation of this transcript during last three stages, *i.e.* from 31 to 45 DAP. In both cultivars, the greatest accumulation of this transcript was observed at 31 DAP. Afterward, the *AAO3* mRNA level decreased, but in mature grains (45 DAP) of Sława, this transcript accumulation was significantly higher than that of Piko. An increase in accumulation was also detected in Piko at 17 DAP and in Sława at 20 DAP, but observed differences were not significant. In both cultivars, the lowest accumulation of mRNA was observed at the first analyzed developmental stage (10 DAP) (Fig. 2a).

The *CPS3* transcript accumulation showed two distinct peaks during grain development in both cultivars. In Piko, the highest levels of *CPS3* mRNA were observed at 14 and 20 DAP. However, in Sława the first peak was observed at 17 and the second at 31 DAP. Relatively low expression values (relative to 10 DAP) were found for Piko grains at 17, 31, 38, and 45 DAP and for Sława grains at 20, 24, 38, and 45 DAP. However, for both cultivars at each developmental stage the level of expression was higher than that at 10 DAP. It is also worth mentioning that in Piko, the expression level was higher at 20 DAP than at 17 DAP, while in Sława at those stages an opposite relationship was observed. Moreover, at 24 and 31 days after pollination, the tested cultivars showed differences in the accumulation of the examined transcript. In Piko, at 24 and 31 DAP, the relative accumulation decreased from 7.8 to 2.8, while in Sława it increased from 3.0 to 6.5. At the last two analyzed stages of grain development, transcript accumulation showed similar values in both cultivars (Fig. 2b).

Table 2. ABA responsiveness expressed as the germination index[a] (GI) of wheat grains five weeks after harvest at physiological maturity (45 DAP). Grains were incubated in water and in different ABA concentration for 7 days, at 20 and 25°C. Water was used as a control

Treatment	Piko	Sława	Piko	Sława
	20°C		25°C	
Control	0.81±0.01	0.79±0.03	0.82±0.03	0.75±0.03
ABA, 10 µmol/L	0.79±0.02	0.78±0.01	0.81±0.01	0.64±0.05
ABA, 50 µmol/L	0.81±0.01	0.72±0.02	0.72±0.02	0.61±0.05
ABA, 100 µmol/L	0.75±0.02	0.72±0.00	0.77±0.03	0.59±0.02
ABA, 150 µmol/L	0.72±0.05	0.71±0.05	0.73±0.04	0.59±0.01
$LSD_{0.05\ (0.01)}$	0.047 (0.064)			

[a] The presented results are the mean values from independent replicates (n = 3) ±SE

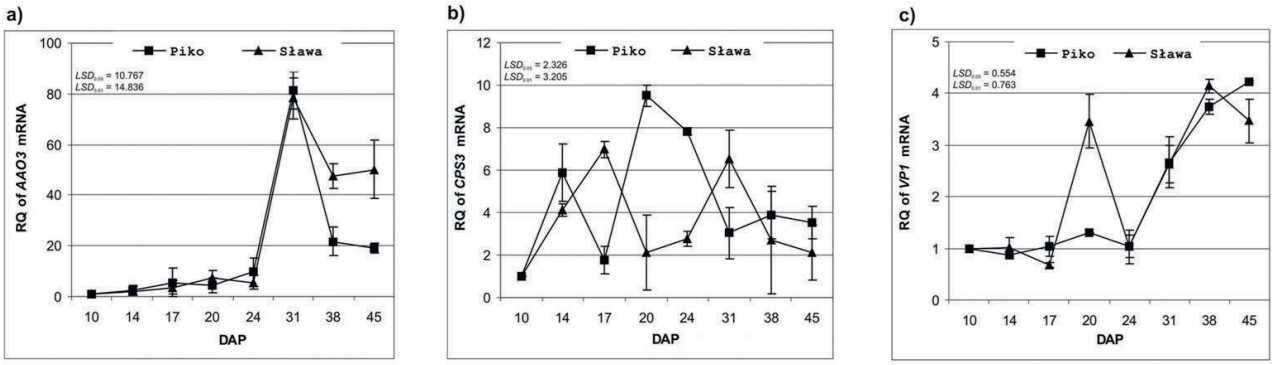

Figure 2. Relative quantity (RQ) of the *AAO3* (a), *CPS3* (b) and *VP1* (c) transcripts, during grain development of cultivars Piko and Sława. The relative mRNA level for each sample was normalized to that in the 10 days after pollination (DAP) of the respective cultivar. *Actin* mRNA was used as an internal control. Each value represents the mean of three independent replicates. Bars represent standard deviation

The expression analysis of the *VP1* gene showed two peaks in both examined cultivars. In Piko (the PHS-susceptible cultivar), the relative *VP1* gene expression was low at the beginning and during the middle stages of grain development, and the first peak was observed at 20 DAP. After 24 DAP, the expression level significantly increased and reached the second peak at 45 DAP. In Sława (the PHS-tolerant cultivar), the relative *VP1* gene expression was also low at the beginning of grain development, but it significantly increased during the middle stages. The first peak was observed at 20 days after pollination. Transcript accumulation then dropped down at 24 DAP and peaked again at 38 DAP. The relative accumulation of the *VP1* mRNA remained high in mature grains of both cultivars (Fig. 2c).

Transcript accumulation in grains induced to sprout in spikes

For this analysis, sprouting grains collected on the third day and non-sprouting ones collected on the sixth day of sprouting induction were used (Table 3). The relative amount of the *AAO3* transcript in sprouting grains was similar in both examined cultivars. In non-sprouting grains, a higher expression level was observed in Piko than in Sława but observed difference was not significant. It is also worth mentioning that in non-sprouting grains of both cultivars, the expression level was higher than that in sprouting grains. This difference for Piko was significant.

The relative *CPS3* expression exhibited differences between the two cultivars. In both sprouting and non-sprouting Piko grains, the *CPS3* expression was significantly higher than for both samples of Sława. On the other hand, in both samples of Sława, a similar relative quantity value of the examined gene was observed.

The relative *VP1* expression showed an accumulation profile similar to that for the *AAO3* gene for both cultivars: in grains without visible signs of external sprouting, the amount of transcript was greater than that in sprouting grains. The cultivars did not differ significantly in the levels of the *VP1* transcript in

Table 3. Relative expression of the *AAO3*, *CPS3* and *VP1* genes in wheat grains (45 DAP) after sprouting induction within spikes. *Actin* was used as a reference gene. The relative mRNA level for each sample was normalized to that in the sprouting grains of Piko

Gene	Piko		Sława	
	Sprouting[a] grains	Non-sprouting[b] grains	Sprouting[a] grains	Non-sprouting[b] grains
AAO3	1.00±0.00	3.20±0.86	1.11±0.14	2.24±0.40
$LSD_{0.05\ (0.01)}$		1.339 (2.222)		
CPS3	1.00±0.00	1.64±0.37	0.37±0.08	0.39±0.07
$LSD_{0.05\ (0.01)}$		0.528 (0.875)		
VP1	1.00±0.00	5.03±0.04	1.27±0.34	2.42±0.96
$LSD_{0.05\ (0.01)}$		1.415 (2.347)		

[a] Sprouting – grains with visible sprouting damages after three days of sprouting induction. [b] Non-sprouting – grains without visible sprouting damages after six days of sprouting induction. The presented results are the mean values from independent replicates (n = 3) ±SE

sprouting grains. However, in non-sprouting grains of Piko, the level of this mRNA was about 2.5 times higher than that observed in Sława.

Discussion

In the literature, there are some reports characterizing differences between PHS-susceptible and PHS-resistant genotypes of wheat (Walker-Simmons, 1987; Corbineau *et al.,* 2000; Gerjets *et al.,* 2010; De Laethauwer *et al.,* 2012). In this study, we analyzed expression of two genes involved in the biosynthesis of ABA and GAs (*AAO3* and *CPS3*, respectively), and one gene (*VP1*) for a transcription factor of ABA-regulated genes. For the studies, grains at various developmental stages as well as mature grains after induction of sprouting were used. The evaluation of grain germination at successive developmental stages and after storage showed that in Piko, the readiness of grains to germinate was greater (from 31 DAP) than in Sława (from 38 DAP). At physiological maturity (45 DAP), Sława still showed decreased germination ability which clearly indicated deeper dormancy. This feature was confirmed after storage of physiologically mature grains, using germination assay in the presence of ABA. Several studies described the correlation between susceptibility to PHS and response of wheat grains to ABA treatment. Cultivars that are resistant / tolerant to PHS are more sensitive to ABA than susceptible cultivars (Walker-Simmons, 1987; Corbineau *et al.,* 2000; Utsugi *et al.,* 2008). In our studies, the grains tested after a 5-week storage showed weaker dormancy when compared to grains examined directly after harvest (45 DAP). Stronger response to ABA – inhibition of germination – was most visible for Sława grains at 25°C. Noda *et al.* (1994) indicated that temperature may affect the break of dormancy and responsiveness of the embryo to ABA. Sława also showed greater tolerance to sprouting in spikes. One of the objectives of the molecular studies in this work was to characterize expression of the *AAO3* gene in both cultivars. In developing grains, the *AAO3* transcript reached the maximum level at 31 DAP in both examined cultivars. Until 24 DAP, no significant differences were observed between these cultivars with respect to accumulation of this transcript. Only at the final developmental stage in Piko we observed a significantly lower level of transcript accumulation in comparison to Sława. This may indicate a significant role of the *AAO3* gene in controlling the amount of endogenous ABA and grain dormancy. The grain ABA content was not addressed in our studies. However, some authors reported two peaks of ABA content in grains. The first peak was noted at 25 DAP and the second from 35 to 40 DAP; and this was correlated with cultivar susceptibility to PHS (Suzuki *et al.,* 2000). In turn, Walker-Simmons (1987) reported one peak of ABA at 40 DAP, irrespective of cultivar susceptibility to sprouting. In that study, the author also noted a slightly higher level of endogenous ABA in the PHS-tolerant wheat cultivar in comparison to the PHS-susceptible one. In the present study, in mature grains of Sława (the PHS-tolerant), higher *AAO3* mRNA accumulation was found in comparison to Piko grains (the PHS-susceptible). The accumulation of *AAO3* transcript is also related to grain dormancy. In grains with visible signs of sprouting (after sprouting induction in spikes), the level of *AAO3* mRNA was distinctly lower than in grains without visible signs of sprouting. Surprisingly, in non-sprouting Piko grains (the PHS-susceptible cultivar), accumulation of this transcript was higher than that in the PHS-tolerant cultivar (Sława). However, some reports indicate an interrelationship between the accumulation of ABA and GA in grains as a key factor determining the degree of grain dormancy and their predisposition to pre-harvest sprouting / germination (Kucera *et al.,* 2005; Finkelstein *et al.,* 2008). The studies performed by Seo *et al.* (2006) showed that ABA is involved in inhibition of GA biosynthesis, both in soaked and developing seeds. In some plant species, including *Arabidopsis* and tobacco, increased ABA biosynthesis occurs in soaked seeds, which is related to the maintenance of dormancy (Grappin *et al.,* 2000; Ali-Rachedi *et al.,* 2004). In our experiment, it was shown that in non-sprouting Piko grains induced to sprout, *i.e.* placed in a high humidity environment, this gene expression was higher than in Sława grains. This seems to be related to the *CPS3* transcript accumulation in grains of both cultivars. In Piko, the relative accumulation of *CPS3* mRNA was significantly higher than in Sława, both in sprouting and non-sprouting grains. It seems that in non-sprouting grains under conditions that favor germination, the amount of the *AAO3* transcript should be higher to balance the high level of the *CPS3* transcript, which determines, to a great extent, the amount of GA synthesized by the embryo. The *CPS3* gene takes part in ent-kaurene synthesis in the metabolic pathway of GAs. The present studies, showed that accumulation of this transcript in developing grains exhibited two peaks. However, in relation to 10 DAP, the accumulation profile for each cultivar is different. Clear differences between the two cultivars can be seen from 17 to 31 DAP. The comparison of grains induced to sprout in spikes showed that accumulation of the *CPS3* transcript in the cultivar susceptible to PHS (Piko) was higher, irrespective of grain dormancy, in comparison to the tolerant cultivar (Sława). In the present studies, the gene encoding the *VP1* transcription factor was also analyzed. The results of many reported experiments showed that in wheat cultivars which are resistant / tolerant to PHS, the level of *VP1* mRNA was higher than in susceptible cultivars (Nakamura & Toyama, 2001; McKibbin *et al.,* 2002; Rikiishi & Maekawa, 2010; De Laethauwer *et al.,*

2012). In turn, in sorghum, the expression level of the *VP1* gene in the embryos of PHS-susceptible genotypes was higher than for PHS-resistant genotypes (Carrari *et al*., 2001). The expression of this gene may occur already during early stages of grain development. In wheat, the highest expression level of the *VP1* gene is observed at about 50 DAP, later on —in the ripening stage (60 DAP)— it drops down (Utsugi *et al.,* 2008). However, Wilson *et al.* (2005) found that the expression of the *VP1* gene peaked about 21 DAP, and at further stages, until 40 DAP, decreased. In our experiments in both examined cultivars, two peaks of the *VP1* gene expression were observed. The first peak was visible at 20 DAP and the second peak at 45 DAP and 38 DAP for Piko and Sława, respectively. In mature grains of both cultivars, relatively high level of this transcript accumulation was observed in relation to 10 DAP (Fig. 2c). For both cultivars, in non-sprouting grains we found a higher expression level of the *VP1* gene in comparison to sprouting grain but only in Piko this difference was significant. A similar correlation was found by Utsugi *et al.* (2008) for isolated wheat embryos of dormant and dormancy-broken grains. In our research, the *VP1* transcript was more abundant in non-sprouting grains of Piko (PHS-susceptible) than in non-sprouting grains of Sława (PHS-tolerant). In the experiment performed by Nakamura & Toyama (2001), the expression level of the *VP1* gene in ABA-treated embryos of deeply dormant and non-dormant wheat cultivars was positively correlated with the level of grain dormancy.

In the presented study, the tested cultivars, characterized by different susceptibility to pre-harvest sprouting, showed different expression patterns of *AAO3*, *CPS3* and *VP1* genes. The correlation between PHS and the expression of the examined genes could be disputed. However, the expression of *AAO3* during grain development corresponds to ABA accumulation described in the literature and observed in the PHS phenotype. We discuss the usefulness of these findings for manipulation of germination in wheat.

References

Ali-Rachedi S, Bouinot D, Wagner MH, Bonnet M, Sotta B, Grappin P, Jullien M, 2004. Changes in endogenous abscisic acid levels during dormancy release and maintenance of mature seeds: studies with the Cape Verde Islands ecotype, the dormant model of Arabidopsis thaliana. Planta 219: 479-488. https://doi.org/10.1007/s00425-004-1251-4

Cadman CS, Toorop PE, Hilhorst HW, Finch-Savage WE, 2006. Gene expression profiles of Arabidopsis Cvi seeds during dormancy cycling indicate a common underlying dormancy control mechanism. Plant J 46: 805-822. https://doi.org/10.1111/j.1365-313X.2006.02738.x

Carrari F, Perez-Flores L, Lijavetzky D, Enciso S, Sanchez R, Benech-Arnold R, Iusem N, 2001. Cloning and expression of a sorghum gene with homology to maize vp1. Its potential involvement in pre-harvest sprouting resistance. Plant Mol Biol 45: 631-640. https://doi.org/10.1023/A:1010648420669

Corbineau F, Benamar A, Côme D, 2000. Changes in sensitivity to abscisic acid of the developing and maturing embryo of two wheat cultivars with different sprouting susceptibility. Isr J Plant Sci 48: 189-197. https://doi.org/10.1560/HG09-MCP0-GF6Y-3TD6

De Laethauwer S, Reheul D, De Riek J, Haesaert G, 2012. Vp1 expression profiles during kernel development in six genotypes of wheat, triticale and rye. Euphytica 188: 61-70. https://doi.org/10.1007/s10681-011-0613-9

Finkelstein RR, Rock CD, 2002. Abscisic acid biosynthesis and response. In: The Arabidopsis Book; Somerville CR & Meyerowitz EM (eds.). pp: 1-52. Am Soc Plant Biol, Rockville, MD, USA. https://doi.org/10.1199/tab.0058

Finkelstein RR, Tenbarge KM, Shumway JE, Crouch ML, 1985. Role of ABA in maturation of rapeseed embryos. Plant Physiol 78: 630-636. https://doi.org/10.1104/pp.78.3.630

Finkelstein RR, Reeves W, Ariizumi T, Steber C, 2008. Molecular aspects of seed dormancy. Annu Rev Plant Biol 59: 387-415. https://doi.org/10.1146/annurev.arplant.59.032607.092740

Gao Y, Liu J, Zhang Z, Sun X, Zhang N, Fan J, Niu X, Xiao F, Liu Y, 2013. Functional characterization of two alternatively spliced transcripts of tomato ABSCISIC ACID INSENSITIVE3 (ABI3) gene. Plant Mol Biol 82: 131-145. https://doi.org/10.1007/s11103-013-0044-1

Gerjets T, Scholefield D, Foulkes MJ, Lenton JR, Holdsworth MJ, 2010. An analysis of dormancy, ABA responsiveness, after-ripening and pre-harvest sprouting in hexaploid wheat (Triticum aestivum L.) caryopses. J Exp Bot 61: 597-607. https://doi.org/10.1093/jxb/erp329

González-Guzmán M, Abia D, Salinas J, Serrano R, Rodríguez P, 2004. Two new alleles of the abscisic aldehyde oxidase 3 gene reveal its role in abscisic acid biosynthesis in seeds. Plant Physiol 135: 325-333. https://doi.org/10.1104/pp.103.036590

Grappin P, Bouinot D, Sotta B, Miginiac E, Jullien M, 2000. Control of seed dormancy in Nicotiana plumbaginifolia: post-imbibition abscisic acid synthesis imposes dormancy maintenance. Planta 210: 279-285. https://doi.org/10.1007/PL00008135

Hsu FC, 1979. Abscisic acid accumulation in developing seeds of Phaseolus vulgaris L. Plant Physiol 63: 552-556. https://doi.org/10.1104/pp.63.3.552

Jacobsen JV, Pearce DW, Poole AT, Pharis RP, Mander LN, 2002. Abscisic acid, phaseic acid and gibberellin contents associated with dormancy and germination in barley. Physiol Plantarum 115: 428-441. https://doi.org/10.1034/j.1399-3054.2002.1150313.x

Karssen C, Brinkhorst-van der Swan D, Breekland A, Koornneef M, 1983. Induction of dormancy during seed development by endogenous abscisic acid: studies on abscisic acid deficient genotypes of Arabidopsis thaliana (L.) Heynh. Planta 157: 158-165. https://doi.org/10.1007/BF00393650

King RW, 1976. Abscisic acid in developing wheat grains and its relationship to grain growth and maturation. Planta 132: 43-51. https://doi.org/10.1007/BF00390329

Kucera B, Cohn MA, Leubner-Metzger G, 2005. Plant hormone interactions during seed dormancy release and germination. Seed Sci Res 15: 281-307. https://doi.org/10.1079/SSR2005218

McCarty DR, 1995, Genetic control and integration of maturation and germination pathways in seed development. Annu Rev Plant Physiol Plant Mol Biol 46: 71-93. https://doi.org/10.1146/annurev.pp.46.060195.000443

McCarty DR, Hattori T, Carson CB, Vasil V, Lazar M, Vasil IK, 1991. The Viviparous-1 developmental gene of maize encodes a novel transcriptional activator. Cell 66: 895-905. https://doi.org/10.1016/0092-8674(91)90436-3

McKibbin RS, Wilkinson MD, Bailey PC, Flintham JE, Andrew LM, Lazzeri PA, Gale MD, Lenton JR, Holdsworth MJ, 2002. Transcripts of Vp-1 homeologues are misspliced in modern wheat and ancestral species. Proc Natl Acad Sci USA 99: 10203-10208. https://doi.org/10.1073/pnas.152318599

Nakamura S, Toyama T, 2001. Isolation of a VP1 homologue from wheat and analysis of its expression in embryos of dormant and non-dormant cultivars. J Exp Bot 52: 875-876. https://doi.org/10.1093/jexbot/52.362.1952

Nambara E, Marion-Poll A, 2003. ABA action and interactions in seeds. Trends Plant Sci 8: 213-217. https://doi.org/10.1016/S1360-1385(03)00060-8

Noda K, Kawabata C, Kawakami N, 1994. Response of wheat grain to ABA and imbibition at low temperature. Plant Breeding 113: 53-57. https://doi.org/10.1111/j.1439-0523.1994.tb00701.x

Rikiishi K and Maekawa M, 2010. Characterization of a novel wheat (Triticum aestivum L.) mutant with reduced seed dormancy. J Cereal Sci 51: 292-298. https://doi.org/10.1016/j.jcs.2010.01.003

Seo M, Peeters AJM, Koiwai H, Oritani T, Marion-Poll A, Zeevaart JAD, Koornneef M, Kamiya Y, Koshiba T, 2000. The Arabidopsis aldehyde oxidase 3 (AAO3) gene product catalyzes the final step in abscisic acid biosynthesis in leaves. Proc Natl Acad Sci USA 97: 12908-12913. https://doi.org/10.1073/pnas.220426197

Seo M, Aoki H, Koiwai H, Kamiya Y, Nambara E, Koshiba T, 2004. Comparative studies on the Arabidopsis aldehyde oxidase (AAO) gene family revealed a major role of AAO3 in ABA biosynthesis in seeds. Plant Cell Physiol 45: 1694-1703. https://doi.org/10.1093/pcp/pch198

Seo M, Hanada A, Kuwahara A, Endo A, Okamoto M, Yamauchi Y, North H, Marion-Poll A, Sun TP, Koshiba T, Kamiya Y, Yamaguchi S, Nambara E, 2006. Regulation of hormone metabolism in Arabidopsis seeds: phytochrome regulation of abscisic acid metabolism and abscisic acid regulation of gibberellin metabolism. Plant J 48: 354-366. https://doi.org/10.1111/j.1365-313X.2006.02881.x

Simlat M, Stobiecki M, Szklarczyk M, 2013. Accumulation of selected phenolics and expression of PAL genes in carrots differing in their susceptibility to carrot fly (Psila rosae F.). Euphytica 190: 253-266. https://doi.org/10.1007/s10681-012-0836-4

Simsek S, Ohm J-B, Lu H, Rugg M, Berzonsky W, Alamri MS, Mergoum M, 2014. Effect of pre-harvest sprouting on physicochemical changes of proteins in wheat. J Sci Food Agric 94: 205-212. https://doi.org/10.1002/jsfa.6229

Singh G, Kumar S, Singh P, 2003. A quick method to isolate RNA from wheat and other carbohydrate-rich seeds. Plant Mol Biol Rep 21: 93a-93f. https://doi.org/10.1007/BF02773401

Sugliani M, Brambilla V, Clerkx EJM, Koornneef M, Soppe WJJ, 2010. The conserved splicing factor SUA controls alternative splicing of the developmental regulator ABI3 in Arabidopsis. Plant Cell 22: 1936-1946. https://doi.org/10.1105/tpc.110.074674

Suzuki T, Matsuura T, Kawakami N, Noda K, 2000. Accumulation and leakage of abscisic acid during embryo development and seed dormancy in wheat. Plant Growth Regul 30: 253-260. https://doi.org/10.1023/A:1006320614530

Toyomasu T, Kagahara T, Hirose Y, Usui M, Abe S, Okada K, Koga J, Mitsuhashi W, Yamane H, 2009. Cloning and characterization of cDNAs encoding ent-copalyl diphosphate synthases in wheat: insight into the evolution of rice phytoalexin biosynthetic genes. Biosci Biotechnol Biochem 73: 772-775. https://doi.org/10.1271/bbb.80781

Utsugi S, Nakamura S, Noda K, Maekawa M, 2008. Structural and functional properties of Viviparous 1 genes in dormant wheat. Genes Genet Syst 83: 153-166. https://doi.org/10.1266/ggs.83.153

Walker-Simmons M, 1987. ABA levels and sensitivity in developing wheat embryos of sprouting resistant and susceptible cultivars. Plant Physiol 84: 61-66. https://doi.org/10.1104/pp.84.1.61

Walker-Simmons M, Sesing J, 1990. Temperature effects on embryonic ABA levels during development of wheat grain dormancy. J Plant Growth Regul 9: 51-56. https://doi.org/10.1007/BF02041941

Wilkinson M, Lenton J, Holdsworth M, 2005. Transcripts of VP-1 homoeologues are alternatively spliced within the Triticeae tribe. Euphytica 143: 243-246. https://doi.org/10.1007/s10681-005-7856-2

Wilson I, Barker G, Lu C, Coghill J, Beswick R, Lenton J, Edwards K, 2005. Alteration of the embryo transcriptome of hexaploid winter wheat (Triticum aestivum cv. Mercia) during maturation and germination. Funct Integr Genomic 5: 144-154. https://doi.org/10.1007/s10142-005-0137-2