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Stability and instability processes in the calli of *Fagopyrum tataricum* that have different morphogenic potentials

Alexander Betekhtin¹ · Artur Pinski¹ · Anna Milewska-Hendel² · Ewa Kurczynska² · Robert Hasterok¹

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Abstract

The morphogenic callus (MC) of *Fagopyrum tataricum* contains a large amount of flavonoids, especially rutin, and exhibits a high level of antioxidant activity. A non-morphogenic callus (NC) may appear on the surface of the MC after two to three years of cultivation and is then subjected to a consistently high level of oxidative stress. The elucidation of the molecular background of this instability is essential for gaining a better understanding of the somaclonal variation mechanisms in tissue cultures that have different morphogenic potentials. Thus, in this study we show that continuous oxidative stress in a NC might be connected with a rapid senescence process and as a result, in the upregulation of the genes that are connected with the telomere complexity, ethylene biosynthesis and the expression of DNA methyltransferases. Moreover, we analysed the presence of the hydroxyproline-rich glycoproteins in the calli and demonstrated the differences between the MC and NC. The LM2 antibody can be useful as a marker of the cells in the MC that are embryogenically determined, while the MAC207 antibody seems to be a positive marker of a MC as its signal was absent in the NC. This study also provides the first report on the effect of trichostatin A on the DNA methyltransferases and demethylases in a MC.

Key message

LM2 can be used as a marker of the embryogenically determined cells. Overproduction of ethylene is present in the NC.

Keywords *Fagopyrum tataricum* · Calli · Morphogenic potential · Hydroxyproline-rich glycoproteins · Ethylene · The maintenance of telomere · DNA methyltransferases · Trichostatin A

Introduction

Buckwheat (*Fagopyrum*) are dicotyledonous plants of the Polygonaceae family. The genus consists of 26 annual and perennial species that primarily occur in the highlands of Eurasia. The two species that are the most cultivated are common buckwheat (*Fagopyrum esculentum* Moench) and Tartary buckwheat (*Fagopyrum tataricum* Gaertn) (Zhou

2018). *F. tataricum* contains a large amount of flavonoids, especially rutin, which due to its antioxidant activity, is well known for its beneficial medicinal effect in the treatment of the fragility of blood vessels and hypertension (Kreft 2016). Compared to *F. esculentum*, *F. tataricum* contains more phenolic compounds in every part of the plant as well as at different growth stages (Gupta et al. 2011). The seeds of *F. tataricum* contain higher amounts of rutin, i.e. 0.8–1.7% dry weight (d.w.), compared to the 0.01% d.w. in *F. esculentum* (Fabjan et al. 2003). The quantity and quality of protein in buckwheat is higher than in wheat, rice, maize and sorghum (Fabian and Ju 2011; Khan and Shewry 2009).

Recently, we demonstrated that the morphogenic callus (MC) of *F. tataricum* can retain nuclear genome stability during as many as ten years of its cultivation (Betekhtin et al. 2017). The appearance of a non-morphogenic callus (NC) on the surface of an MC seems to be connected with the endoreduplication cycles. As was demonstrated earlier, an MC consists of proembryogenic cell complexes (PECCs) and a ‘soft’

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callus. During the cyclical callus development, the mature PECCs disintegrate, thereby giving rise to young PECCs and 'soft' callus cells. The NCs, which are formed exclusively from the parenchymous type cells of a callus, differ from the MCs due to their friable structure, high growth rate and complete loss of the capacity for morphogenesis (Betekhtin et al. 2017; Rumyantseva et al. 2003, 2004). The NC is a good example of the total instability and fast senescence in a plant tissue culture. The tissue culture research demonstrated that the MC of *F. tataricum* contains a large amount of phenolics as well as having a high antioxidant activity compared to the NC (Akulov et al. 2018). Moreover, the same authors highlighted the use of an *F. tataricum* tissue culture to study the metabolism of phenolic compounds as well as their being a promising alternative source of rutin and other biologically active phenolic compounds. A better understanding of the basis of any tissue culture-induced genetic variation should be helpful in developing more stable cell culture systems.

The primary cell wall plays an important role in regulating the morphogenetic processes (Knox 1992) and its chemical composition is crucial for establishing and/or maintaining the cellular differentiation status of cells (Corral-Martinez et al. 2016; Ikeuchi et al. 2013; Potocka et al. 2018). The main cell wall polysaccharides are pectins, and hydroxyproline-rich glycoproteins (HRGPs, arabinogalactan proteins (AGPs) and extensins), which all participate, though to a different extent, in cell expansion, adhesion and as regulatory molecules, and therefore, they are key determinants of the physical properties of the cell wall (Willats et al. 2001). It is well established that somatic embryogenesis (SE) is accompanied by modifications of the structure and molecular composition of the cell wall as well as specific gene expression (Kikuchi et al. 1995; Smertenko and Bozhkov 2014; Verdeil et al. 2001). The importance of HRGPs was shown for the embryogenic and non-embryogenic callus of sugarcane (Suprasanna et al. 2004). The authors demonstrated the HRGPs expression at all of the stages of callus development except the non-embryogenic one. Analysis of HRGPs during banana cultures also suggests that they play an important role in the regeneration process during SE (Pan et al. 2011). The correlation of changes between different HRGPs and reactive oxygen species is well documented (Cosi et al. 2007). HRGPs play a defensive role in host-pathogen interactions as well as changes in their presence under diverse biotic and abiotic factors (Basavaraju et al. 2009; Mareri et al. 2018). Despite the large number of reports about changes in the chemical composition of cell walls and changes in gene expression during SE, such analyses are still very limited for *F. tataricum* calli.

The aim of this study was to perform a complex analysis of the stability/instability in calli of *F. tataricum* that had different morphogenic potentials. We used immunolocalisation techniques against selected cell wall epitopes to compare

whether they can be markers that are diagnostic for the MC and NC. Moreover, because of differences in the growth rate (very fast senescence in the NC, we hypothesised that the ethylene biosynthesis genes are overexpressed in *F. tataricum* NC), genome stability/instability and, most importantly, in their morphogenic potential. Therefore, RT-qPCR was used to assess the expression of the genes that are connected with ethylene biosynthesis, telomere function and DNA methyltransferases/demethylases in relation to the different callus types. Trichostatin A (TSA) was used to demonstrate the affect of histone deacetylase (HDAC) inhibition on the DNA methyltransferases/demethylases.

Materials and methods

The lines of calli from immature embryos of *F. tataricum* were obtained in the Department of Plant Anatomy and Cytology, University of Silesia in Katowice, Poland. Both the MC and NC were cultivated in an incubator at $25\text{ }^{\circ}\text{C} \pm 1$ on an RX medium according to Betekhtin et al. (2017) with some minor modifications that are connected with the application of a ready-to-use B5 medium that includes vitamins (Duchefa, Haarlem, The Netherlands) for callus cultivation rather than a homemade medium.

Histological and immunostaining procedures

The MC and NC were fixed in a mixture of 4% paraformaldehyde and 1% glutaraldehyde in phosphate buffered saline (PBS, pH 7.2) overnight at $4\text{ }^{\circ}\text{C}$. Next, the samples were washed three times in PBS for 15 min each, followed by a dehydration series in increasing ethanol concentrations and then gradually embedded in London Resin (LR White resin, St. Louis, USA). The samples were then cut into $1.5\text{ }\mu\text{m}$ thick cross sections using an EM UC6 ultramicrotome (Leica Biosystems, Wetzlar, Germany) and the sections were placed on glass slides that had been coated with poly-L-lysine. For the general histology, the slides were stained with 0.05% (aqueous solution) Toluidine Blue O (Sigma-Aldrich, St. Louis, MO, USA) for 5 min. For the immunocytochemical analyses, the sections were treated with a blocking buffer (2% foetal calf serum and 2% bovine serum albumin in PBS) for 30 min at room temperature (RT). Next, the samples were incubated with primary monoclonal antibodies (Plant Probes, Leeds, UK) that had been diluted 1:20 in a blocking reagent at $4\text{ }^{\circ}\text{C}$ overnight. The sections were then washed in the blocking buffer three times and incubated with the secondary antibody, labelled with AlexaFluor 488 goat anti-rat IgG (Jackson ImmunoResearch Laboratories, Cambridgeshire, UK) and diluted 1:100 in the blocking buffer for 2 h at RT. The antibodies that were used are listed in Table 1. Next, the samples were washed in the blocking buffer followed by

Table 1 The antibodies that were used for the immunocytochemistry with the relevant references

Antibodies	Epitopes	References
	<i>Hydroxyproline-rich glycoproteins (HRGPs)</i>	
JIM8	Arabinogalactan, AGP glycan	Pennell et al. (1991)
JIM13	Arabinogalactan, arabinogalactan protein, β -D-GlcpA-(1→3)- α -D-GalpA-(1→2)-L-RhA	Knox et al. (1991), Yates and Knox (1994) and Yates et al. (1996)
LM1	Extensin, HRGP, the LM1 epitope most likely includes extensin	Smallwood et al. (1995)
LM2	Arabinogalactan protein, recognises a carbohydrate epitope containing β -linked glucuronic acid	Smallwood et al. (1996) and Yates et al. (1996)
MAC207	Arabinogalactan protein, (beta)GlcA1→3(alpha)GalA1→2Rha	Bradley et al. (1988), Pattathil et al. (2010), Pennell et al. (1989) and Yates et al. (1996)

staining with 0.01% (w/v) fluorescent brightener 28 (FB28) (Sigma-Aldrich, St. Louis, MO, USA) in PBS, which was used to visualise cell walls due to its affinity to cellulose. Subsequently, the samples were rinsed in PBS and distilled water and dried sections were mounted on the slides with Fluoromount (Sigma-Aldrich, St. Louis, MO, USA). As a negative control, the primary antibody was omitted and the blocking buffer was applied along with all of the other steps of the procedure. All of the observations were made and the photographs were taken using an AxioImager Z2 epifluorescence microscope equipped with an AxioCam Mrm monochromatic camera (Zeiss, Oberkochen, Germany) that were equipped with narrow-band filters for AlexaFluor 488 and DAPI.

Real time PCR

A NucleoSpin® RNA Plant (Macherey-Nagel, Düren, Germany) was used to isolate the total RNA from both types of calli. The RNA was isolated on the 11th day of cultivation when it was accompanied by the appearance of PECCs on the surface of the MC along with the high mitotic activity of the NC (Rumyantseva et al. 2004). TSA (Sigma-Aldrich, St. Louis, MO, USA) was used in the RX medium at concentrations of 2.5 and 5 μ M. The concentration and quality of the isolated RNA was assessed using an ND-1000 NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). First-strand cDNA was produced using a Maxima H Minus First Strand cDNA Synthesis Kit with dsD-Nase (Thermo Scientific, Waltham, MA, USA). The product of the reverse transcription was diluted with water at a 4:1 ratio and 2 μ L of this solution and was used for the Real Time RT-qPCR reactions using a LightCycler® 480 SYBR Green I Master in a LightCycler® 480 Real-Time PCR System (Roche, Mannheim, Germany). The primers that were relevant to the genes that were studied are listed in Table 2. The genomic sequence of *F. tataricum* cv. Pinku1 was downloaded from <http://www.mbkbase.org/Pinku1/> (online access 4 February 2018) and loaded into Geneious 11.1.2 software (<http://www.geneious.com>, Kearse

et al. 2012). The genes of interest were found using a keyword search in the Buckwheat Genome DataBase (BGDB), which contains the draft genome sequence of *F. esculentum* Moench (<http://buckwheat.kazusa.or.jp/index.html>, online access 4 February 2018, Yasui et al. 2016). The identity of the targeted genes was additionally confirmed through BLAST searches using protein sequences as a query with respect to their similarities to *Arabidopsis thaliana* (Arabidopsis). The coding DNA sequences from the database were used as the query to search for the homologous genes in the *F. tataricum* cv. Pinku1 genome using a BLAST search in Geneious. The alignments of the gene sequences from *F. tataricum* cv. Pinku1 and *F. esculentum* Moench were performed in Geneious using the Needleman–Wunsch algorithm. The gene-specific primers for the RT-qPCR analysis were designed from the identical sites in the genes of both species using PrimerQuest Tool software (<https://eu.idtdna.com/PrimerQuest/Home/Index>).

The amplification efficiency of each gene was checked according to Taylor et al. (2010). The stability of the actin expression in the calli was checked using the BestKeeper Excel-based tool (Pfaffl et al. 2004). The relative changes in gene expression were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001) and were normalised to the expression level of the actin. The calculations and statistical analysis were performed in the R (v 3.4.3) package pcr and the Student's t-test was used for the statistical analysis.

Results

HRGPs in the MC and NC

To test whether the selected HRGP epitopes were different between the types of calli that were studied, immunocytochemical analyses were performed using specific monoclonal antibodies: JIM8, JIM13, LM1, LM2 and MAC207 (Table 1). The representative histological character of the calli and cell features that were subjected to the immunocytochemical analyses are presented in Fig. 1a, e, i. The

Table 2 Oligonucleotide primers used for RT-qPCR reaction with the relevant description of the genes

Genes	Description of the genes	Primer sequence (5'–3')
<i>FtPinG0002124000.01</i>	<i>Actin</i>	pF-GGAAGTATAGCGTCTGGATTG pR-CTGGACCGGATTCATCATAC
<i>FtPinG0009372500.01</i>	<i>Ethylene response factor 1 (ERF1)</i>	pF-AGAGTTAAGGAGGAAGAGGAG pR-ACGTGTCTGAATCTCGTATCT
<i>FtPinG0004556400.01</i>	<i>1-Amino-cyclopropane-1-carboxylate synthase 6 (ACS6)</i>	pF-GGCCATGGTGAAGAATCTG pR-GCAAGACCCATCTGGATAAC
<i>FtPinG0004154200.01</i>	<i>1-Amino-cyclopropane-1-carboxylate synthase 2 (ACS2)</i>	pF-GATCCACCGGAGCTAATGA pR-GCGGTCAAATGCAGGATAA
<i>FtPinG0008351200.01</i>	<i>Aminocyclopropanecarboxylate oxidase 1 (ACO1)</i>	pF-CGGGACTTGAGTTCTACAAA pR-CTCAGCACTTCCAGTTGAT
<i>FtPinG0000974100.01</i>	<i>Telomerase activating protein Est1 (EST1)</i>	pF-GCTGCACCATTACAAGAGA pR-CACTTCCTCTCTCACCATTAG
<i>FtPinG0008479800.01</i>	<i>Telomerase reverse transcriptase (TERT)</i>	pF-TCAGCATGGGATCTAGTCTTAC pR-CTGGTATTTACCACAAGGAATGG
<i>FtPinG0009136200.01</i>	<i>Protection of telomeres protein 1 (POT1)</i>	pF-CGTACCAGGTTTCTTCAAAC pR-TGTTTTGACACTGTACCAAGG
<i>FtPinG0008019600.01</i>	<i>Protection of telomeres protein 2 (POT2)</i>	pF-CTGGTTTGTGGTATGGTATCT pR-GCACATTAAGTCACTTAGGC
<i>FtPinG0006631600.01</i>	<i>DNA (cytosine-5)-methyltransferase (CMT3)</i>	pF-CCACTGATCCCAAGATTATGC pR-GGTTTCAGCTCTCCCAATAAC
<i>FtPinG0009406600.01</i>	<i>DNA methyltransferase (MET1)</i>	pF-GCTGAAGAGGAAATAGAAGAGG pR-CTGGGTGGATATTGAAGGAAG
<i>FtPinG0006897400.01</i>	<i>DNA methyltransferase (MET2)</i>	pF-GTTGAAGATGAGGCAGAAGAA pR-TGAGAGGATACTGATGGAGAC
<i>FtPinG0009482900.01</i>	<i>DNA glycosylase DEMETER (DME1)</i>	pF-CCACACCAGAACAAGACATC pR-GGTGAACCTTCAACATCCAG
<i>FtPinG0007109000.01</i>	<i>DNA glycosylase DEMETER (DME2)</i>	pF-GGAAGCATTGATTTGGAATGG pR-AAAGCCTAACACACTCTACAC
<i>FtPinG0003657700.01</i>	<i>DNA glycosylase DEMETER (DME3)</i>	pF-CCACAGATTCAACAGTCTCTC pR-GGCCTATGCTTCTTTCTTCT
<i>FtPinG0007109000.01</i>	<i>DNA glycosylase, repressor of transcriptional gene silencing (ROS1)</i>	pF-GAAGTAACGAGAGCCCTTATG pR-CCTGGGAGTTTGTGATGTT

MC consisted of PECCs that had phenolic-containing cells (Fig. 1a, arrows) and a ‘soft’ callus (Fig. 1a, asterisks; e) compared to the NC, which consisted of only parenchymous-type cells (Fig. 1i; due to the difficulty in separating the fluorescence signals that were present in the cell wall and plasma membrane using light microscopy, these two compartments are described together. The signals inside the cells were localised in the intracellular compartments).

The JIM8 epitope in the embryogenic cells of the MC calli was distributed in the cell walls and in the cytoplasm in a dotted manner (Fig. 1b–d). The presence and distribution of this epitope in the “soft” callus was similar to that described above (Fig. 1f–h). Compared to the MC, the epitope labelled with JIM8 antibody in the NC cells was abundantly present in the cytoplasm compartments, in the cell wall and in the newly formed cell walls in particular. This signal was uniformly distributed along the entire length of the walls (Fig. 1j–l, l arrows demonstrate the newly appearing cell walls). The main differences between the MC

and NC calli rely on the amount of the AGP epitope that are recognised by the JIM8 and its cellular localisation, which indicated that the occurrence of this epitope in the NC callus cells was more abundant in both the cytoplasm and the wall. Although the JIM13 signal was present in the cytoplasm compartments in both calli, it was more abundantly present in the NC cells (Fig. 2). The distribution of this epitope was also observed in the cell walls in a dotted manner in the MC cells (Fig. 2b, c, e, f) and was uniformly stained in the NC cells (the reverse of the case of the JIM8 epitope).

Unlike the two previous epitopes, the LM1 epitope was present in the intercellular spaces and on the surface of the phenolic-containing cells (Fig. 3b, e, arrows) and embryogenic cells in the MC cells (Fig. 3a–f). In the NC, it was localised on the surface and in the intercellular spaces (Fig. 3g–l). In the MC, the LM2 antibody was localised in the intracellular compartments (Fig. 4a–c), and in some cases, it was probably also localised in the plasmalemma of the embryogenic and the “soft” callus cells but in very

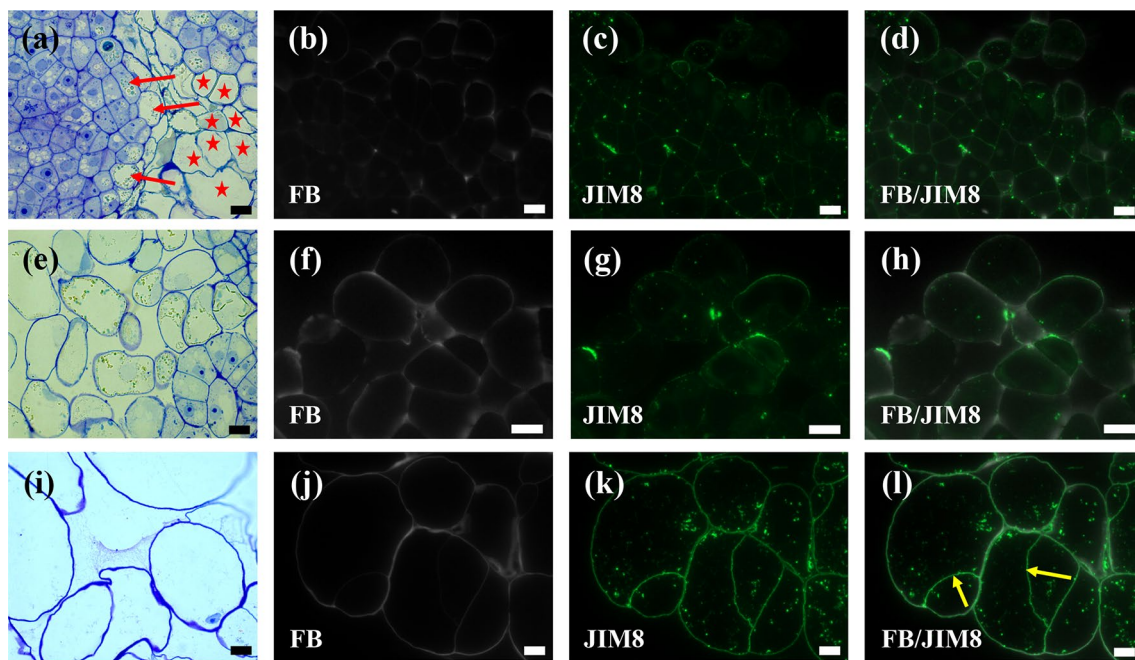


Fig. 1 Histological sections of the *F. tataricum* MC (**a**, **e**) and in NC (**i**). Immunolocalisation of JIM8 in the MC (**b–d**, **f–h**) and NC (**j–l**). FB fluorescent brightener. **a** Arrows demonstrate the phenolic-containing cells, asterisks ‘soft’ callus cells. **l** Arrows demonstrate newly

appeared cell walls. The greenish background visible on some photomicrographs is due to the autofluorescence. Scale bars 10 µm. (Color figure online)

low amounts (Fig. 4f). The signals of this antibody were absent or almost absent in the plasmalemma of the phenolic-containing cells (Fig. 4b, e, asterisks). Compared to the MC, in the NC calli, LM2 was detected in the cell wall and in the intracellular compartments (Fig. 4g–l). The immunolocalisation of MAC207 demonstrated the presence of this epitope in the intercellular spaces of the “soft” callus cells (Fig. 5a–f) and in the intracellular compartments in some cells of the MC (Fig. 5a–c). The NC callus cells were completely negative for the MAC207 signals (Fig. 5g–i).

RT-qPCR of selected genes

In order to characterise the differences that are present between the MC and NC at the molecular level, the genes that are connected with the biosynthesis of ethylene and with the metabolism of ethylene precursors as well as with the telomere length homeostasis and chromosome end protection were assessed (the descriptions of the genes are listed in Table 2). The similarities of the selected genes on the protein level to the proteins encoded by the genes from Arabidopsis are shown in Table 3. The expression of the actin gene was found to be stable in the buckwheat tissue culture material, thus it was used as the reference gene. Moreover, the amplification efficiency for each gene was determined to be close to 100%, thereby permitting the calculations according to the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). The

relative expression level of *ACS2*, *ACS6* and *ERF1* were high in the NC. The *ACS2* and *ACS6* genes were 55- and 30-fold more expressed in the NC. The *ERF1* demonstrated a five times higher expression in the NC (Fig. 6a). The expression level of *ACO1* was lower in the NC (Fig. 6a). The relative expression levels of *TERT*, *EST1* and *POT1* were higher in the NC (Fig. 6b). The *EST1* was characterised by a 20-fold time higher expression in the NC compared to the MC. The *POT2* expression level was slightly higher in the MC than in the NC (Fig. 6b).

The expression levels of the DNA methyltransferases such as *MET1* and *MET2* differed to various degrees between the MC and NC, with the exception of *CMT3*, for which no statistically significant differences were observed (Fig. 6c). The highest level of expression was characteristic for *MET1* and it was six times higher in the NC than in the MC. In contrast, the expression of *MET2* was only slightly higher in the NC compared to the MC. The expression of all of the demethylases (*DME1*, *DME2*, *DME3* and *ROS1*) that were analysed was consistently lower in the NC. In order to analyse the influence of the HDAC inhibition on the on the DNA methyltransferases and demethylases in the MC, we used two different concentrations of TSA (2.5 µM and 5 µM), which was added to the cultivation medium. The results demonstrated an increase in the expression level of all of the analysed methyltransferases and demethylases compared to the untreated MC and that the use of TSA at

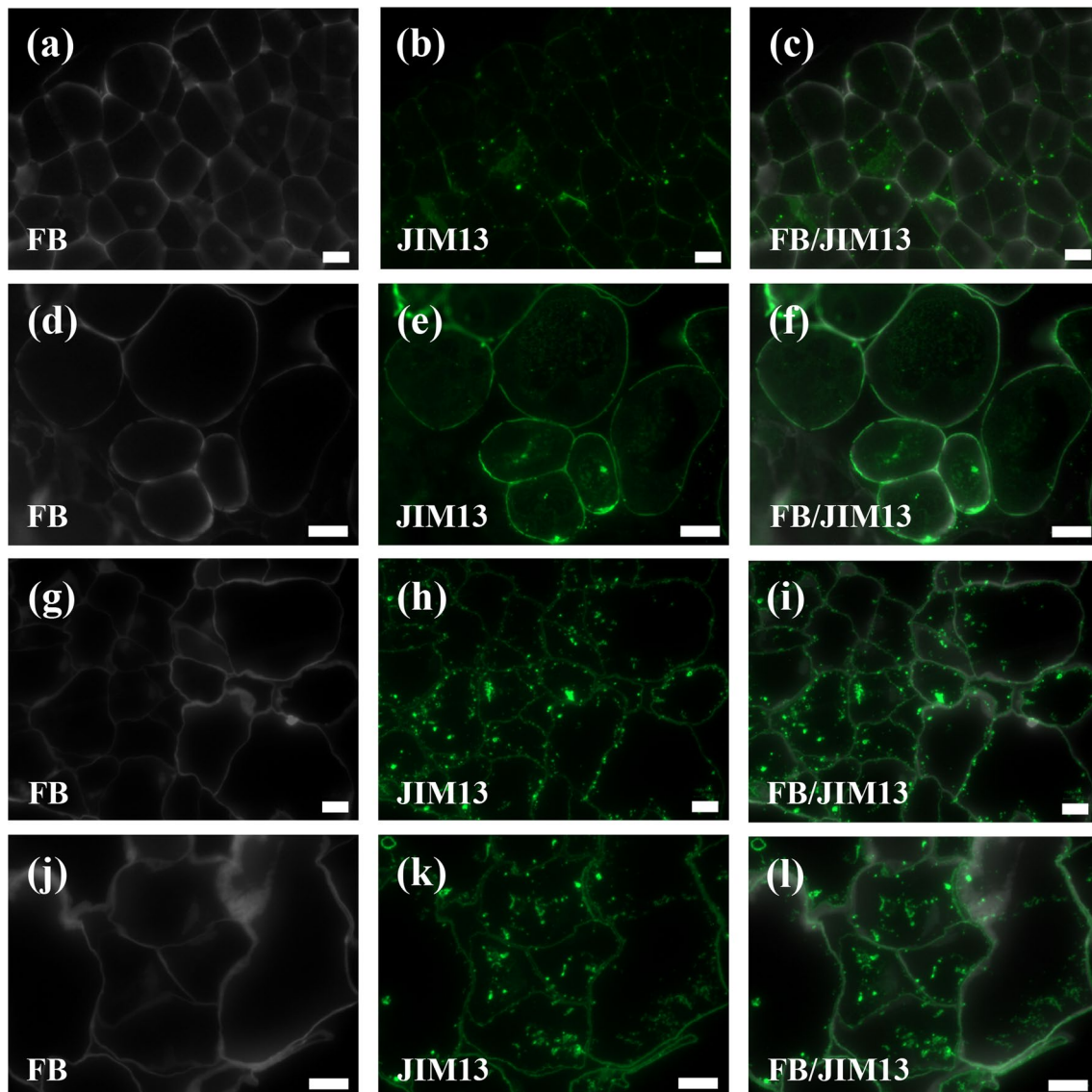


Fig. 2 Immunolocalisation of JIM13 in the *F. tataricum* MC (a–f) and NC (g–l). The greenish background visible on some photomicrographs is due to the autofluorescence. Scale bars 10 μ m. (Color figure online)

a lower concentration resulted in a more prominent effect (Fig. 6d). However, the dynamics of the gene expression clearly decreased at a concentration of 5 μ M.

Discussion

In our work, selected AGPs and extensins were investigated because of their well-known involvement in different developmental processes, including plant growth and development, plant defence, cell proliferation, cell expansion, cell differentiation, cell extension and SE (Pennell 1998; Smith 2001). AGPs are one of the extracellular matrix surface network components that are pivotal for the cell division

and expansion, programmed cell death, SE and many other crucial aspects of the plant cell functions (Betekhtin et al. 2016; Chapman et al. 2000; Romyantseva 2005; Samaj et al. 2008). The studies on *F. tataricum* suggested the presence of this matrix in the MC and its total absence in the NC (Romyantseva et al. 2003). It was shown that the *F. tataricum* MC secreted much more extracellular polymers into the medium compared with the NC (Romyantseva et al. 2004). A significant increase in the secretion of extracellular polymers was connected with the formation of the PECCs. However, these authors did not specify the AGPs or extensins that were secreted. The experiments that were performed in the present study demonstrated the presence of ‘patchy’ signals of LM1 on the surface of a PECC in both the MC and NC.

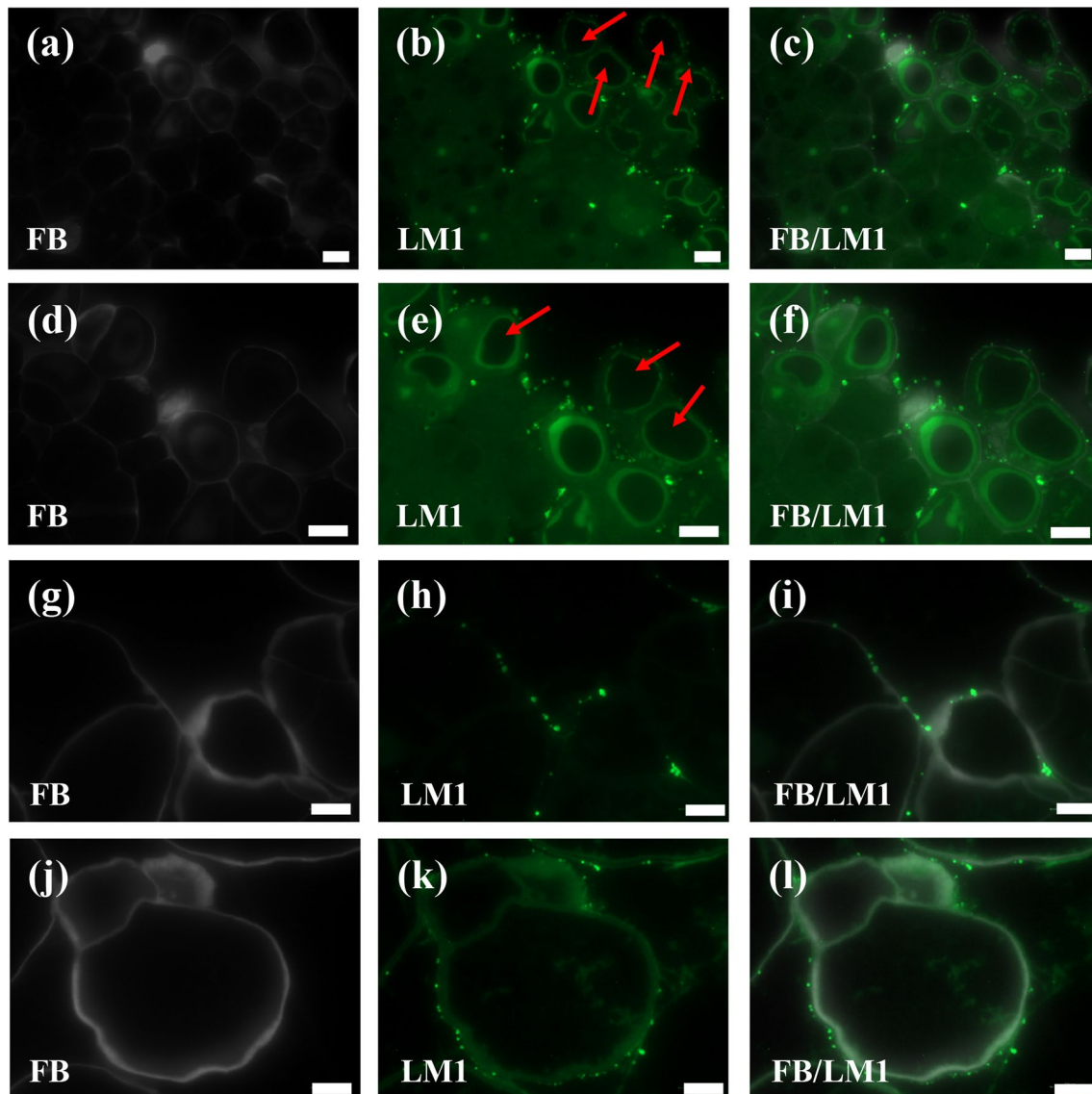


Fig. 3 Immunolocalisation of LM1 in the *F. tataricum* MC (a–f) and NC (g–l). The greenish background visible on some photomicrographs is due to the autofluorescence. **b** and **e** Arrows: the phenolic-containing cells. Scale bars 10 μ m. (Color figure online)

Thus, this type of extensins may be crucial for some developmental processes in both types of the callus. MAC207 seems to be a marker of the MC of *F. tataricum* as it was only present in the intercellular spaces of the “soft” callus cells and in the intracellular compartments (very rarely in some cells) of the MC and was totally absent in the NC. The involvement of this epitope was analysed during *in vitro* cultures, including microsporogenesis (El-Tantawy et al. 2013; Showalter 2001), but most often during zygotic embryogenesis (Leszczuk and Szczuka 2018), thus a comparison of our studies with the literature data is difficult. Nevertheless, the prominent function of these AGPs seems to be the cell–cell interactions or cellular signalling during the formation of a new PECC. The possibility of mediating signal transduction

at the cell wall-plasma membrane interface by AGPs was demonstrated earlier in several studies (Kohorn 2001, 2016; Showalter 2001).

For carrot, maize and *Arabidopsis*, it was shown that the embryogenic callus is marked by its presence in cell walls of the AGP epitopes that are recognised by the JIM8 antibody (Majewska-Sawka and Nothnagel 2000; Potocka et al. 2018). Other studies have shown that this epitope is necessary for somatic embryo formation (McCabe et al. 1997). In the AGPs in the embryogenic calli of *F. tataricum* that have been analysed to date, the presence of JIM4, LM2, JIM13 and JIM16 epitopes has been suggested (Rumyantseva 2005). In *Arabidopsis* explants, LM2 gave either no signal or a very weak signal in the cells that displayed typical embryogenic

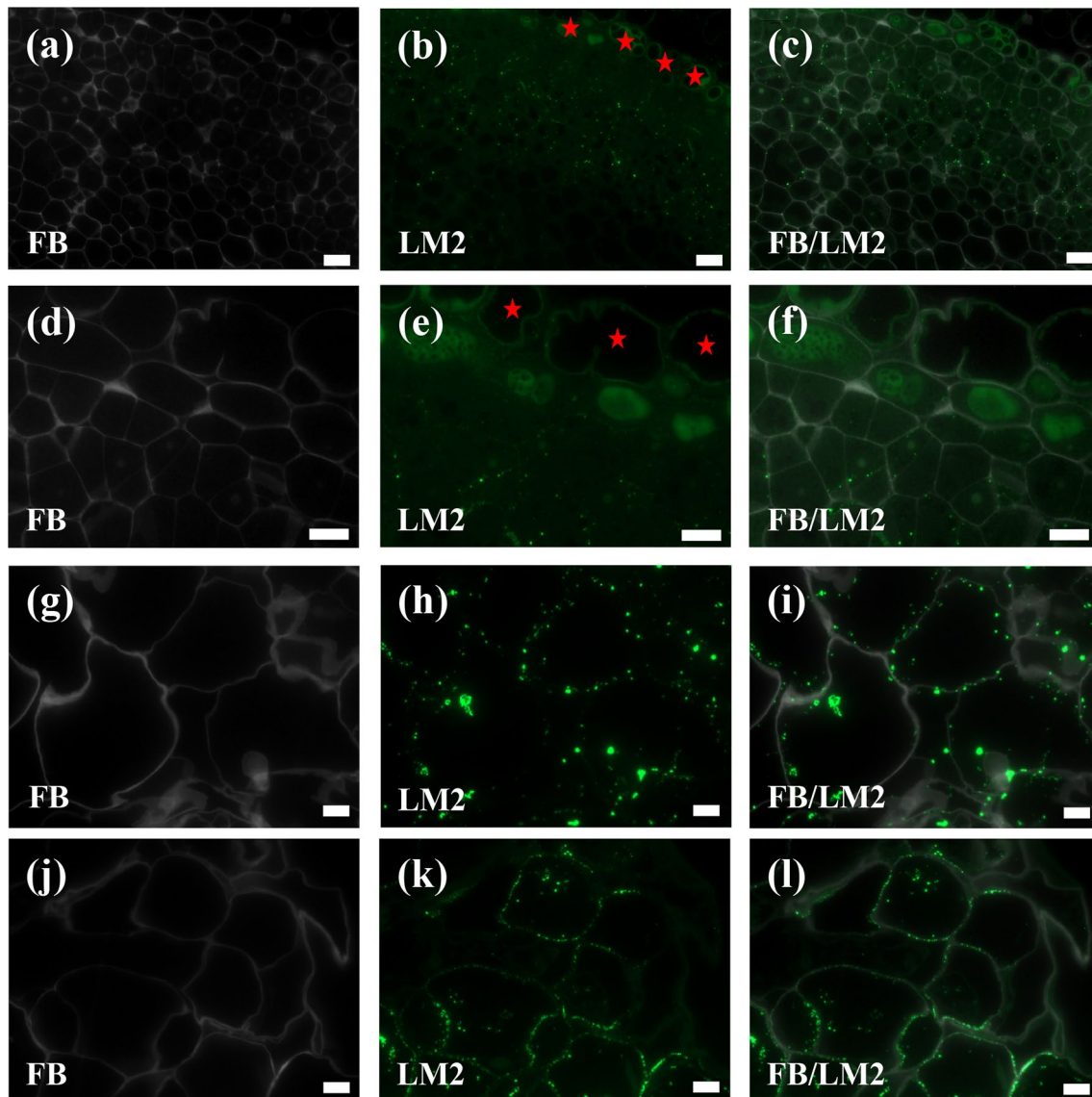


Fig. 4 Immunolocalisation of LM2 in the *F. tataricum* MC (a–f) and NC (g–l). Asterisks: the phenolic-containing cells. The greenish background visible on some photomicrographs is due to the autofluorescence. Scale bars 10 μm (d–l), 20 μm (a–c). (Color figure online)

features, but in all of the neighbouring cells, its presence was abundant in different intracellular compartments (Potocka et al. 2018). A similar pattern of LM2 epitope distribution was reported for the highly embryogenic cell line of a hybrid fir (*Abies alba* \times *Abies cephalonica*) in which the signals were weak in the cell walls of the embryogenic cells and much stronger in the cytoplasm compartments of the large vacuolated compartments of the cells that were not engaged in SE (Samaj et al. 2008). Thus, our results for *F. tataricum* are consistent with those that have been obtained for other species to date.

Interestingly, for both JIM8 and JIM13, we found a visible increase of the signal intensity in the NC. As was

demonstrated in some earlier studies, the NC is under constant oxidative stress (Kamalova et al. 2009) and AGPs are known to be involved in the salt stress response (Lamport et al. 2006; Olmos et al. 2017). Thus, it is likely that during such stress conditions, they may act as a plasticiser to loosen the pectin network or as a cell wall stabiliser. Gong et al. (2012) demonstrated that the overexpression of *GhAGP31*, which encodes a nonclassical AGP in cotton, significantly improved the freezing tolerance of yeast cells and the cold tolerance of *Arabidopsis* seedlings.

All of the AGP epitopes that were analysed in the present study indicate the role of AGPs, as the key constituents of the cell wall that are involved in the diverse reactions of a

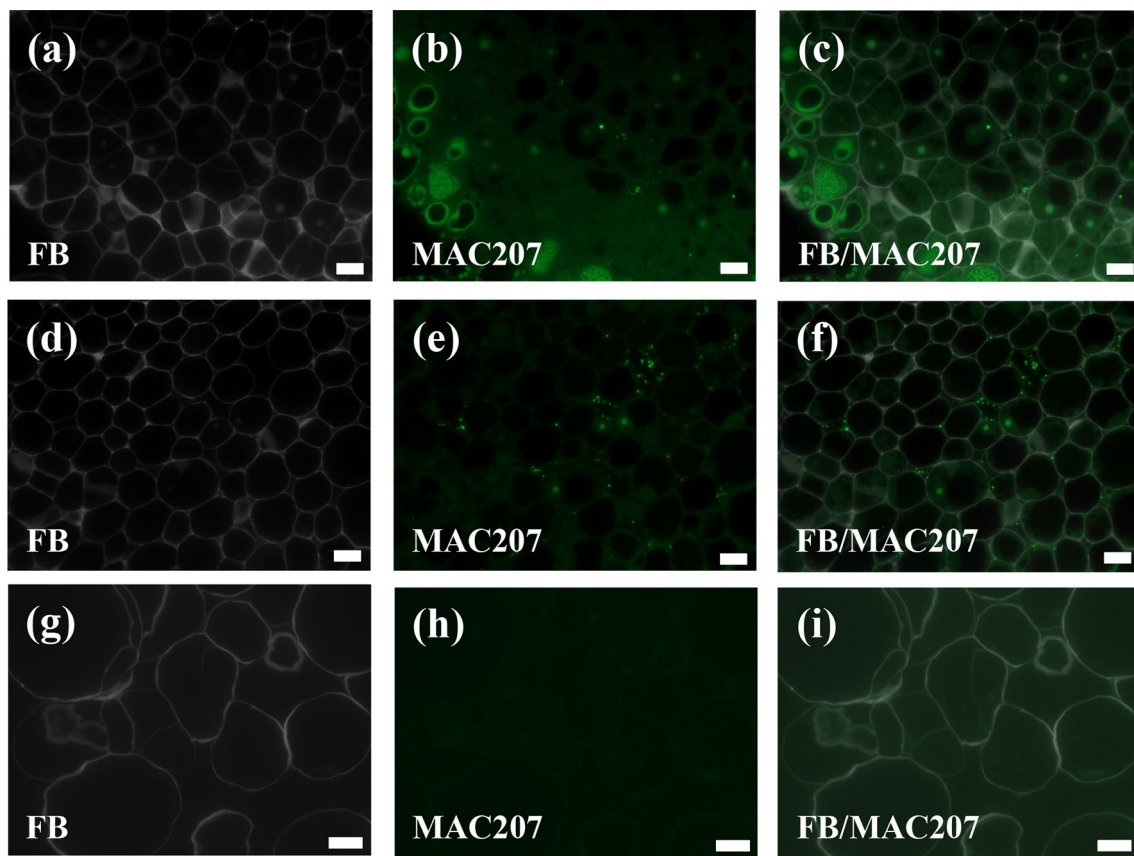


Fig. 5 Immunolocalisation of MAC207 in the *F. tataricum* MC (a–f) and NC (g–i). The greenish background visible on some photomicrographs is due to the autofluorescence. Scale bars 10 μ m. (Color figure online)

cell. Observations of the distribution of AGPs in embryogenic tissue of winter oilseed rape suggested that AGPs may play a specific role in embryogenic competency or in determining the embryogenic cells fate (Namasivayam et al. 2010). The MAC207 and LM2-binding epitopes are involved in plant morphogenesis, which is suggested by the correlation between their presence and the embryogenic potential of the callus tissue (Wiśniewska and Majewska-Sawka 2008). Extensive studies have revealed that AGPs, including those that are recognised by the antibodies that were used in present work are specific markers of the cell identity and the direction of cell differentiation in in vitro cultures (Konieczny et al. 2007). AGPs that contain JIM13 and LM2 epitopes as well as pectins seem to be important for the formation and proper assembly of the wall and providing a network for the attachment of other cell wall components (Majewska-Sawka and Munster 2003). To summarise, AGPs and extensins are broadly distributed in tissue and callus cultures, and their function in various aspects of plant growth and development, including the participation of these wall components in the formation of the plant cell surface continuum, has been postulated (Borassi et al. 2016).

As we demonstrated in this study, the high level of oxidative stress in the NC is connected with a significant increase in the expression of the *EST1* gene. *EST1* is a multifunctional protein that plays a critical role in the maintenance of telomeres as well as in the stimulation of the telomerase activity in yeasts (Renfrew et al. 2014). Mutations in *EST1* lead to telomere shortening (Lundblad and Szostak 1989). Moreover, the analysis of the expression of the *F. tataricum* protection of telomere genes, *FtPot1* and *FtPot2*, in the present study revealed a higher expression of *FtPot1* in the NC compared to the MC. We demonstrated that the upregulation of *FtPot1* is correlated with the upregulation of *TERT* in the NC. The downregulation of both of these genes was a characteristic feature of the MC. The buckwheat calli did not show any strong differences in the expression of *FtPot2* but the exact functions and binding properties of POT1 and POT2 are still under debate (Prochazkova Schrupfova et al. 2016). The genome of *Arabidopsis* contains two POT1-like proteins, AtPOT1 and AtPOT2 (Shakirov et al. 2005). It was demonstrated that AtPOT1 appears to be an important positive regulator of telomere length, while AtPOT2 negatively regulates the telomerase activity and participates in protecting the chromosome ends. Shakirov et al. (2005)

Table 3 The identity between *F. tataricum* and Arabidopsis genes on the protein level

Genes	Description of the genes	Amino acids length (aa)	Arabidopsis best hit	Arabidopsis gene description	Amino acids identity (%)	Query cover (%)	E-values
<i>FtPinG0009372500.01</i>	Ethylene response factor 1 (<i>ERF1</i>)	218	<i>AT3G23240.1</i>	Ethylene-responsive transcription factor 1B	52	99	8E–66
<i>FtPinG0004556400.01</i>	1-Amino-cyclopropane-1-carboxylate synthase 6 (<i>ACS6</i>)	573	<i>AT4G11280.1</i>	1-Aminocyclopropane-1-carboxylate synthase 6	65	82	0.0
<i>FtPinG0004154200.01</i>	1-Amino-cyclopropane-1-carboxylate synthase 2 (<i>ACS2</i>)	491	<i>AT1G01480.1</i>	1-Aminocyclopropane-1-carboxylate synthase 2	63	97	0.0
<i>FtPinG0008351200.01</i>	Aminocyclopropane-1-carboxylate oxidase 1 (<i>ACO1</i>)	308	<i>AT2G19590.1</i>	1-Aminocyclopropane-1-carboxylate oxidase 1	69	95	6E–156
<i>FtPinG0000974100.01</i>	Telomerase activating protein Est1 (<i>EST1</i>)	969	<i>AT5G19400.1</i>	Telomerase activating protein Est1	50	90	0.0
<i>FtPinG0008479800.01</i>	Telomerase reverse transcriptase (<i>TERT</i>)	718	<i>AT5G16850.1</i>	Telomerase reverse transcriptase	30	90	3E–71
<i>FtPinG0009136200.01</i>	Protection of telomeres protein 1 (<i>POT1</i>)	468	<i>AT2G05210.1</i>	Protection of telomeres 1	40	97	1E–96
<i>FtPinG0008019600.01</i>	Protection of telomeres protein 2 (<i>POT2</i>)	467	<i>AT5G06310.1</i>	Protection of telomeres 2	37	97	1E–88
<i>FtPinG0006631600.01</i>	DNA (cytosine-5)-methyltransferase (<i>CMT3</i>)	829	<i>AT1G69770.1</i>	DNA (cytosine-5)-methyltransferase <i>CMT3</i>	52	96	0.0
<i>FtPinG0009406600.01</i>	DNA methyltransferase (<i>MET1</i>)	1553	<i>AT4G08990.1</i>	DNA methyltransferase 1	56	97	0.0
<i>FtPinG0006897400.01</i>	DNA methyltransferase (<i>MET2</i>)	1564	<i>AT5G49160.1</i>	DNA methyltransferase 2	58	95	0.0
<i>FtPinG0009482900.01</i>	DNA glycosylase <i>DEMETER</i> (<i>DME1</i>)	607	<i>AT5G04560.1</i>	DNA glycosylase <i>DEMETER</i>	69	93	0.0
<i>FtPinG0007109000.01</i>	DNA glycosylase <i>DEMETER</i> (<i>DME2</i>)	1856	<i>AT5G04560.1</i>	DNA glycosylase <i>DEMETER</i>	66	46	0.0
<i>FtPinG0003657700.01</i>	DNA glycosylase <i>DEMETER</i> (<i>DME3</i>)	1598	<i>AT5G04560.1</i>	DNA glycosylase <i>DEMETER</i>	46	44	1E–55
<i>FtPinG0007109000.01</i>	DNA glycosylase, repressor of transcriptional gene silencing (<i>ROS1</i>)	1856	<i>AT2G36490.1</i>	DNA glycosylase, repressor of transcriptional gene silencing, <i>ROS1</i>	46	66	0.0

demonstrated that the overexpression of these proteins in Arabidopsis led to telomere dysfunction and serious morphological defects. The constant monitoring of the *EST1* and cyclin-dependent kinase (CDK) gene expression levels can shed more light on the participation of these genes in the cell cycle, and as a result, on their contribution to the stability/instability of the MC and NC. Thus, in the future research, it would be interesting to measure the telomere length in the MC and NC using the terminal restriction fragments. This could help in gaining an understanding of the reason for the high expression level of *TERT* and *FtPot1* in the NC.

As was mentioned earlier, the NC has a very fast growth rate and, as a result, very fast senescence processes compared to the MC. We hypothesise that the ethylene

biosynthesis genes are overexpressed in the *F. tataricum* NC. Together with other hormones, ethylene plays a key role in the senescence processes (Iqbal et al. 2017). To compare the level of the gene expression that is connected with the ethylene biosynthesis, we selected the main genes that are connected with this process such as 1-aminocyclopropane-1-carboxylic synthase (*ACS2* and *ACS6*), 1-aminocyclopropane-1-carboxylic acid-oxidase (*ACO*) and ethylene response factor (*ERF*). We demonstrated a higher expression of both *ACS2* and *ACS6* in the NC, and an increase in the *ACO1* level in the MC. Such differences in the *ACS2* and *ACS6* expression can lead to the overproduction of ethylene and the induction of fast senescence processes in the NC. The higher expression level of the *ACO1* in the MC may

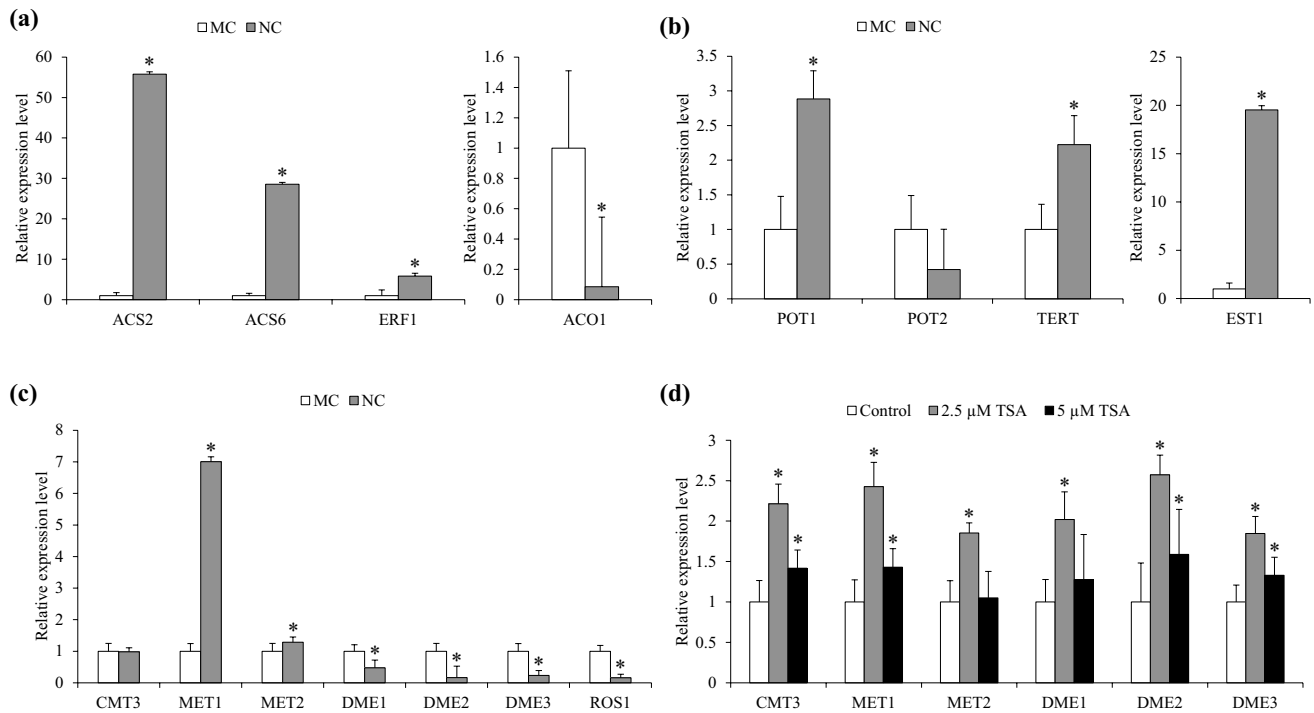


Fig. 6 **a** Relative expression levels of selected genes connected with ethylene biosynthesis and metabolism of ethylene precursors: *ACS2*, *ACS6*, *ACO1*, *ERF1*, **b** relative expression levels of selected genes connected with the telomere maintenance: *TERT*, *EST1*, *POT1*, *POT2*, **c** relative expression levels of selected genes connected with DNA methyltransferases and demethylases in the MC and NC and **d**

subjected to treatment with 2.5 and 5 μM TSA. Relative expression levels were normalised to an internal control (*FiPinG0002124000.01*, the gene encoding for actin) and calibrated to the control culture (MC cultivated on RX medium). *Value is significantly different from the control culture ($p < 0.05$, $n = 3 \pm SD$)

be associated with the PECCs that appeared on the surface of the MC on the 11th day of cultivation. Moreover, in the present study we observed an increase in the *ERF1* expression that may be connected with the oxidative stress present in the NC. *ERF1* is upregulated during the stress response to abiotic stressors such as water deficit, cold and salt treatment (Lestari et al. 2018; Makhloufi et al. 2014). The ethylene and jasmonate pathways converge in the transcriptional activation of *ERF1* in order to prevent the progression of a disease in Arabidopsis (Lorenzo et al. 2003). To summarise, the reaction to the ethylene production or overproduction can differ among the species. It was demonstrated that the addition of ACC, which is an ethylene precursor that promotes ethylene production in the barley genotype Morex, increased the green plant regeneration compared to another genotype, Golden Promise, which had a reduced regeneration after the addition of ACC (Jha et al. 2007). Such differences seem to be connected with higher production of ethylene in Golden Promise than is required to increase the plant regeneration rates. The use of ethylene inhibitors such as aminoethoxyvinylglycine, cobalt chloride and silver thiosulphate in *Sinningia speciosa* and *Picea glauca* significantly improved the frequency of regeneration (Chae et al. 2012).

There is no information in the literature about the expression of the DNA methyltransferase genes in the genus *Fagopyrum*. We demonstrated that the expression of DNA methyltransferase is higher in the NC. On the other hand, DNA demethylases had higher expression levels in the MC. Similar results were reported on the transcriptome-based characterisation of a habituated and non-habituated cell culture of Arabidopsis by Pischke et al. (2006). These authors showed the upregulation of *MET1*, *CMT1* and *CMT3* in habituated calli. In their analyses of the global DNA methylation that had been isolated from embryogenic and non-embryogenic calli of *Eleuterococcus senticosus*, Chakrabarty et al. (2003) demonstrated a significantly lower level of DNA methylation in the embryogenic calli. It should be noted that ROS1/DEMETER family gene expression is lower in the NC compared to the MC. It was shown in Arabidopsis that a mutation in the *ros1* gene leads to DNA hypermethylation and enhances transcriptional gene silencing (Agius et al. 2006; Gong et al. 2002). Moreover, the authors stressed that the overexpression of ROS1 in transgenic plants leads to a reduced level of cytosine methylation, and to a higher target gene expression. Tang et al. (2016) showed that ROS1 preferentially targets the transposable elements (TEs) and

intergenic regions. Thus, ROS1 may prevent the spread of DNA methylation from the TEs to nearby genes.

In order to determine the affect of the HDAC inhibition on the DNA methyltransferases/demethylases, we used TSA, which is known to be effective in inhibiting HDACs by displacing the zinc ion from the active centre of class 1 and 2 of the family of enzymes, including plant HDACs. When optimising doubled haploidy in wheat, Jiang et al. (2017) demonstrated that TSA increases the regeneration of an embryo and green plant. They indicated that using this agent can be useful in the rapid improvement of the wheat germplasm as well as in dealing with *in vitro* recalcitrance. The experiments on *Brassica napus* showed that HDACs regulate the switch to the haploid embryogenesis, and that blocking their activity by TSA in cultured male gametophytes leads to an increase in the proportion of that switch from pollen to embryogenic growth (Li et al. 2014). In our experiments, we observed an increase in the expression of both DNA methyltransferases and demethylases after the TSA treatment at a concentration of 2.5 μM .

Conclusion

In this study, we investigated the stability vs instability processes in the MC and NC of *F. tataricum*. The immunolocalisation of five antibodies showed differences between diverse types of callus. We found that: (1) the MAC207 antibody seems to be a marker of the MC, which is due to the lack of its signal in the NC, (2) LM2 can be used as a marker of the embryogenically determined cells in the MC, (3) the constant oxidative stress in NC may be connected with the fast senescence process and as a result the upregulation of the genes that are connected with telomere complexity, ethylene biosynthesis and DNA methyltransferases expression and (4) TSA at a concentration of 2.5 μM leads to a significant increase in the expression of both DNA methyltransferases and demethylases.

In a future study, the continuous measurement of the *EST1* and CDKs expression as well as the telomere length should be considered. The measurements of the ethylene concentration, treatments with inhibitors of HDAC and ethylene biosynthesis should also be considered.

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Author contributions Authors' contributions AB conceived of and designed the study and supervised its execution. AB, AMH and AP carried out the experiments: AB and AMH performed

immunocytochemistry analyses; AB and AP performed the RT-qPCR experiments. AB, EK, RH drafted the manuscript with contributions from all of the authors. All of the authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The authors declare that the study was carried out according to the accepted professional conduct. However, no ethical approval was needed for the study as it did not involve the use of animals or human subjects.

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