**The varied ability of grains to synthesize and catabolize ABA is one of the factors affecting dormancy and its release by after-ripening in imbibed triticale grains of cultivars with different pre-harvest sprouting susceptibilities**

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**Abstract**

 Abscisic acid (ABA) is a phytohormone involved in acquisition of primary dormancy and dormancy maintenance in imbibed seeds. After imbibition, ABA content decreased to a much lower level in embryos of freshly harvested triticale grains of the Leontino cultivar, which is more susceptible to pre-harvest sprouting (PHS) than embryos of the Fredro cultivar. Lower ABA content in the Leontino cultivar resulted from increased expression of *TsABA8’OH1* and *TsABA8’OH2*, which encode ABA 8’-hydroxylase and are involved in ABA catabolism. Higher ABA content and maintenance of dormancy in Fredro grains were correlated with intensified ABA biosynthesis, which resulted from higher expression of *TsNCED1*, which encodes9-*cis*-epoxycarotenoid dioxygenase. These results suggest that grains of triticale cultivars with different resistance to PHS vary in their ability to metabolize ABA after imbibition.

 After-ripening did not affect the ABA content in embryos of dry grains of either triticale cultivar. However, after-ripening caused dormancy release in Fredro grains and significantly affected the ABA content and the rate of its metabolism after imbibition. A more rapid decline in ABA content inimbibed Fredro grains was accompanied by decreased transcript levels of *TsNCED1* as well as increased expression of *TsABA8’OH1* and *TsABA8’OH2*. Thus, after-ripening may affect dormancy of grains through reduction of the ABA biosynthesis rate and intensified ABA catabolism.

 Overexpression of *TsNCED1* in tobacco increases ABA content and delays germination, while overexpression of *TsABA8’OH2* decreases ABA content, accelerates germination, and reduces the sensitivity to ABA of transgenic seeds compared to seeds of wild-type plants. Therefore, these genesmight play an important role in the regulation of triticale grain dormancy, thus affecting susceptibility to PHS.

*Keywords:* Abscisic acid; ABA 8'-hydroxylase; after-ripening; 9-*cis*-epoxycarotenoid dioxygenase; dormancy; pre-harvest sprouting.

*Abbreviations:* ABA, abscisic acid, TsABA8’OH, *Triticosecale* ABA 8'-hydroxylase; AR, after-ripened; D, dormant; FH, freshly harvested; TsNCED, *Triticosecale* 9-*cis*-epoxycarotenoid dioxygenase; PHS, pre-harvest sprouting.

**Introduction**

Following maturation, seeds of many plant species exhibit primary dormancy and do not germinate even if placed under optimal conditions (Finch-Savage and Leubner-Metzger, 2006). The dormancy state at harvest may directly affect the quality of crops; therefore, it is considered a trait of great economic importance. For cereals, a dormancy that is too strong delays germination and causes uneven emergence, thus interfering with proper crop management and, in the case of industrial use, the malting process (Gao et al., 2013; Rodríguez et al., 2015). On the other hand, a low dormancy may lead to pre-harvest sprouting (PHS), in which grains germinate while they are still on the mother plant (Kulwal et al., 2010). Especially prone to this adverse process are the grains of wheat, rye, and triticale. Primary dormancy is lost through the process of after-ripening, dry storage at room temperature, which can last for several days to several months (depending on the species and the cultivar) (Finch-Savage and Leubner-Metzger, 2006). However, grains of the cereal varieties particularly susceptible to PHS may exhibit germinability before the dry storage period (Gerjets et al., 2010).

Seed dormancy is controlled by components of intrinsic hormonal and metabolic pathways that are influenced by external environmental signals (Fidler et al., 2015; Gerjets et al., 2010; Nambara et al., 2010; Rodríguez-Gacio et al., 2009). It is believed that the plant hormone abscisic acid (ABA) is required for induction of primary dormancy, while dormancy release is accompanied by a decrease in the level of and/or sensitivity to this phytohormone with a simultaneous increase in the concentration of and/or sensitivity to gibberellins (GA) (Gubler et al., 2005; Matilla et al., 2015; Rodríguez-Gacio et al., 2009; Tutlle et al., 2015). Although genetic analyses have confirmed the involvement of embryonic ABA in the onset and maintenance of primary dormancy (Karssen et al., 1983; Fang and Chu, 2008), the mechanisms associated with dormancy release remain largely unexplained. Gibberellins are increasingly attributed to promoting germination once dormancy is broken, rather than contributing to dormancy release (Jacobsen et al., 2002). Additionally, the correlation between the ABA content and the dormancy status in mature dry seeds is not always observed. For example, after-ripened (AR) dry seeds of barley and Arabidopsis do not show significant differences in the embryo ABA content from dormant (D) seeds that were not after-ripened, even though they germinate much faster (Ali-Rachedi et al., 2004; Gubler et al., 2008; Jacobsen et al., 2002; Millar et al., 2006). Significant differences in the ABA levels between D and AR seeds were, however, observed after imbibition. During the first few hours of imbibition, ABA content was reduced in the embryos of both types of seeds, although ABA content was reduced to a much lower level in the embryos of AR seeds (Ali-Rachedi et al., 2004; Jacobsen et al., 2002; Millar et al., 2006). Thus, the ABA content in dry seeds it is not always a reliable indicator of dormancy status, but rather there is a varied capacity for ABA biosynthesis and catabolism in seeds (Millar et al., 2006; Gubler et al 2008).

It is believed that the reduction of ABA content observed after imbibition is a result of increased catabolism of this phytohormone (Rodríguez-Gacio et al., 2009). On the other hand, studies using fluridone, an ABA biosynthesis inhibitor, indicated that *de novo* ABA synthesis is required to maintain dormancy during imbibition of D seeds. Thus, the precise balance between ABA biosynthesis and catabolism may play a crucial role in the maintenance and release of seed dormancy. It is believed that the key enzyme regulating ABA biosynthesis is 9-*cis*-epoxycarotenoid dioxygenase (EC 1.13.11.51, NCED), whereas the dominant ABA catabolic pathway is the reaction catalysed by ABA 8'-hydroxylase (EC 1.14.13.93, ABA8'OH) (Iuchi et al., 2001; Kushiro et al., 2004). In barley, after-ripening induces changes in the expression of some genes coding for NCED and ABA8’OH. Lower ABA levels, observed in embryos of imbibed AR grains compared with imbibed D grains, were accompanied by higher transcript levels of *HvABA8’OH1*, while high expression of *HvNCED1* correlated with high ABA content and dormancy maintenance in imbibed D seeds (Millar et al., 2006).

ABA metabolism in cereal grains, especially in relation to dormancy release through after-ripening, was studied mainly in barley, a species which requires long periods of dry storage (Chono et al., 2006; Gubler et al., 2008; Jacobsen et al., 2002; Millar et al., 2006), while little is known about this process in wheat or triticale, which are particularly susceptible to PHS (Gerjets et al., 2010; Jacobsen et al., 2013). Moreover, the very few results obtained for some wheat cultivars are often inconclusive (Jacobsen et al., 2013; Liu et al., 2013). Therefore, the goal of this study was to investigate whether the varied ability of grains to synthesize and catabolize ABA can be one of the factors affecting triticale PHS susceptibility. Since a comparative analysis of ABA metabolism in response to after-ripening in cultivars with different PHS susceptibilities has not been done, we also aimed to clarify how postharvest storage affects ABA metabolism in grains of two triticale cultivars that differ in their resistance to PHS. Therefore, ABA content in dry and imbibed triticale grains in response to after-ripening was determined. Then, the expression levels of genes encoding NCED and ABA8’OH in imbibed grains of the two cultivars that differ in their susceptibility to PHS were analysed. Based on the analysis of these gene expression profiles in combination with ABA content and germinability, genes encoding enzymes that are potentially involved in the regulation of ABA metabolism in triticale grains were selected and functional analyses were performed.

**Material and methods**

*Plant material and experimental conditions*

Grains of two winter triticale (× *Triticosecale* Wittm.) cultivars (Leontino and Fredro) that differ in their resistance to PHS were provided by Danko Plant Breeders Ltd., Laski, Poland. Leontino is considered one of the most susceptible cultivars to PHS, while Fredro is one of the most resistant. Grains were sown in October in pots containing 10 kg of soil. The soil was supplemented with nitrogen (0.75 g KNO3 and 1.75 g NH4NO3 per pot), potassium (0.5 g KNO3 per pot), phosphorus (0.5 g KH2PO4 per pot), calcium (0.8 g Ca(H2PO4)2 × H2O per pot), magnesium (0.65 g MgSO4 × 7H2O) and micronutrients. Plants were grown in a greenhouse with natural illumination and an average temperature of 25 °C during the day and 15 °C during the night. After 6 weeks in the greenhouse, pots with seedlings (13 per pot) were transferred to a vernalization chamber (2 °C) for 8 weeks. The vernalized seedlings were then transferred back to the greenhouse, where they were kept until the end of the experiment in late June. Plants were irrigated twice per week. Grains were harvested at full maturity (50 days after pollination) and half of the harvested grains were stored at room temperature for 3 months to after-ripen.

*Germination tests*

Freshly harvested or after-ripened grains were sterilized as previously described (Fidler et al. 2016). Thirty grains were placed in 9-cm-diameter Petri dishes with two sheets of Whatman No. 2 filter paper watered with 5 ml of sterile water and allowed to germinate at 23 °C in darkness. Germination tests were performed in triplicate. Grains were considered to have germinated when the coleorhiza protruded from the seed coat.

*Total RNA extraction*

Total RNA was isolated from the embryos of collected grains as recommended by the PureLink RNA Mini Kit protocol (Life Technologies), including the DNAse treatment.

*Relative semi-quantitative RT-PCR*

The transcript levels of the triticale genes in embryos of collected grains were analysed using Titanium One-Step RT-PCR Kit (Clontech), and aliquots of 100 ng of total RNA were used as templates in each reaction. RT-PCRs were performed with gene-specific primers for *18S rRNA* (F: 5’- CCAGGTCCAGACATAGTAAG -3’; R 5’-GTACAAAGGGCAGGGACGTA -3’), *TsNCED1* (F: 5’-CTCCATCTTCAACGACACGGACGACC -3’; R 5’- CAGGTGTGGTAAATGAACCAAGGAATCG -3’), *TsNCED2* (F: 5’-GTGGAGAGGCAGGAGAAGAAGCTC-3’; R 5’- CTGCTGCAGGCGCTCCGTCTC -3’), T*sABA8’OH1* (F: 5’- CTTCGGGGAGGAGGAGATGCAG -3’; R 5’-GTTGTCGCCGAGGAACTTGACCATC -3’) and *TsABA8’OH2* (F: 5’-GATGGCCTTCTTCCTCCTCCTGTGCATC -3’; R 5’-GTAGTCGCCCTGGTGGAAGAAGAGC -3’) under the following conditions: 60 min at 50 °C; 5 min at 94 °C; 7 (*18S rRNA*), 33 (*TsNCED1*), 36 (*TsNCED2*), 30 (*TsABA8’OH1*), or 32 (*TsABA8’OH2*) cycles of 30 s at 94 °C, 30 s at 61 °C (*TsNCEDs*), 60 °C (*TsABA8’OH1*), or 62 °C (*18S rRNA, TsABA8’OH2*), 1 min at 68 °C; and a final extension of 2 min at 68 °C.

The gene specific primers were designated to the triticale sequences cloned previously (Fidler at al., 2016) and deposited in Genbank under following accession numbers: *TsNCED1 - KF923955.1*, *TsNCED2 - KF923956.1*, *TsABA8’OH1* - KF923953.1and *TsABA8’OH2*; - KF923954.1). The amplification of the *18S rRNA* gene was performed as an internal control. The generated RT-PCR products were cloned into a pJET1.2/blunt cloning vector (Thermo Scientific), and at least 6 clones of each insert were sequenced at the DNA Sequencing and Oligonucleotide Synthesis Laboratory at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, to confirm specific amplification. At least two technical replicates of RT-PCR reaction were performed for each of the two RNA isolations

*Endogenous ABA measurements*

The ABA content in embryos of collected triticale grains was measured using the Phytodetek ABA enzyme immunoassay test kit (Agdia). A sample of 150 mg of frozen embryos was homogenized in liquid nitrogen, and then 1.5 ml of extraction buffer (80% methanol, 2% acetic acid and 20 mg / l butylated hydroxytoluene) was added to the powdered tissue. The ABA was then extracted for 18 h at 4 °C in the dark with gentle agitation. The homogenate was centrifuged at 13 000 rpm at 4 °C, and the supernatants were collected and stored at −80 °C until further use. Before measurement, supernatants were diluted 40-fold in TBS-buffer (Tris-buffered saline), and ABA content was measured at least in triplicate as described by the Phytodetek protocol.

*Construction of plant expression vectors*

The full-length cDNA of *TsNCED1* and *TsABA8’OH2* was amplified by PCR using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) with gene-specific primers containing restriction sites for *BamH*I at the 5’ end (5’ -ATAGGATCCATGCAGACTCTGTCCG -3’) and *EcoR*I at the 3’ end (5’ -CGAGGCCCAGCACTAAGAATTCACA -3’) of *TsNCED1* and *HindIII* at the 5’ end (5’ -AAATAAGCTTATGGCCTTCTTCCTCC -3’) and *EcoR*I at the 3’ end (5’ -GACGAAGTGATAGTGTGAGAATTCACA -3’) of *TsABA8’OH2*. The amplification was performed under the following conditions: 3 min at 95 °C; 3 cycles of 30 s at 95 °C, 30 s at 50 °C, 3 min at 72 °C; 30 cycles of 30 s at 95 °C, 30 s at 60 °C, 3 min at 72 °C; and a final extension of 10 min at 72 °C. The PCR products were digested with the restriction enzymes listed above and ligated to a 35S cassette containing a 35S CaMV promoter, and subsequently inserted into a pGreen 0029 binary vector (provided by Dr. Roger Hellens and Dr. Phil Mullineaux of the John Innes Centre, Norwich, UK; Hellens et al., 2000). The constructs *35S::TsNCED1* and *35S::TsABA8’OH2* were introduced by electroporation into *Agrobacterium tumefaciens* strain C58C1, which contained the virulence helper plasmid pCH32 (Hamilton et al., 1996).

*Generation of transgenic tobacco plants*

Seeds of *Nicotiana tabacum* L. cv. LA Burley 21 (Legg et al., 1970) were surface sterilized and germinated on ½ MS medium (Murashige and Skoog, 1962) in growth chamber at temperature 26 °C for 16 hours (day) and 22 °C for 8 hours (night) . Three-week-old tobacco seedlings were transformed with *Agrobacterium*,carrying one of the recombinant plasmid: *35S::TsNCED1*, *35S::TsABA8’OH2* or 35S cassette, according to the methods described by Rossi et al. (1993). Plants were grown on MS medium supplemented with MS vitamins and kanamycin (50 µg/ml). The regenerated plants with confirmed presence of transgenes were then transferred to soil and grown in a greenhouse at 25-30 °C. T1 seeds of the transgenic plants were germinated on ½ MS medium with kanamycin (150 µg/ml). Seedlings of lines that showed approximately 3:1 segregation of kanamycin resistance were chosen to generate subsequent generations (T2, T3). The T3 seeds from the homozygous T2 generation were used for further analysis. Presence of the transgenes in kanamycin-resistant plants was confirmed by PCR using KAPA HiFi HotStart PCR Kit (Kapa Biosystems) with the primers specific for the 35S cassette (F 5’- CCCACTATCCTTCGCAAGAC -3’; R 5’ -CCCAAAGCGAGTACACAAC -3’) and genomic DNA as a template (Edwards et al., 1991) under the following conditions 5 min at 95 °C; 35 cycles of 20 s at 98 °C, 15 s at 64 °C, 2 min at 72 °C and a final extension of 2 min at 72 °C. Presence of *TsNCED1* and *TsABA8’OH2* transcripts in the transgenic lines was confirmed by semi-quantitative RT-PCR, which was performed with 100 ng of total RNA isolated from seeds and gene-specific primers for *TsNCED1* and *TsABA8’OH2*, under the conditions described above. The exception was the use of the following reverse primer for *TsNCED1*: 5’-CCTCGAGGTTCACCTGGCTCTCC-3’.

*Analysis of transgenic tobacco seeds*

Transgenic tobacco seeds were germinated on ½ MS medium containing 0, 5 or 10 µM ABA (one hundred per Petri dish in triplicate) under the conditions described above for wild-type seeds. The endogenous abscisic acid level in the whole transgenic tobacco seeds was determined as described above.

**Results**

*Effect of after-ripening on germination and ABA content of dry grains*

We began our research with a comparison of the effect of after-ripening (AR, 3 months of dry storage) on the germination of grains of two triticale cultivars (Fredro and Leontino), which differ in the susceptibility to PHS. We have previously shown that after 48 hours of imbibition, the freshly harvested FH) grains of the Leontino cultivar, which is more susceptible to PHS, germinated in almost 100%, while the FH grains of the Fredro cultivar, which is less susceptible to PHS, germinated only in approximately 10% (Fidler et al., 2016). Dry storage significantly affected the germinability of the Fredro cultivar grains and after 16 hours of imbibition almost 100% germinated, as did the grains of the Leontino cultivar (Supplementary Fig. S1).

 Postharvest storage did not significantly change the ABA content in embryos of dry grains and the ABA content in the after-ripened (AR) grains of the Fredro cultivar was almost twice as high as in the grains of the Leontino cultivar (Fig. 1).

*ABA content during imbibition of freshly harvested and after-ripened grains*

 In order to examine whether the differences in ABA content observed in dry grains of triticale cultivars with different PHS susceptibility persist after imbibition, the concentration of this phytohormone in imbibed grains was also measured.

After imbibition of freshly harvested grains, a significantly higher (3-4 times) ABA content was observed in the embryos of the Fredro than in the Leontino cultivar throughout the whole experimental period (Fig. 1). In the first 4 hours of imbibition, the ABA content decreased in the embryos of both cultivars. However, in the embryos of Leontino, ABA content decreased 2.5-fold, while in the embryos of Fredro, ABA content decreased 1.5-fold. Thereafter, the embryos of Leontino maintained a relatively low level of ABA content, while in the embryos of Fredro, an increase in the ABA content was observed from the 16th hour of imbibition and lasted until the end of the experiment. At the 48th hour of imbibition, the ABA content in embryos of Fredro has reached a similar level as in dry grains and was four times higher than in embryos of Leontino.

After imbibition of after-ripened grains, the ABA content in the embryos of Fredro remained almost twice as much as it was in the embryos of Leontino, throughout the whole experimental period (Fig. 1). In the first 4 hours of imbibition, an approximately 2.5-fold decrease in ABA content was observed in the embryos of both cultivars. In the following hours of imbibition, the ABA content was maintained in embryos of both cultivars at a constant, relatively low level. In the AR grains of Fredro, the increase in ABA content, which occurred from the 16th hour of imbibition in FH grains, was not observed.

*Expression of genes encoding ABA metabolic enzymes during imbibition of freshly harvested and after-ripened grains*

To investigate whether differences in the ABA content observed after imbibition between triticale cultivars result from the varying ability of grains to synthesize and/or catabolize ABA, an analysis of the expression level of genes encoding enzymes involved in ABA metabolism was performed.

After imbibition of freshly harvested grains, the mRNA level of genes encoding *Triticosecale* 9-*cis*-epoxycarotenoid dioxygenase (*TsNCED*) was higher in the embryos of the Fredro than in the Leontino cultivar. The greatest differences between cultivars were observed in the transcript level of *TsNCED1* (Fig. 2A). The expression of this gene was much higher in the embryos of Fredro than Leontino already at the 2nd hour of imbibition and lasted until the end of the experiment. In Leontino, a decrease in *TsNCED1* mRNA level was observed after 1 day of imbibition. A high transcript level of *TsNCED1* in embryos of Fredro was correlated with high ABA content, observed throughout the whole experimental period (Fig. 1). The transcript level of *TsNCED2* increased at the 2nd and 4th hours of imbibition in the embryos of both cultivars; however, the increase was more significant in embryos of Fredro (Fig. 2A). Thereafter, the level of *TsNCED2* mRNA was significantly reduced, and no differences in the expression of this gene between cultivars were observed. Expression of genes encoding *Triticosecale* ABA 8'-hydroxylase (*TsABA8'OH*) increased in the embryos of freshly harvested grains of both cultivars in the early hours of imbibition, however the increase was more significant in the embryos of Leontino than Fredro (Fig. 2A). An increased level of *TsABA8'OH1* mRNA was observed between the 2nd and 8th hours of imbibition in Leontino and between the 2nd and 6th hours of imbibition in Fredro. An elevated level of *TsABA8'OH2* transcript was observed between the 2nd and 12th hours of imbibition in Leontino while in Fredro, expression of this gene transiently increased only at the 2nd hour. In the later hours of imbibition, mRNA levels of both *TsABA8'OHs* decreased and maintained relatively low until the end of the experiment.

 After imbibition of after-ripened grains, the expression of *TsNCED1* was much higher in the embryos of Fredro than Leontino (Fig. 2B), which was previously observed in imbibed FH grains (Fig. 2A). However, after-ripening changed the profile of *TsNCED1* expression, especially in the embryos of Fredro. The high transcript level of *TsNCED1* in this cultivar was observed only between the 4th and 16th hours of imbibition. After 1 day of imbibition, a decrease in the expression of *TsNCED1* was observed in the embryos of both cultivars. The expression of *TsNCED2* was maintained at a constant, low level throughout the whole experimental period in the embryos of both cultivars. After-ripening also influenced the expression profile of both *TsABA8'OH*s, especially in the Fredro cultivar (Fig. 2B). The elevated mRNA levels of *TsABA8'OH1* and *TsABA8'OH2*, which were observed from the 2nd hour of imbibition, were more pronounced and lasted longer in the AR than FH grains of both cultivars (Fig. 2A, Fig. 2B).

*Constitutive expression of TsNCED1 in tobacco increases ABA level in seeds and delays germination*

 Transgenic tobacco seeds, overexpressing the triticale gene encoding NCED, *TsNCED1*, were obtained after transformation of wild-type plants witha *35S::TsNCED1* construct. The presence of *TsNCED1* transcripts in tobacco seeds of the transgenic lines was confirmed by RT-PCR (Fig. 3A).

To investigate the effect of *TsNCED1* overexpressionon the germinability of tobacco seeds, germination tests were conducted (Fig. 4A). Seeds of all tested *35S::NCED1* transgenic lines germinated later than seeds from wild-type plants. Six days after the start of imbibition, the seeds from transgenic plants germinated between 20% (*35S::NCED1-27*) and 40% (*35S::NCED1-13*), while the seeds of wild-type plants germinated in more than 80% (Fig. 4A, Supplementary Fig. S2).

 In dry seeds, the ABA content in *35S::TsNCED1* transgenic lines was by 35% (*35S::NCED1-13*) to 55% (*35S::NCED1-27*)higher than in seeds of wild-type plants (Fig. 4B, Supplementary Fig. S2). After 6 hours of imbibition, the differences in ABA content were even more pronounced, and the ABA content in seeds of transgenic lines was higher by approximately 50% (*35S::NCED1-13*) and 80% (*35S::NCED1-2, 35S::NCED1-27*) than in seeds of wild-type plants.

*Seeds of transgenic tobacco plants with overexpression of TsABA8’OH2 exhibit earlier germination and less sensitivity to ABA*

Transgenic tobacco seeds, overexpressing the triticale gene encoding ABA 8’-hydroxylase, *TsABA8’OH2*, were obtained after transformation of wild-type plants witha *35S::TsABA8’OH2* construct. The presence of *TsABA8’OH2*  transcripts in tobacco seeds of the transgenic lines was confirmed by RT-PCR (Fig. 3B).

To determine the effect of *TsABA8’OH2* overexpressionon the germinability of tobacco seeds, germination tests were performed (Fig. 5A). Seeds of all tested *35S::TsABA8’OH2* transgenic lines started to germinate earlier than seeds of wild-type plants. Four days after the imbibition started, the seeds of *35S::TsABA8’OH2* transgenic lines germinated between 25% (*35S::TsABA8’OH2-10*) and 40% (*35S::TsABA8’OH2-12*), while the wild-type seeds germinated in less than 5% (Fig. 5A, Supplementary Fig. S3).

In dry seeds, the ABA content in *35S::TsABA8’OH2* transgenic lines was by 15% (*35S::TsABA8’OH2-10*) to 25% (*35S::TsABA8’OH2-12*)higher than in wild-type plants (Fig. 5B, Supplementary Fig. S3). After imbibition, these differences in ABA content between the transgenic and wild-type plants were maintained through the entire experimental period.

Furthermore, seeds of transgenic *35S::TsABA8’OH2* lines were less sensitive to exogenous ABA in comparison to seeds of wild-type plants (Fig. 5C, Fig. 5D, Supplementary Fig. S4). At the 14th day of imbibition on medium with 5 µM ABA, the seeds of *35S::TsABA8’OH2* transgenic lines germinated between 25 and 35%, while the wild-type seeds germinated only in 13%. At the 21st day of imbibition on medium with 5 or 10 µM ABA, the number of germinated seeds was about 1.6-fold higher in the transgenic lines compared to wild-type plants.

**Discussion**

It is already well documented that the PHS susceptibility of cereal grains directly correlates with the dormancy level (Gao et al., 2013; Mohan et al., 2009). Similarly, in triticale, while imbibed grains of the Leontino cultivar germinated when freshly harvested, grains of the Fredro cultivar germinated only after three months of postharvest storage (Supplementary Fig. S1). Thus, less susceptibility to PHS of the Fredro cultivar correlates with slower grain dormancy release. Also in wheat, grains of cultivars less susceptible to PHS required a longer postharvest period, during which dormancy was broken (Gerjets et al., 2010; Tuttle et al. 2015).

Despite the fact that after-ripening greatly changes dormancy status, especially of the Fredro cultivar, the ABA content of dry triticale grains in both analysed cultivars was not affected and was twice higher in Fredro than Leontino for both FH and AR grains (Fig. 1). The different ABA content in dry grains of triticale cultivars differing in the PHS susceptibility might result from the varied rate of ABA biosynthesis and catabolism during grain maturation, as previously described (DeLaethauwer et al, 2014; Fidler et al., 2016). Postharvest storage did not change significantly the ABA content also in dry seeds of other species such as Arabidopsis, barley, and wheat (Ali-Rachedi et al., 2004; Jacobsen et al., 2002; Liu et al., 2013; Millar et al., 2006). It was suggested, thereafter, that the ABA content in dry seeds it is not always a reliable indicator of dormancy status but rather there is a varied ability of seeds to synthesize and catabolize ABA, which affects the ABA content after imbibition (Millar et al., 2006; Gubler et al., 2008). Therefore, it can be assumed that the differences in germinabilty of FH grains of two triticale cultivars that differ in their resistance to PHS might result not only from different ABA content in dry grains but mainly from changes in ABA content after grain imbibition. Indeed, in the first hours of imbibition of FH grains, a decrease in ABA content was observed in the embryos of both triticale cultivars, but to a much lower level in Leontino, a cultivar less susceptible to PHS than Fredro (Fig. 1). The high level of ABA content in imbibed FH Fredro grains prevents germination of this cultivar. The reduction of ABA content, which enables germination, was observed in imbibed Fredro grains only after postharvest storage (Fig. 1). At 4th hour of imbibition, the ABA content in AR Fredro grains decreased almost 3 times, while in FH grains only by 35%. Thus, after- ripening greatly altered the dynamic of ABA changes observed in the embryos of imbibed Fredro grains and allowed germination. To date, studies describing changes in ABA content during imbibition of grains of cultivars with different PHS susceptibility have not been reported. However, changes in ABA content during imbibition of dormant or non-dormant (after-ripened) wheat and barley grains were described and were similar to that observed in imbibed Fredro and Leontino grains, respectively (Jacobsen et al., 2002; Jacobsen et al., 2013; Millar et al., 2006).

 Among genes related to ABA biosynthesis, the greatest differences between Leontino and Fredro grains were observed in the expression of *TsNCED1* (Fig. 2). After imbibition of FH grains, the expression of *TsNCED1* was up-regulated in embryos of both cultivars, but to a much higher level in Fredro than in Leontino, where it remained high until the end of the experiment. Thus the elevated expression of *TsNCED1* seems to be essential for maintaining a high ABA concentration during imbibition of dormant FH grains of the Fredro cultivar. Also in barley and wheat, the maintenance of dormancy of imbibed dormant grains was accompanied by increased expressionofABA biosynthetic genes, *HvNCED1* and *TaNCED1*, respectively (Gubler et al. 2008; Jacobsen et al., 2013; Millar et al., 2006). Furthermore, the *TsNCED1* expression in triticale was greatly affected by after-ripening, especially in imbibed grains of Fredro. After imbibition of AR Fredro grains, the transcript level of *TsNCED1* decreases much earlier and to a greater extent than in FH grains, which was correlated with a greater decrease in ABA content (Fig. 1) and dormancy release (Supplementary Fig. S1). However, the effect of after-ripening on the expression of genes involved in ABA biosynthesis is not unequivocal. For example, after imbibition of non-dormant AR Arabidopsis seeds, the changes in expression of genes encoding NCED compared to dormant seeds were not observed (Millar et al., 2006). In turn, in cereals, after imbibition of barley and wheat grains, the transcript levels of *HvNCED1* and *TaNCED1* were lower in embryos of non-dormant AR grains than in dormant FH grains (Ishibashi et al., 2017; Kashiwakura et al., 2016; Jacobsen et al., 2013; Millar et al., 2006), which was in line with the results obtained for triticale. Thus, the essential role of *NCED1* in dormancy maintenance after grains imbibition may apply not only to triticale but also to other cereals. In cereals, the role of *NCED2*, the second gene encoding 9-*cis*-epoxycarotenoid dioxygenase, in the regulation of ABA content and grain dormancy in imbibed grains is unclear. In triticale, a temporal up-regulation of *TsNCED2* expression was observed during early hours of imbibition only of FH grains in both cultivars, and was higher in Fredro than in Leontino. During imbibition of AR grains the expression of *TsNCED2* was at constant, low level. Contrary, during early hours of imbibition of barley and wheat grains, the expression of *HvNCED2* and *TaNCED2,* respectively, was higher in AR than in FH grains (Gubler et al., 2008; Jacobsen et al., 2013; Millar et al., 2006). However, in other wheat varieties, the *TsNCED2* expression in imbibed grains was either suppressed (Kashiwakura et al., 2016) or remained unchanged (Liu et al., 2013) due to post-harvest storage. Thus, it can be assumed that depending on the species or even cultivar, postharvest storage may differentially affect *NCED2* expression after imbibition.

It is believed that the decrease in ABA content, observed after imbibition, may be the result of a more intensive catabolism of this phytohormone (Nambara et al., 2010). Indeed, the elevated transcript levels of *TsABA8'OH1* and *TsABA8'OH2* encoding ABA 8'-hydroxylase, an enzyme involved in ABA inactivation, were observed in the first hours after imbibition of FH and AR grains of both triticale cultivars (Fig. 2). However, higher expression of these genes was observed in the embryos of Leontino than Fredro. Futhermore, the increased transcript levels of both genes in embryos of Fredro were more pronounced and lasted longer in AR than in FH grains that correlated with the dormancy release of this cultivar. Previous studies have indicated that only homologous of *TsABA8'OH1*, the barley *HvABA8'OH1* and wheat *TaABA8'OH1*, are mainly responsible for an intensified ABA catabolism and ABA decline that enable the dormancy release after imbibition. Moreover, the transcript levels of *HvABA8'OH1* and *TaABA8'OH1* in imbibed grains were strongly increased as a result of postharvest storage (Ishibashi et al., 2017; Jacobsen et. al., 2013; Millar et al. 2006). The expression of the *TsABA8'OH2* homologues(the second triticale *TsABA8'OH* gene), the *Brachypodium distachyon BdABA8'OH2*, the barley *HvABA8'OH2* and wheat *TaABA8'OH2* was observed at very low, constant level in imbibed grains (Barrero et al., 2012; Gubler et al. 2008; Jacobsen et al., 2013; Millar et al., 2006). Thus, the involvement of these genes in the regulation of ABA content in imbibed grains has not been suggested so far.. Only recently a temporary induction of both *TaABA8’OH1* and *TaABA8’OH2* expression has been observed during the early imbibition of wheat grains and, as in case of triticale, an important role of both genes in the regulation of ABA catabolism during early imbibition has been suggested (Kashiwakura et al., 2016).

Based on our results, it can be assumed that changes in ABA content in imbibed triticale FH and AR grains are the result of a balance between ABA biosynthesis, controlled by *TsNCED1,* and ABA catabolism, regulated by *TsABA8'OH1* and *TsABA8'OH2.* Furthermore,the rate of ABA biosynthesis and catabolism during imbibition of triticale grains varies between cultivars with different PHS susceptibility. After-ripening induces changes in the dormancy state of triticale grains, especially the Fredro cultivar, with concomitant changes in the dynamics of ABA metabolism, both biosynthesis and catabolism. However, it should be noted that after-ripening can also affect ABA embryo sensitivity (Matilla et al., 2015). In embryos of the Fredro grains, a decrease in the sensitivity to ABA occurred only after postharvest storage and was accompanied by grain dormancy release (Zdunek-Zastocka et al., 2016).

 Both NCED and ABA8’OH are encoded by a gene family whose members can be expressed in different tissues and are involved in different physiological processes (Bak et al., 2011; Finkelstein et al., 2008; Zdunek-Zastocka and Sobczak, 2013). Therefore, in order to confirm the contribution of *TsNCED1* and *TsABA8'OH2* in ABA metabolism and dormancy regulation, these genes were constitutively expressed in tobacco. Among the ABA catabolism genes, *TsABA8'OH2* was chosen for further study because, as mentioned above, it had not previously been associated with the ABA content regulation in grains. Plants with overexpression or silencing of genes encoding NCED have so far been studied primarily for their response to abiotic stress (Iuchi et al., 2001; Zhang et al., 2008). Only few studies indicate that overexpression or silencing of genes encoding NCED also affects the dormancy of transgenic plant seeds. Overexpression of *LeNCED1* in tomato and rice *NCED3* in Arabidopsis caused increased ABA content in seeds of transgenic plants and delays in germination compared to seeds of wild-type plants (Hwang et al., 2010; Thompson et al., 2000). Similarly, *TsNCED1* overexpression in tobacco plants affected both ABA content in seeds as well as their germination. In all independent transgenic lines, germination was delayed and ABA content in dry and imbibed seeds was about 1.6- fold higher than in wild-type seeds (Fig. 4A, Fig. 4B). Overexpression of triticale *TsABA8'OH2* in tobacco plants accelerated germination and decreased ABA content of transgenic seeds compared to wild-type seeds (Fig. 5A, Fig. 5B). Moreover, the transgenic *35S::TsABA8’OH2* seeds were less sensitive to exogenous ABA and their germination was inhibited to a lesser extent by the presence of 5 µM and 10 µM ABA when compared to wild-type seeds (Fig. 5C, Fig. 5D). Analysis of transgenic seeds confirmed that triticale *TsNCED1* and *TsABA8'OH2* are involved in the regulation of ABA metabolism, and the expression of these genes may affect seed dormancy.

PHS is one of the factor that limit the production and quality of cereals. Thus, search for new molecular markers that will facilitate the selection of PHS-resistant cultivars is an important goal of breeding programs. The results obtained in the presented work indicate that one of the factors determining the dormancy and therefore, the susceptibility to PHS of triticale grains, might be their varied ability to synthesize and catabolize ABA, which influences the ABA content after imbibition. Therefore, the identification of genes which determine the rate of ABA synthesis and catabolism during imbibition of cereal grains, should be of interest. In cereals such as wheat, barley and now also in triticale, the key role in the regulation of ABA synthesis after imbibition of grain is attributed to *NCED1*, whereas the ABA catabolism is mainly controlled by *ABA8'OH1* (Gubler et al., 2008; Jacobsen et al., 2013; Millar et al., 2006). However, the results obtained in this study indicate that in addition to the ABA metabolic genes committed so far to play an important role in dormancy regulation (*NCED1, ABA8’OH1*), and thus susceptibility to PHS, also manipulations within *TsABA8'OH2* may be effective in improving resistance to PHS.

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**Figures**



**Fig. 1.** Abscisic acid content in embryos of dry and imbibed freshly harvested (FH) and after-ripened (AR) grains of Leontino and Fredro cultivars. Vertical bars indicate the mean ± SD of at least three independent measurements. The dry grains are these at 0 hour after imbibition.



**Fig. 2.** Transcript levels of *TsNCEDs* and *TsABA8'OHs* in embryos of imbibed freshly harvested (A) and after-ripened (B) grains of Leontino and Fredro cultivars. Relative transcript levels were quantified using the ImageJ software, and the value corresponding to the expression of an individual gene in a Leontino variety at the start of the experiment (0 hour) was set as 1. Vertical bars indicate the mean ± SD of at least two technical replicates of RT-PCR reaction performed for each of two biological replicates.



**Fig. 3.** Semiquantitative RT-PCR analyses of tobacco seeds of plants overexpressing *TsNCED1* (A) or *TsABA8'OH2* (B). RT-PCR reactions were performed with *TsNCED1* or *TsABA8'OH2* gene-specific primers. WT – wild-type plants; 13, 27, 31 - tobacco transgenic lines *35S::TsNCED1*; 10, 12, 15 – tobacco transgenic lines *35S::TsABA8'OH2*.



**Fig. 4.** Germination (A) and abscisic acid content (B) of tobacco seeds of plants overexpressing *TsNCED1*. Vertical bars indicate the mean ± SD of three independent replicates. WT – wild-type plants; *35S::TsNCED1-13, -27, -31* – tobacco transgenic lines.



**Fig. 5.** Germination (A, C, D) and abscisic acid content (B) of transgenic tobacco seeds of plants overexpressing *TsABA8'OH2*. Seeds were germinated on ½ MS (A, B) or on ½ MS containing 5 µM ABA (C) or 10 µM ABA (D). Vertical bars indicate the mean ± SD of three independent replicates. WT – wild-type plants; *35S::TsABA8'OH2-10*, *-12*, *-15* – tobacco transgenic lines.