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RESEARCH NOTE

Changes in gene expression patterns associated with microspore embryogenesis in hexaploid triticale (×*Triticosecale* Wittm.)

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Abstract To gain a better understanding of the molecular mechanisms controlling microspore embryogenesis (ME) in triticale (×*Triticosecale* Wittm.), the expression patterns of 13 genes, previously identified in bread wheat to be associated with microspore-derived embryo development, were analysed. Four triticale doubled haploid (DH) lines, significantly different with respect to embryogenic potential, were studied. The gene expression profile was dissected at different points of the ME induction procedure up to the 8th day of in vitro culture (dc). RT-PCR revealed that these 13 genes were expressed during triticale ME. Variations in gene expression profiles were observed between the studied DH lines. DH28 (highly embryogenic) was the only one in which all analysed genes (*Ta.TPD1-like*, *TAA1b*, *GSTF2*, *GSTA2*, *CHI3*, *Tad1*, *XIP-R1*, *Ta-AGL14*, *TaNF-YA7*, *SERK2*, *SERK1*, *TaEXPB4*, *TaME1*) were up-regulated during the first 8dc. In the less embryogenic DH31, *TAA1b*, *GSTA2* and *TaEXPB4* were already induced on 4dc. In DH25, ME was initiated quite efficiently but soon inhibited, which coincided with the lack of gene expression (*TaEXPB4*, *TaME1*) or down-regulation (*Tad1*, *XIP-R1*, *TaAGL14*, *TaNF-YA*, *SERK2*, *SERK1*) on 8dc. In the recalcitrant DH50 line, the majority of genes were expressed at a lower level or not at all, indicating disturbances in ME initiation. In this study, the molecular mechanisms involved in triticale ME induction were analysed for the first time, laying the foundation for further characterisation of specific genes controlling microspore-derived embryo development.

Keywords Androgenesis · Microspore reprogramming · Gene expression · Triticale

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Introduction

Due to its yield potential, good grain quality and high tolerance for environmental conditions, triticale (×*Triticosecale* Wittm.) is a very promising crop candidate for modern agricultural systems, especially bio-organic and sustainable farming. The continued growth of the economic importance of this cereal generates a strong interest in its genetics and genome organisation and in biotechnological tools which can be used for its further improvement (Góral et al. 2005; Tams et al. 2005; Alheit et al. 2011; Badea et al. 2011; Tyrka et al. 2011; Krzewska et al. 2012; Żur et al. 2012). Among others, the process termed 'microspore embryogenesis' (ME) or 'androgenesis', as a method for fast production of totally homozygotic, doubled haploid (DH) lines, can significantly accelerate breeding progress.

Since the first report describing anther-derived triticale plant formation (Wang et al. 1973), considerable progress in DH technology has been made, but great genotype dependency, the rather poor regeneration ability of the produced embryo-like structures (ELS) and a high rate of albino plant formation continue to limit wide application of this technology (Tuvesson et al. 2003). Any improvements that might increase the effectiveness of triticale DH production would be highly valued, especially by triticale breeders for whom the instant production of true breeding lines would bring considerable profits.

It is well known that ME is accompanied by many physiological, metabolomical and molecular changes, but it is very difficult to distinguish those directly involved in embryogenesis induction. Over the past decade many efforts have been undertaken to obtain a better understanding of the mechanisms that induce microspore reprogramming from the gametophytic to the sporophytic pathways. Despite extensive studies, the knowledge concerning the molecular and physiological background behind this switch is still fragmentary. The expression patterns of genes associated with microspore reprogramming have been examined widely in model plant species such as tobacco, rapeseed and barley (for review see Hosp et al. 2007; Soriano et al. 2013). In barley, a number of candidate genes have been identified and associated with ME induced after stress mannitol treatment (Maraschin et al. 2006; Muñoz-Amatriaín et al. 2006, 2009). Also, in wheat, five genes have been identified in microspore-derived embryogenic structures, one of them corresponding to an early cysteine-labelled metallothionein (EcMt) (Reynolds and Kitto 1992; Reynolds and Crawford 1996). Recently, a collection of wheat genes induced during microspore-derived embryo development has been expanded and connected to early, middle and late stages of microspore embryogenesis (Sánchez-Díaz et al. 2013).

In order to gain a better understanding of the molecular mechanisms that control triticale ME, the expression profile of wheat orthologous genes controlling intra-embryo communication (*Ta.TPD1-like*), early cell pattern formation (*TaAGL14*), cell wall modification (*XIP*-R1), signalling (*TAA1b*, *SERK1*, *SERK2*), defence reactions (*GSTF2*, *GSTA2*, *CHI3*, *Tad1*) and embryo differentiation (*TaEXPB4*, *TaNF-YA7*, *TaME1*) were analysed in four triticale DH lines with different androgenic responses. To our knowledge this is the first study concerning the expression of genes connected with ME in triticale.

Materials and methods

Plant material

The population of 146 DH lines of winter triticale was derived from the F1 generation of a cross between German inbred line ‘Saka 3006’ and Polish cv. ‘Modus’ by the maize method (Wędzony 2003) in the State Plant Breeding Institute, Hohenheim University in Stuttgart (Germany), and kindly provided by Dr. Eva Bauer. Four DH lines selected from this population were used in this study: two high- (DH28 and DH31) and two low-embryogenic (DH25 and DH50). Germinating triticale kernels were vernalised and donor plants were grown as described earlier by Krzewska et al. (2012).

Protocol for ME induction

Tillers with central florets at the mid- to late-uninucleate microspore stage were pre-treated at 4 °C in the dark for 3 weeks. The protocol for ME induction was described previously by Żur et al. (2009). Collected microspores were re-suspended in a volume of 190-2 medium modified according to Pauk et al. (2000, 2003) to produce a final suspension density of 70,000 microspores per ml. The microspore suspensions were co-cultured with immature triticale ovaries (10 ovaries per ml; 1.5 ml per 35 × 10 mm Petri dish) and incubated in darkness at 26 °C.

Sample collection and cytological analysis

The samples of microspores were directly isolated from freshly-cut tillers (0dp) and low-temperature-treated tillers (21dp) and collected with the use of a Pasteur pipette from Petri dishes after 4 and, finally, after 8 days of in vitro culture (4dc, 8dc).

At the same time points, the course of ME was monitored with the use of a Nikon Eclipse TS100 inverted microscope equipped with Hoffman modulation contrast and a DS-Ri1 digital camera and processed by Laboratory Imaging Ltd. NIS-Elements AR 2.10 programme (Fig. 1). The percentages of microspores at each stage of development were calculated for the total of 500 objects per analysis. The experiment was based on five biological replicates (each Petri dish containing 1.5 ml of microspore suspension was considered one replicate).

RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol Reagent (Gibco BRL) and cleaned with an RNeasy MinElute Cleanup kit (Qiagen). cDNA synthesis was performed using an MMLV RT Reverse Transcriptase kit (Promega).

Selection of genes

Genes were selected according to their expression pattern in wheat ME (Sánchez-Díaz et al. 2013). The following genes were analysed: TaAffx.3154.1 (*Ta.TPD1-like*), Ta.9528.1 (*TAA1b*), Ta.1775.1 (*GSTF2*), Ta.303.2 (*GSTA2*), Ta.21342.1 (*CHI3*), Ta.28319.1 (*Tad1*), Ta.13785.1 (*XIP-R1*), Ta.6411.1 (*TaAGL14*), Ta.10047.1 (*TaNF-YA7*), Ta.6832.1 (*SERK2*), Ta.12817.1 (*SERK1*), Ta.3749.1 (*TaEXPB4*) and Ta.7773.1 (*Ta.ME1*). The 18S ribosomal wheat gene was used as a control.

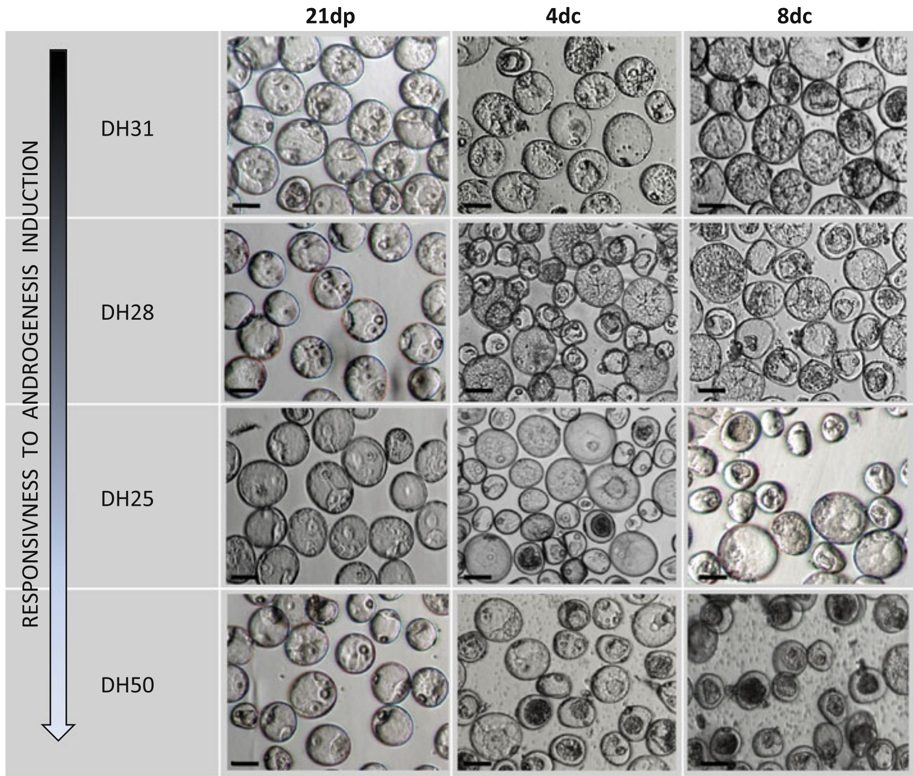

Fig. 1 The progressive stages of embryogenesis in isolated microspore cultures of four doubled haploid (DH) lines of triticale (×*Triticosecale* Wittm.) of high (*DH31*, *DH28*) and low (*DH25*, *DH50*) androgenic potential. The morphology of microspores: after 3 weeks of low temperature (4 °C) embryogenesis-inducing treatment (*21dp*); after 4 days of in vitro culture (*4dc*); and after 8 days of in vitro culture (*8dc*). Hoffman contrast, *bars* 20 µm

Table 1 PCR primer sequence and annealing temperature for semiquantitative RT-PCR

Unigen	Forward 5′–3′	Reverse 5′–3′	Tm
TaAffx.3154.1	AAGTTTCAGCGTCTTCCTCGCT	TCGATGCAGGTGTTGGTGAACT	58
Ta.9528.1	TGCTCTCCATCCTCTGTTGC	GGGATGTGGACCTTGAAGAAGT	56
Ta.1775.1	CGGCAAAGCTGACGAATCTGTT	ACTTCTCTGCCTTCTTTCCGAACC	58
Ta.303.2	GCGGAATCGAAGAATTAGCAATGG	AAGGTGAACGGGATGTGGTT	56
Ta.21342.1	TTCAAGACGGCGTTGTGGTTCT	TGGTTGTAGCAGTCCAGGTTGT	58
Ta.28319.1	AACGCCTTCTACCAGGTCCCTT	ACGTTGGTGAACTGGCTCGTGTT	60
Ta.13785.1	GGCTCTGGAACTTCAACAAGGACT	TTTGGGTGAACGTAACCGACCT	58
Ta.6411.1	AAGCTGAGCGCTACGGCCTA	CACGAATTGTCCCATTGACG	58
Ta.10047.1	AGGCAAGATGATGTCGGCTTTG	GCATGGTATTGCTTCGCGTTCA	58
Ta.6832.1	AGCTTCGATTCCTCCGTCTT	AGGCACCTGCTGATTGAGTT	56
Ta.12817.1	AACTGTCAGGTGCATTGGTGTC	TCCAGAACTTGGAGGGTGCTAA	57
Ta.3749.1	GGCTACAAGCACACTAACCAGT	TTGCATGGCACCCTTTGGAA	57
Ta.7773.1	ACCAAAGCTCGTGTGATGAGGA	TTCGTTGAGGAAGGCCCAGTT	58
18S rDNA	CGGCTACCACATCCAAGGAA	TGTCACTACCTCCCCGTGTCA	57

Expression analysis by RT-PCR

Standard RT-PCRs were performed using samples and primers indicated in Table 1. Primer pairs were designed based on the wheat gene consensus sequence on HarvEST:Wheat version 1.59 (Sánchez-Díaz et al. 2013). The PCR conditions were as follows: 94 °C for 2 min and 35 cycles at 94 °C for 60 s, 55–60 °C (depending on primers) for 60 s, 72 °C for 60 s, and 72 °C for 10 min using 190 ng of template cDNA. Fragments were visualised by agarose gel electrophoresis with SYBR staining (Invitrogen).

Results and discussion

The progress in the course of ME in suspension cultures of the examined triticale DH lines and final effectiveness of the process is presented in Table 2 and Fig. 1.

Table 2 The progress in the course of microspore embryogenesis and the final effectiveness of the process in four DH lines of triticale (×*Triticosecale* Wittm.)

	DH25	DH50	DH31	DH28
% Embryogenic microspores at 21dp*	13.0	3.4	19.1	18.4
% Embryogenic microspores at 4dc**	9.5	4.4	18.5	15.7
% Embryogenic microspores at 8dc***	2.2	4.2	17.0	9.4
Final effectiveness of ME				
ELS/10^5 MCS	4.4	21.0	139.8	146.5
R/10^5 MCS	0.6	1.0	19.3	32.0
GR/10^5 MCS	0.6	0	17.4	31.4

The percentages of microspores at each stage of development were calculated for the total 500 objects per analysis. The experiment was based on five biological replicates (each Petri dish containing 1.5 ml of microspore suspension was considered one replicate)

21dp*—microspores isolated from low temperature-treated tillers (21 days at 4 °C); embryogenic microspores include star-like structures (SLSs)

4dc**—microspores collected after 4 days of in vitro culture; embryogenic microspores include SLSs and microspores after symmetrical division of the nucleus

8dc***—microspores collected after 8 days of in vitro culture; embryogenic microspores include SLSs, microspores after symmetrical division of the nucleus and multicellular structures

ELS/10^5 MCS—androgenic structures produced per 10^5 microspores

R/10^5 MCS—total number of regenerated plants per 10^5 microspores

GR/10^5 MCS—the number of green plants regenerated per 10^5 microspores

The RT-PCR assay revealed that primer sets designed for genes induced during bread wheat anther culture turned out to be homologous to the triticale cDNA sequence. Differences between DH lines in the level and the pattern of expression were observed in all genes studied and are presented in Fig. 2. Genes identified as induced at 5 days of culture in wheat, namely *TaTPD1-like*, *TAA1b*, *GSTF2* and *GSTA2* (Sánchez-Díaz et al. 2013), were also activated in triticale at the corresponding phase of sporophytic development (4–8dc). Of these four genes, only the glutathione *S*-transferase gene *GSTF2* was induced on 4dc in all studied genotypes. However, differences in expression profile were observed, showing the highest level on 4dc in the low-responding lines (DH25 and DH50) and the highest or equal level on 8dc in the high-responding lines DH28 and DH31, respectively. The other induced *GST* (*GSTA)* presented the same pattern of expression in all lines except DH28, in which it was specifically expressed on 8dc. For the first time, Vrinten et al. (1999) identified *GST* transcripts in mannitol-treated barley microspores in the early stages of embryogenesis, although its expression was not associated with the acquisition of embryogenic potential. The up-regulation of *GST* genes after stress treatment has also been observed in barley by Maraschin et al. (2006), Muñoz-Amatriaín et al. (2006, 2009) and Jacquard et al. (2009). In triticale, their induction after stress treatment was observed for *GSTA* as previously described in wheat (Sánchez-Díaz et al. 2013) and also for *GSTF2*. It seems that high expression of *GSTs* is especially important starting on the 8th day of in vitro culture, which may be associated with the first multicellular structure formation. It might be supposed that *GST*s, the enzymes that catalyse the conjugation of glutathione (GSH) to a wide variety of substrates and can also act as GSH-dependent peroxidase, play an important role in further phases of androgenic structure development. Such a conclusion seems to be confirmed by Gong et al. (2005), who showed that GST expression determined in vitro shoot regeneration from leaf explants of transgenic *Arabidopsis thaliana.*

In highly 'responsive' lines, *TaTPD1-like* was expressed specifically on 4dc in DH28, whereas in DH31 the expression was first observed on 4dc, with the highest level of expression on 8dc. This gene was expressed specifically on 8dc in DH25 and at a low level on 4dc in DH50. Another gene, *TAA1b*, was expressed mainly on 8dc; however, in lines DH31 and DH25, it was also expressed at a low level on 4dc. Initially, the functions of both these genes (*Ta.TPD1-like* and *TAA1b*) were connected to early anther development. *TAPETUM DETERMINANT1 (TPD1)* encodes for a small protein that is required for the maintenance of tapetum cell fate (Yang et al. 2003). However, *Ta.TPD1-like* was also expressed in two-celled proembryos and in wheat embryos excised 10, 12 and 14 days after pollination, and a role in intra-embryogenic cell-to-cell communication was recently proposed for it (Leljak-Levanić et al. 2013; Sánchez-Díaz et al. 2013). The *TAA1b* gene codes for a fatty acil-coA reductase that mediates the biosynthesis of long chains of fatty alcohols (VLCFA) and was expressed within the sporophytic tapetum cells (Wang et al. 2002). The expression profile of *TAA1b* in both wheat and triticale ME and the high level of expression of this gene in excised zygotic wheat embryos suggested another function which could be related to VLCFA signalling (Worrall et al. 2003; Sánchez-Díaz et al. 2013).

Genes characterised previously as middle or late ME genes in wheat were also induced in triticale at the moment of multicellular structure formation (8dc). These genes showed major differences in the expression pattern between lines. Three of them, *CHI3*, *Tad1* and *XIP-R1*, have been associated with stress-induced responses.

In planta, chitinases play an important role inducing defence reactions and in plant growth and development e.g. in zygotic and somatic embryogenesis (review in Grover 2012). In triticale microspore cultures, the expression pattern of the *CHI3* gene was highly genotype-specific and

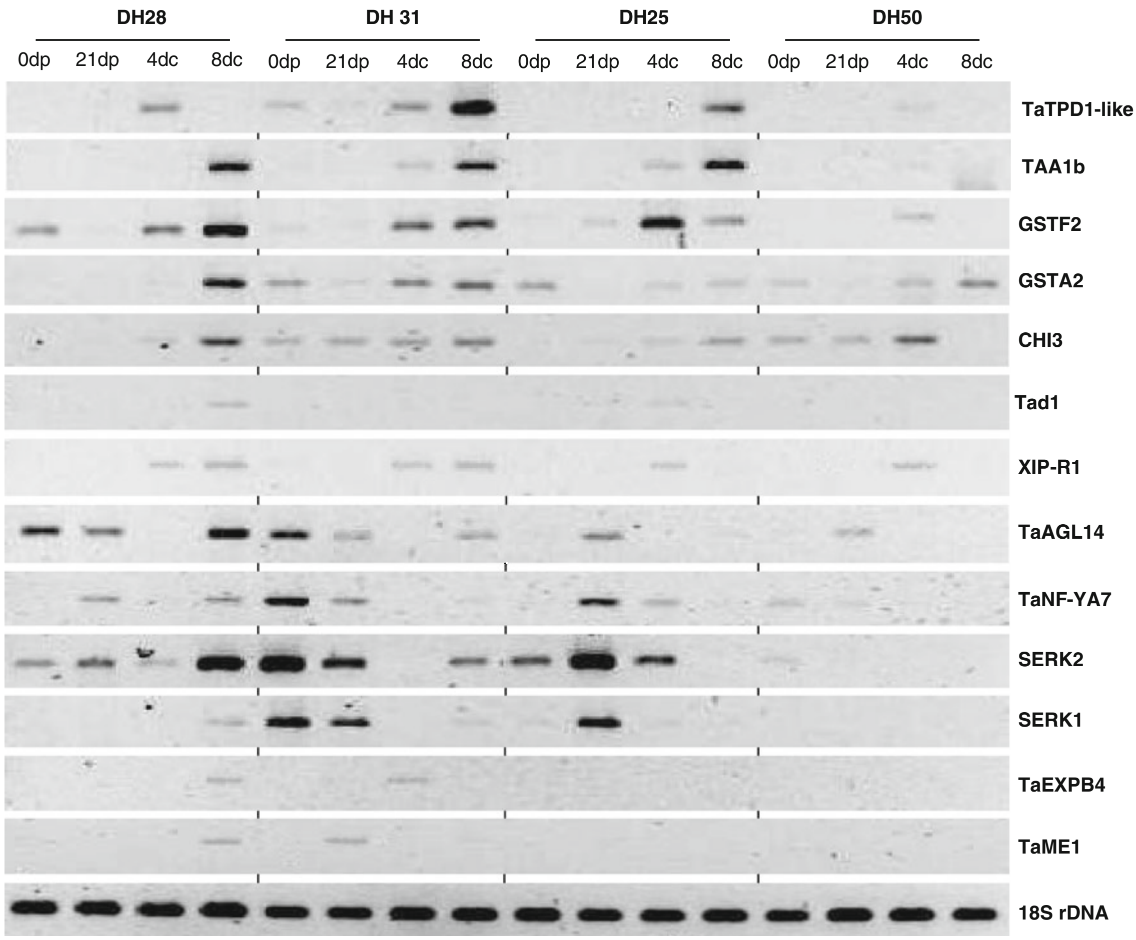

Fig. 2 Expression analyses by semiquantitative RT-PCR of thirteen genes associated with microspore embryogenesis in triticale. Four different stages were assayed in each of the four lines (*DH28*, *DH31*, *DH25* and *DH50*): uni-nucleated microspores before stress treatment (*0dp*), microspores after 21 days of low temperature treatment (*21dp*), microspores after 4 days of in vitro culture (*4dc*), and microspores after 8 days of in vitro culture (*8dc*). 18S rDNA was used as a control

inconsistent. Only in the highly responding DH28 was this gene specifically expressed on 8dc. In wheat, *CHI3* was highly induced in the anther culture phase but its expression was also inconsistent between cultivars, being almost constitutive in the low-responding one.

Although *Tad1* induction was reported earlier in seedlings treated with low temperature (Koike et al. 2002), in this experiment no expression was observed in cold-treated triticale microspores. Instead, *Tad1* was expressed in in vitro cultured microspores, on 4dc or 8dc, but only in lines DH25 and DH28, respectively. It coded for a protein that showed a certain similarity to plant defensins or δ-thionins, small cysteine-rich proteins that play important roles in plant defence against pathogenic fungi (Koike et al. 2002).

Next in this group, *XIP-R1* can be considered an early gene in triticale, as its expression on 4dc was observed in all studied DH lines. Depending on its embryogenic potential, this gene was up- or down-regulated on 8dc. *XIP-R1* is involved in the degradation of arabinoxylans (AXs), the main non-starch polysaccharides from grain cell walls (Dornez et al. 2010). In wheat, a near-specific expression in ME was observed in *Tad1* and *XIP-R* (Sánchez-Díaz et al. 2013).

In highly-responding lines DH28 and DH31, a group of genes associated with developmental control, namely *TaAGL14*, *TaNF-YA7*, *SERK2* and *SERK1*, was induced on 8dc. These genes were also expressed in uni-nucleated microspores of DH31 and two of them (*TaAGL14*, *SERK2*) in DH28. However, the level of expression in the majority of these genes decreased after the stress treatment. Moreover, these genes were not expressed during culture in the recalcitrant DH50 line and were down-regulated on 8dc in DH25, which was associated with inhibited ME. Different developmental programs are represented by these genes: *TaAGL14* is a MADS-box type II (MIKC-type) gene (Zhao et al. 2006) which controls determination of cell identity in plants (Masiero et al. 2011 and references therein); *TaNF-YA7* is a nuclear factor that binds to the CCAAT-box element(s) (Stephenson et al. 2007) as the *Arabidopsis LEAFY*

COTYLEDON1 (LEC1) gene, one of the major regulators of embryogenesis (Harada 1999, and references therein); *SERK* proteins are involved in signalling pathways associated with cell pluripotency and reprogramming (Hecht et al. 2001). In many plant species their expression has been associated with the early stages of zygotic and somatic embryogenesis (Savona et al. 2012, and references therein). In highly-responding cultivars of wheat, *SERK1* and *SERK2* were expressed only before the stress treatment (Sánchez-Díaz et al. 2013).

A low expression level of *TaEXPB4* and *TaME1*, identified in wheat as associated with late phases of ME, was observed only in the highly 'responsive' DH lines of triticale. In wheat, a role for *TaEXPB4* in cell extensibility associated with active growth at the globular phase of embryo development was proposed by Sánchez-Díaz et al. (2013). The low level of expression of this gene in triticale may be due to the lower demand of cell extensibility, since the structures on 4–8dc are still confined inside the exine. The expression of gene *TaME1* (*MICROSPORE EMBRYOGENESIS-1*) was first observed in the work of Sánchez-Díaz et al. (2013). In wheat, it was identified and characterised as an embryo-specific gene, whereas its expression in triticale was observed on 21dp in DH31 and on 8dc in DH28. None of these genes was expressed at any stage of microspore development in the recalcitrant lines (DH25 and DH50).

In attempting to associate gene expression profiles, cytological observations of the ME process and final plant production efficiency, it should be remembered that final ME efficiency is also modulated by later events along the developmental pathway. For example, proper exine rupture and successful release of multicellular structure are very important for the final efficiency of the process. Considering this, DH28, the line with the highest final androgenesis efficiency, is the only one in which all genes were expressed during the microspore culture phase. In line DH31, several genes were expressed earlier than in the other DH lines, confirming morphological observations indicating faster initiation of ME. The differences in gene expression between these lines suggest that DH28 possesses a more precise early signalling regulation (*TaTPD1-like*). Moreover, DH31 was characterised by lower activation of genes related to cell identity and embryogenesis (*TaAGL14*, *TaNF-YA7* and *SERK2*). Altogether, this can result in lower final efficiency of ME. The gene expression pattern in line DH25 also seems to be in agreement with the morphological characterisation of this genotype, indicating a high induction rate on 4dc and severe disturbances in ME progress on 8dc. Finally, in line DH50, the analysed genes were not expressed or expressed at very low levels, indicating that initiation of ME was faulty from the beginning.

The morphological characterisation of isolated microspore cultures of four DH lines with different rates of ME induction, together with the description of expression profile of genes related to mechanisms such as signalling, cell fate determination, and cell wall modification, enabled the first step in identification of the molecular mechanisms involved in triticale ME induction. These results confirm that despite different procedures used for triticale and wheat microspore reprogramming (cold vs. mannitol stress treatment) and various androgenesis methods (isolated microspore vs anther culture) at least some molecular mechanisms are the same. The obtained results should be considered as preliminary and the presented study should be seen as the starting point for further characterisation of genes associated with androgenesis induction in triticale.

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