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ORIGINAL PAPER

Dimethyl tyrosine conjugated peptide prevents oxidative damage and death of triticale and wheat microspores

Rakesh Kumar Sinha[1] · François Eudes[1]

Abstract Functional damage of mitochondria and chloroplasts under stress contributes to reactive oxygen species (ROS) induced cell death. A high percentage of cell mortality during microspore culture is a limiting factor in haploid and doubled haploid plant production. In the present study, we studied the contribution of dimethyl tyrosine (DMT) conjugated short peptides to microspore embryogenesis. These DMT-peptides, which are known to translocate to subcellular targets and scavenge ROS, mitigate the effect oxidative stress plays on microspore viability and embryogenesis. The number of viable microspores was significantly higher in the presence of SS-31 and caspase-3-inhibitor (Ac-DEVD-CHO). In particular, the total number of green plant regeneration was increased by 42 % in the presence of SS-02, and by 55 % in the presence of SS-31, in triticale. Conversely, lower caspase-3-like activities were observed in the presence of SS-31 and Ac-DEVD-CHO, and intracellular ROS was reduced in the presence of SS-31, supporting the involvement of SS-31 in reducing microspore cell death by mitigating ROS and caspase-3-like activity. This study further supports the concept that antioxidant conjugated peptides offer a useful strategy for reducing ROS in plant cells.

Keywords Oxidative stress · Cell death · Microspore · Cell penetrating peptides · Microspore embryogenesis · Dimethyl tyrosine

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Introduction

Microspore embryogenesis starts with the reprogramming of the development of the male gametophyte to a haploid embryo developmental pathway, which is routinely induced under a stress treatment. After chromosome doubling these haploid plants become double haploid (DH). This process produces complete homozygous breeding lines in a considerably shorter period of time, which is valuable in breeding programs and research areas like genome engineering (Maraschin et al. 2005; Wurschum et al. 2012) transformation (Kumlehn et al. 2006) and embryological studies (Wrobel et al. 2011). Stress treatments are necessary for the embryogenic response of microspores (Maraschin et al. 2005; Touraev et al. 1996). Cold and nutrient starvations are among the most common stresses applied in wheat and triticale microspore tissue culture (Eudes and Amundsen 2005; Oleszczuk et al. 2004). It has been revealed that the increased stress intensity plays an important role in the frequency of microspore embryogenesis initiation, but on the other hand, it reduces the proportion of microspore cell viability and green plant regeneration by damaging the nuclear and chloroplast genomes (Rodriguez-Serrano et al. 2012; Shariatpanahi et al. 2006a).

Reactive oxygen species (ROS) are well known as stress signaling molecules in plants (Baxter et al. 2014; Kreslavski et al. 2012). It is established that organelles such as chloroplasts, mitochondria with a highly oxidizing metabolic activity and intense rate of electron flow are a major source of ROS in plant cells during abiotic stresses such as high intensity light stress (Sinha et al. 2012) or heat stress (Reape and McCabe 2010). An excessive amount of ROS has been involved in progressive oxidative damage exacerbating cellular damages (Roldan-Arjona and Ariza

2009; Van Breusegem and Dat 2006), ultimately causing cell death (Imlay 2008). Rodriguez-Serrano et al. (2012) have observed an increased activity of ROS in the cytoplasm of stress treated microspores and linked this increase with programmed cell death (PCD). Plants have developed a defense mechanism involving enzymes such as superoxide dismutases, catalases and peroxidases to remove ROS as well as antioxidants in the form of ascorbic acid, glutathione, α-tocopherols, and carotenoids, to protect against ROS (Krieger-Liszkay and Trebst 2006; Yadav et al. 2010).

Despite their destructive activity, ROS also play an important role in triggering embryogenic and plant development (Goyal et al. 2013; Żur et al. 2014). ROS generation and the activity of enzymatic and non-enzymatic antioxidants during low temperature stress treatment on embryogenic potential in triticale anthers were recently reported by Żur et al. 2014. They demonstrated that the genetically controlled, but environmentally modified cell tolerance to oxidative stress plays an important role in triticale microspore embryogenesis (Żur et al. 2014). Oxidative stress signaling may depend on the redox milieu of a cell, which is governed by the equilibrium between ROS formation and scavenging (Fortes et al. 2011; Mittler et al. 2004). There is a correlation between enhanced activity of antioxidants and improved tolerance in plants against a wide variety of environmental stress conditions (Alscher 1989; Herouart et al. 1994). A similar phenomenon might contribute towards the enhanced microspore viability during its isolation and culturing. Several transcriptional analyses on microspore embryogenesis have shown the up-regulation of genes involved in defense reaction against oxidative stress (Jacquard et al. 2009; Maraschin et al. 2005). A recent report showed the plastid antioxidant, glutathione, enhances microspore embryogenesis and green plant production in triticale and the wheat isolated microspore culture (IMC) (Asif et al. 2013).

Engineering a shift in ROS equilibrium or blocking the events of a PCD could potentially increase the efficiency of microspore embryogenesis and DH production.

Caspases are the key regulators of apoptosis in animal cells, but the plant genome is not known to contain true caspase genes. Nonetheless, several closely related proteins having caspase like activity have been reported in plants (Goyal et al. 2013; Vacca et al. 2006; Xu and Zhang 2009). Cell death is a crucial process involved in various aspects of plant life cycle, including abiotic and biotic responses. Caspase 3-like activity and microspore cell death have been shown to be increased after inductive stress in barley microspore and embryogenic suspension cultures (Rodriguez-Serrano et al. 2012). Caspase inhibitors Ac-DEVD-CHO have also been shown to block PCD after pathogen induction in tobacco leaves (Lam and del Pozo 2000) and reduces apoptosis in maize embryonic callus

(Hansen 2000) and in barley microspore suspension culture (Rodriguez-Serrano et al. 2012).

A series of cell-permeable peptide antioxidants SS-31, SS-02 and SkQ1 have been tested and reported to be involved in scavenging ROS in animal cell (Poljsak 2011). Zhao et al. (2004) demonstrated the role of antioxidant peptides (SS-02 and SS-31) involved in scavenging H_2O_2 and inhibiting lipid oxidation. It assisted in overcoming cell death due to release of cytochrome c from the inner mitochondrial membrane and helped to inhibit mitochondrial swelling and oxidative cell death (Zhao et al. 2005). The antioxidant properties of these small peptides are due to the presence of Dimethyl Tyrosine (DMT) which is closely related to a phenolic antioxidant compound 3, 5-dimethylphenol (Wright et al. 1997).

In this study we have tested several DMT conjugated peptides against early death in microspore culture, and embryogenesis in triticale and wheat cultivars (Sunray and AC Andrew). We treated microspores with these DMT conjugated peptides during the wash phase of their isolation, and observed reduced microspore cell death and enhanced formation of embryo like structure. Our results indicate that the stress treatment used for inducing microspore embryogenesis formed excessive ROS leading to cell death, which are partially mitigated by use of DMT conjugated peptides.

Materials and methods

Growing conditions and plant material

Triticale and wheat seeds (cv. Sunray and AC Andrew, respectively) were grown in the Cornell mix as mentioned in Asif et al. (2013). The plants were treated with 2.5 ml l^{-1} Tilt™ (propiconazole, Syngenta) before the tillering stage No. 2 as per Zadok et al. (1974) and Intercept™ (0.004 g per liter of soil, Imidacloprid, Bayer) once sufficient root development was established to control pests. The first 7 tillers from each pot were harvested when the microspores reached mid-to-late uninucleate stage. The remaining spikes were allowed to grow half their length out of the boot and then harvested for ovaries. Triticale and wheat tillers were kept at (4 °C) for 3 weeks with their bases in distilled water and their heads wrapped in aluminum foil. After 3 weeks ± 3 days, the spikes were extracted from their tillers and after evaluation of their general appearance, only the most homogenous spikes were used.

Microspore isolation and treatments

Microspores were isolated as per Asif et al. (2013). The mid-to-late uninucleate microspore stage was verified from

a median floret using acetocarmine staining. Spikes were surface sterilized with 10 % (v/v) bleach (5.25 % sodium hypochlorite) for 3 min followed by four washes with sterile double distilled water with constant agitation. Anthers from eight spikes were aseptically dissected and transferred to a sterile and refrigerated 110 ml Warring blender cup (VWR International) containing 50 ml filter sterilized wash solution at 4 °C. Anthers were blended twice for 7 s at low speed (18,000 rpm). The extract was filtered through 100 µm sterile mesh (VWR International). The cells were then pelleted by centrifugation ($100\times g$ for 5 min at 4 °C) using a swinging bucket rotor (Eppendorf AG, Hamburg, Germany). After removing the supernatant, the pellet was resuspended in 16 ml of ice cold wash solution medium. This homogenous volume was uniformly distributed between eight 15-ml tubes and each aliquot layered over with 10 ml of wash solution. Each tube was supplemented with 10 µl of a 10 mM solution of one of the following peptides SS-31, SS-02, DMT-cTP1, DMT-cTP3, DMT-mTP3, DMT-mTP4 (Table 1) or control made of sterile water. The tubes were gently inverted to homogenize the solutions (1 min) and then the microspores were pelleted by centrifugation ($100\times g$ for 5 min at 4 °C). The pellet was re-suspended in about 1 ml of wash buffer supplemented with 10 µM of DMT peptides. Microspores were Purified using 20 % maltose gradient (13 min) and the microspore concentration in each tube was determined using a hemocytometer, and adjusted to 1×10^5 cells per ml. 20 nM of DMT peptides were present in the final induction media throughout the culture. Five ovaries from similarly sterilized spikes taken directly from the plant were added to each Petri dish. Microspore culture was incubated for 4 weeks in induction media in the dark at the 28 °C and the microspore development of embryo-like structure was observed. The multicellular structures were counted using microscopy in each and every replicate. Embryos were transferred to regeneration medium (Asif et al. 2013). Once the embryos turned green, the number of

green and albino plantlets was recorded and green plants were aseptically transferred into 50 ml of rooting media in Magenta Vessels (VWR International) or disposable food grade transparent containers, and returned to the regeneration chamber. Once plants reached the 2–3 leaf stage, and had sufficient root growth, they are transplanted into 4×8 Spencer-Lemaire root trainers and placed into a growth cabinet with the same conditions as for the donor plants. The number of independent replicates in each experiment is given in Table 2.

Data recording and statistical analysis from embryogenesis and plant regeneration

The development of multicellular structure and embryos were first verified after 10–14 days. The number of embryos or embryo-like structures was determined 27–30 days after isolation when the tissue was large enough to transfer to regeneration medium (Asif et al. 2013). The total number of green and albino plants were determined following germination of the embryos (approximately 2–3 weeks after transfer) and were presented as the mean value of all the replicates (Table 2) per petri dish. The experiments were laid out in completely randomized design with different replications in triticale and wheat. The experimental unit was one Petri dish containing 1×10^5 microspores. The number of replications for each genotype in each experiment is detailed either in Table 2 or in Fig. legend. The data was analyzed separately for each species. For all variables recorded in the measurements, analyses of variance were conducted using the restricted maximum likelihood (REML) mixed model in SAS/STAT. The means were compared using the Tukey's test at $P \leq 0.05$.

Localization of DMT conjugated peptides SS-31

To determine the localization of SS-31, N-terminal labelling with Alexa Fluor® 633 (CRB LTD, UK) was

Table 1 DMT-conjugated peptides used for the studies

Treatment		Sequence	Mol. weight	Hydrophilicity
1	SS-02	DMT-D-Arg-Phe-Lys-NH$_2$	658.69	GWS
2	SS-31	D-Arg-DMT-Lys-Phe-NH$_2$	658.69	GWS
3	DMT-cTP1	DMT-D-MGGCVSTPKSCVGAKLR-NH$_2$	1903.32	GWS
4	DMT-cTP3	DMT-D-MKNPPSSFASGFGIR-NH$_2$	1805.09	PWS
5	DMT-mTP3	DMT-D-MKLLWRLILSRKW-NH$_2$	1952.47	PWS
6	DMT-mTP4	DMT-D-MWWRRSRTNSLRYT-NH$_2$	2122.45	GWS

Peptides were synthesized by CanPeptide

DMT 2,6-DimethylTyrosine, *D-Arg* D-form Arg, *D-M* D-form Met, *GWS* good water soluble, *PWS* poor water soluble

Table 2 Effect of DMT label small Peptides on embryo like structure (ELS) and plant regeneration

Trt	Mean (triticale)				Mean (andrew)			
	N	ELS	GP	AP	N	ELS	GP	AP
SS-02	9	1154.67a	77.57ab	102.00ab	4	479.25a	5.50a	54.75a
SS-31	9	1151.11a	86.71a	109.42a	4	491.00a	5.25ab	55.75a
DMT-cTP1	9	884.67ab	70.71ab	91.71ab	4	408.00a	3.50bc	51.50a
DMT-cTP3	9	669.56b	62.43ab	95.57ab	4	338.25a	3.50bc	47.75a
DMT-mTP3	9	648.22b	60.72b	90.43b	4	288.25a	3.75abc	40.50a
DMT-mTP4	9	796.44ab	60.28b	85.57b	4	372.50a	3.00c	43.25a
Control	18	603.44b	54.86b	93.57b	8	355.50a	4.00abc	47.50a

Values followed by same letter do not differ significantly within each column (Tukey's at 0.05 level of probability)

N represents number of replicates, ELS embryo-like structures, GP green plants, AP albino plants

custom designed by CRB LTD (UK). Fresh microspore cultures were incubated for 15 min at 28 °C in the dark, with 10 µM of Alexa Fluor label SS-31 prepared in sterile double distilled water. For detection of viable microspore 10 µM Fluorescein diacetate (FDA) was incubated for 15 min at 28 °C in the dark and subsequently, microspores were washed twice with wash buffer. Microspores were incubated with FDA along with SS-31 which passively diffused across the plasma membrane and accumulated in active microspores. Microspores were subsequently washed twice with phosphate buffered saline (pH 7.2) and then placed onto a slide with 50 % glycerol in PBS. The samples were observed using confocal laser scanning microscopy (Olympus, FV1000). Both FDA signals (Excitation: 488 nm for peptide, Emission: 519 nm) and the Alexa Fluor® label peptides signal was captured in red fluorescence (Excitation: 633 nm, Emission: 648 nm).

ROS detection by confocal microscopy

To monitor the extent of the ROS level in live cells, imaging of ROS was performed by total ROS detection reagent (ENZO LIFE SCIENCE, INC, US). ROS activity was determined according to the manufacturer's instructions using confocal laser scanning microscopy. Microspores were isolated in the presence and absence of SS-31 and incubated in induction media without ficoll. One day old IMCs were centrifuged at $400\times g$ for 5 min to yield a count of 1×10^5 cells/sample and incubated for 2 h at 28 °C, in the dark, in the presence and absence of ROS 3-Plex Detection Mix with periodic shaking. Subsequently, microspores were washed twice with 2 ml $1\times$ wash buffer (provided in the kit) at $400\times g$ for 5 min to remove the ROS 3-Plex Detection Mix. The cells were treated with SS-31 (10 µM), ROS inducer Pyocyanin or ROS inhibitor N-acetyl-L-cysteine for 30 min at 28 °C. The cells were centrifuged in 2 ml $1\times$ wash buffer at $400\times g$ for 5 min

and the supernatant was removed. The cells were resuspended in 100 µL of $1\times$ wash buffer and 20 µL of aliquot was applied onto the microspore slide. The cells were overlaid with a cover slip and analyzed immediately by confocal laser scanning microscope (Olympus Fluorview 1000 attached to inverted microscope IX81). Excitation was provided by the 488 nm line of an argon ion laser and the signal detected using a 505–525 nm emission filter. 30 µL aliquots were diluted in 280 µL of $1\times$ wash buffer and analyzed for fluorescence intensity measurement using a flow cytometer. The Fluorescence intensity of control and treated microspores was measured using flow cytometer by calculating the mean value of the fluorescence peak of different treatments. The intensity of the lasers was set according to unlabeled samples at the beginning of each experiment. All experiments shown were performed independently with at least three independent biological replicates.

Determination of microspore viability using a flow cytometer

To study the possible effects of DMT on microspore viability within the first 5 days, microspore cultures were treated with SS-31 (10 µM), Ac-DEVD-CHO (20 µM) and compared to control at fixed time intervals of 24 h. Ac-DEVD-CHO (20 µM) was incubated with microspores for 15 min and a final concentration of 1 µM of Ac-DEVD-CHO was used in induction media throughout the viability measurement. The viability was determined by staining microspores with 10 µM FDA for 10 min in the dark at room temperature using a Flow cytometer (Becton–Dickinson, San Jose, CA). The percentage of dead (unstained by FDA) and live (stained by FDA) microspores was quantified for at least 10,000 microspores per treatment after every 24 h till 5 days. For statistical analysis the data from three biological and two technical replicates were used.

Caspase-3-like enzymatic assay

Caspase-3-like activity of triticale cultivar Sunray was observed in two time points, 24 and 96 h. It was measured in cell suspension culture of IMCs using caspase-3 Colorimetric assay kit (Enzo Life Sciences, Inc., Farmingdale, NY, USA), based on spectrophotometric detection of the chromophore p- nitroaniline (pNA) after cleavage from the labeled substrate DEVD-pNA by caspase 3. Comparison of the absorbance (405 nm) of pNA from the treated sample with a control allowed determination of the fold increase in caspase-3 activity. Additional samples were incubated in parallel with 20 µM caspase-3-inhibitor, Ac-DEVD-CHO. All assays were performed in six biological replicates and results were considered statistically significant when $P < 0.05$.

Results

In the present study, we have analyzed six different peptides as carriers covalently bound to DMT, which disarms the ROS by accepting free radicals. In these studies, we demonstrated the effects of DMT conjugated transit peptides for microspore viability and ROS production and its subsequent effects on the formation of green plants in triticale and wheat.

DMT conjugated peptides enhanced the formation of embryo-like structure

The effect of different DMT conjugated peptides on the formation of embryo-like structure (ELS) was observed in 4 weeks old microspore cultures. We performed these experiments first in triticale, and attempted to replicate these findings in wheat cultivar AC Andrew. In triticale, among the six peptides used for the studies, SS-02 and SS-31 produced the highest number of ELS and green plants (Table 2). The average number of ELS increased from 603 in control to 1154 and 1151 when the microspore wash solution was supplemented with SS-02 ($P > 0.0067$) and SS-31 ($P > 0.0071$) (Table 2). Whereas, when the wash solution was supplemented with DMT conjugated cTP1, cTP3, mTP3 and mTP4 produced 884, 669, 648 and 796 ELS respectively (Table 2). Similar results were obtained with respect to green plant production; SS-02 and SS-31 produced a mean of 77 and 86 green plants per petri dish, which is 42 and 55 % respectively higher in comparison to control. DMT conjugated cTP1, cTP3, mTP3 and mTP4 produced 70, 62, 60, and 60 green plants respectively, compared to 54 green plants in control. The total number of green plant regeneration in triticale was increased by 42 %

in the presence of SS-02, and by 55 % in the presence of SS-31, in comparision to control.

Localization of SS-31

Cellular localization of SS-31 was observed in viable FDA stained cells. Autofluorescence in red and green channels, showed fluorescence of microspore exines but not in the cytoplasm of microspores (Fig. 1a, b). Alexa Fluor® 633 labelled SS-31 was observed in the cytoplasm, but not in the vacuole (Fig. 1f). The co-localization of both green and red fluorescent signals indicated both the viability and presence of SS-31 in microspore. The fluorescence of the FDA was brighter than Alexa Fluor®, as confirmed by confocal microscope (Fig. 1), and a bright halo of red fluorescence was also systematically observed on the exine.

Effect of SS-31 on microspore viability and Caspase-3-like activity

The microspore viability of triticale cultivar Sunray was observed at fixed time intervals using flow cytometry. Differences between SS-31 treated and control cultures were first observed after 24 h of incubation. At that time, microspore viability in control established at 48 % (Fig. 2a), while significantly higher viability (57 %) was observed in SS-31 treated microspores. Interestingly, when microspore culture was treated with Ac-DEVD-CHO, a specific inhibitor of caspase-3-like activity, the percentage of microspore viability resembles with the SS-31 treated microspore culture during the first 48 h (Fig. 2a). The percentage of microspore viability kept decreasing to approximately 11, 15.8 and 22.8 % after 120 h of microspore culture in control, SS-31 and Ac-DEVD-CHO treated cultures, respectively. The proportion of dead cells was higher in control microspore culture throughout the observation (Fig. 2). Moreover, a significantly higher percentage of cell viability from 48 to 120 h was observed in microspore culture treated with Ac-DEVD-CHO and SS-31 in comparison to control, suggesting the possible role of caspase-3-like activity in the pathway as well as antioxidant properties of SS-31.

To investigate the type of microspore cell death took place in the culture, we analyzed (Fig. 2b) caspase-3-like activity at two different experimental time points (24 and 96 h). As shown in Fig. 2b, treatment of microspores culture with SS-31 resulted in reduced caspase-3-like activity by 40 % at 24 h and 30 % at 96 h compared to control microspores. The highest levels of caspase-3-like -activity were observed in 96 h control culture followed by SS-31 treated microspore culture (Fig. 2b). As a positive control, microspore culture was treated with caspase-3-inhibitor (Ac-DEVD-CHO), resulting in considerably lower levels
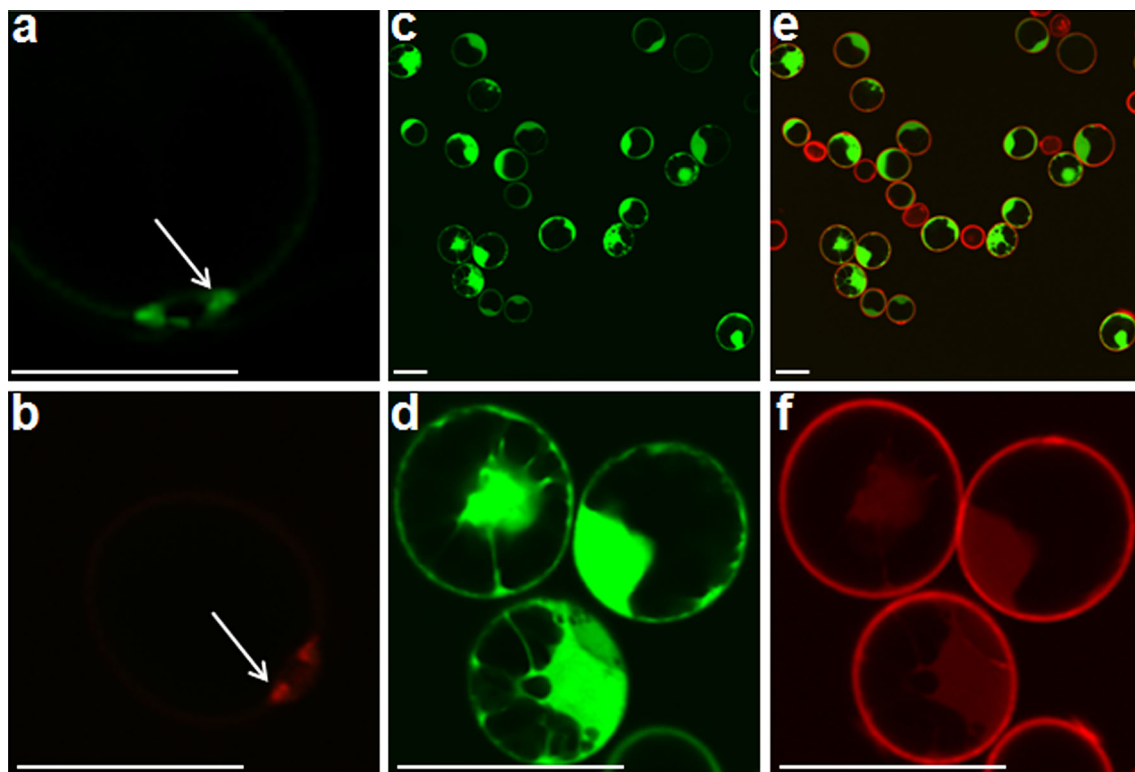
Fig. 1 Confocal microscopy localization of fluorescent labeled SS-31 in triticale microspore. Live cell specific probe FDA was used for viability assay and Alexa Fluor® 633 labeled SS-31 (10 µM) was used for confocal microscopic localization of SS-31; *bars* 50 µM. **a** Control autofluorescence of microspore in *green* channel. **b** Control autofluorescence of microspore in *red* channel. **c–d** *Green* fluorescence in the cytoplasm designates lives microspores caused by FDA (λ 488) staining and the absence of green fluorescence in the cytoplasm designates the dead microspores. **e–f** *Red* fluorescence denotes SS-31 labeled Alexa Fluor® 633; *red* fluorescence signals observed on the exine and *green* signals in cytoplasm designates live microspore with combined *red* and *green* florescence. *Arrow* designates autofluorescence signals in micropore of microspores in the *green* and *red* channel

of caspase-3-activities observed at both 24 and 96 h cultures. The reduced caspase-3-like activities were observed throughout the experiment in microspore culture with caspase-3-inhibitor (Ac-DEVD-CHO) and SS-31 (Fig. 2b); which showed a similar pattern with the proportion of viable microspores detected by FDA staining (Fig. 2a).

Reactive oxygen species in microspore culture

To evaluate the effect of oxidative stress on microspore culture, treatments with specific scavenger, enhancer and SS-31 were carried out in microspore culture. Differential interference contrast images (DIC) show the presence of large vacuolated microspores (Fig. 3a). The presence of large vacuolated microspores is a characteristic feature of the early stage of microspore culture. No specific fluorescence signal was detected in vacuolated microspore in the absence of oxidative stress probe (Fig. 3b), while in the presence of oxidative stress probe the control presented a strong fluorescence signal (Fig. 3c). A decrease in the fluorescence signal was observed in the microspores treated with SS-31 compared to control (3d). As a positive control for the ROS staining, the microspores were treated with an exogenous ROS inducer Pyocyanin, which resulted in an increase in green fluorescence by 30 % (Fig. 3f, g) and nearly disappeared in the presence of ROS inhibitor (N-acetyl-L-cysteine, NAC) (Fig. 3e, g). The ROS scavenging activity of SS-31 was observed with 40 % less signal intensity compared to control (Fig. 3d, g). In all the samples, the green signals were only observed in the cytoplasm, but not in the vacuole.

The reduced frequency of albino plants was observed in DMT conjugated cTP1, mTP3, and mTP4; whereas DMT-cTP3, SS-02 and SS-31 showed a higher number of albino plants compare to control (Table 2). In wheat, DMT conjugated peptides did not result in data statistically different from the control. However, the mean number of ELS increased from an average of 355 in control to 479, 491, 408 and 372 in SS-02, SS-31, DMT-cTP1 and DMT-mTP4, respectively. Treatment with SS-02 and SS-31 also increased the mean number of green plants to 5–6 per Petri dish in comparison to 4 in the control, whereas 3–4 green
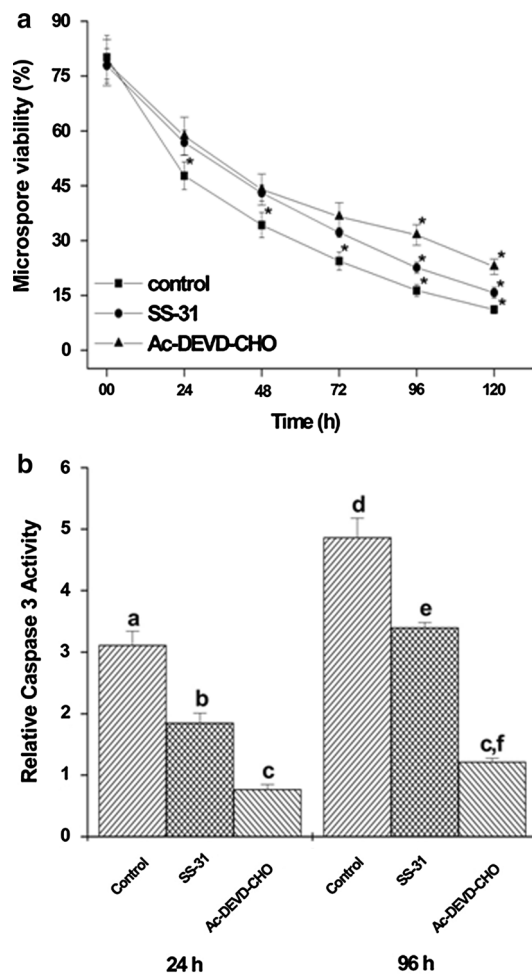
a

b

Fig. 2 Effect of SS-31 on microspore viability and caspase-3-like activity. Microspore viability and caspase-3-like activity was evaluated in triticale microspore suspension cultures. **a** The percent viability of microspore cells treated with SS-31 and in the presence of caspase inhibitor Ac-DEVD-CHO. **b** comparison of caspase-3-like activity in control, SS-31 treated and in the presence of caspase 3 inhibitor (Ac-DEVD-CHO) in microspore cells. The *bars* represent the standard error of the mean of seven independent experiments. The *asterisk* (*) and *letter* indicate significant differences at $P < 0.05$ according to Tukey's test. Same letter do not differ significantly within each column

plants were produced from DMT conjugated cTP1, cTP3, mTP3, and mTP4 (Table 2). Trends are also reported for the number of albino plants; the least number of albino plants was obtained from DMT-mTP3.

Discussion

The present study has made the first reported effort at addressing mitigation of ROS production and cell death through the delivery of DMT conjugated peptides into microspores. We presented evidence that DMT conjugated peptides contributed to reduce ROS abundance and

caspase-3-like activities. The specific subcellular localization of one of the peptides used, SS-31, was determined using covalently bound Alexa Fluor® 633 dyes, in live microspores (Fig. 1). Although the presence of Alexa Fluor® 633 labelled SS-31 was confirmed in cytoplasm of live cells (Fig. 1), this is contrary to earlier reports indicating that the SS-31 peptide contains a sequence motif that targets the inner mitochondria of animal cells (Szeto 2006; Zhao et al. 2004) and can freely pass the plasma membrane in animal cell (Hazell and Wang 2005). SS-31 carries a +3 net charge at physiological pH (Zhao et al. 2003); the mitochondrial uptake may be dependent on electrostatic attraction between cationic and highly anionic cardiolipin molecules of the inner mitochondrial membrane (Reddy et al. 2011). Nevertheless, the integrity and penetration capacity of SS-31 may be affected due to the conjugation with Alexa Fluor® 633, as Alexa dyes carry a net negative charge, and is twice the molecular mass of previously used radioactive labelled ³H SS-31 (Zhao et al. 2004). The negative charge of fluorophores possibly causes a shift in net positive charge of SS-31 and reduces electrostatic interaction with negatively charged cell structures (Mahmudi-Azer et al. 1998). Earlier, Dimethyl sulfoxide (DMSO) was used as a carrier of Colchicine and reported to increase the efficiency of chromosome doubling (Subrahmanyam and Kasha 1975) or to increase the solubility of p-chlorophenoxyisobutyric acid in triticale microspore culture (Żur et al. 2015). However, High concentrations of DMSO may inhibit embryogenesis. The uptake of SS-31 into mitochondria and proplastid is most likely possible via a diffusion process and may depend on the concentration of SS-31 inside the cytoplasm.

Following standard pretreatment and purification, the control triticale microspore culture presented the highest percentage of cell death (Fig. 3a), as well as the highest caspase-3- like activity (Fig. 3b). Prior evidence suggests that metacaspases were involved in the regulation of plant cell death (Uren et al. 2000). It has been demonstrated that metacaspases have been regulated during the process of embryogenesis of Norwegian spruce and in stress induced PCD in Arabidopsis (Reape and McCabe 2010) and in barley microspores (Rodriguez-Serrano et al. 2012). In the first 48 h, samples treated with SS-31 and caspase 3 inhibitor Ac-DEVD-CHO shows similar viability change, whereas after 48 h viability of the SS-31 treated microspore culture was observed to be higher than the control but lower than Ac-DEVD-CHO treated microspore. The relative caspase-3-activity was significantly reduced in SS-31 and Ac-DEVD-CHO treated microspores and a relationship can be established between this reduced activity and the increased cell viability. Although, comparatively less difference was measured for the relative caspase activity between 24 and 96 h in Ac-DEVD-CHO treated microspore
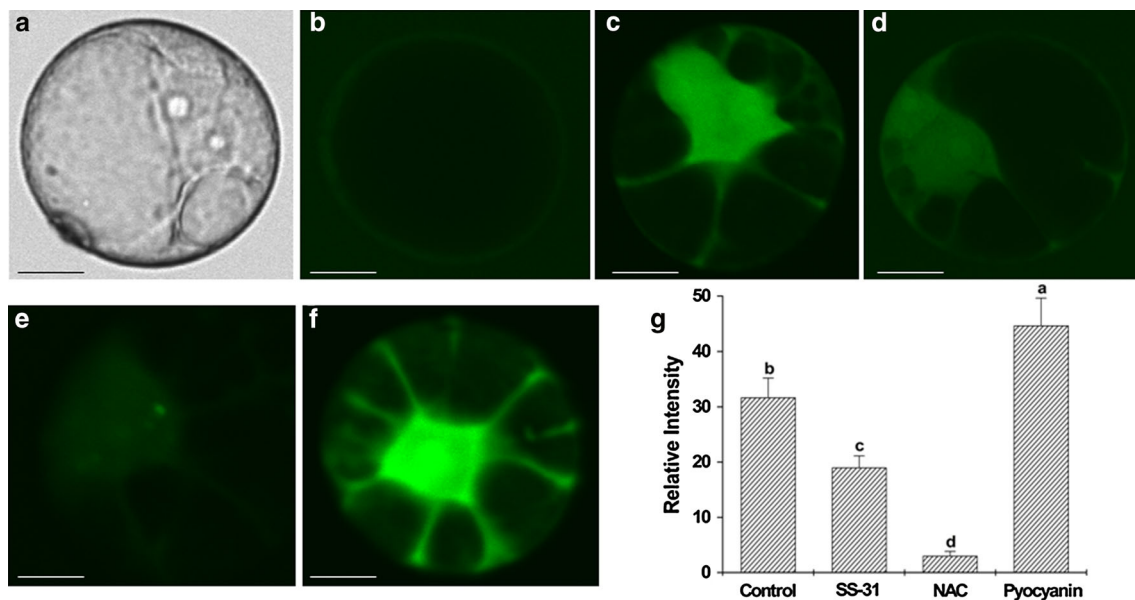
Fig. 3 Imaging of total ROS production. Imaging of total ROS production in Triticale microspore cultures using ROS specific probe 3-Plex Detection Mix under confocal microscopy. **a–f** Micrograph showing a total ROS fluorescence signal: **a** designate differential interference contrast microscopy (DIC) image, **b** autofluorescence of microspore in *green* channel, **c** control microspore with *green* fluorescence, **d** microspore treated with SS-31, **e** microspore treated with ROS inhibitor N-acetyl-L-cysteine (NAC), **f** microspore treated with ROS inducer Pyocyanin, **g** histogram showing relative fluorescence intensity of total ROS production measured using a flow cytometer, *bars* 20 µM

culture, the cell viability was reduced to 31 % over this interval, suggesting the role of caspase-3-like activity but not limited to this pathway for microspore death. The present study revealed that different proteases might be active during PCD in wheat and triticale, having both similar or dissimilar substrates and inhibitory properties compared to the mammalian caspase 3. These results also open possibilities of modulating PCD using different caspase inhibitors to increase cell viability and embryogenesis.

Triticale (Sunray) and Wheat (AC Andrew) DH cultivar used in the study had been selected on the basis of earlier experiments as significantly different in their embryogenic potential. Among the six conjugated peptides, SS-02 and SS-31 significantly enhanced the number of ELS in triticale, while we report a similar trend in wheat (Table 2). The mean number of ELS was also higher in treatments DMT-cTP1 and DMT-mTP4 than the control. The mean number of green plants/100,000 microspores was higher in all DMT conjugated peptide treated triticale microspores and in presence of SS-02 and SS-31 in wheat (Table 2), although significant differences were only observed in SS-02 and SS-31 treated triticale microspores. DMT peptides were used during microspore isolation for a shorter period of time; however, the lower concentration of DMT peptides (1.5 µM) were present for a longer period of time. To prevent DMT peptide accumulation in the cytoplasm, we decreased the exposure period with higher concentration of DMT peptides and increased with lower concentration.

Earlier, cellular experiments showed that SS-02 and SS-31 can reduce intracellular ROS production even at nano molar concentrations (Zhao et al. 2005) and may be cytotoxic at concentrations over 10 µM (Murphy 1997). Furthermore, the direct involvement of DMT peptides in green plant regeneration was less likely, this may be reflected in the increase in the number of ELS per petri plates. The access of DMT-cTPs and DMT-mTPs into the microspores could have been restricted, due to adherence of the peptides to the peripheral exine, as suggested by some observations (data not shown). While most antioxidants require a specific concentration to prevent oxidative cell death (Jauslin et al. 2003), adherence to exine would affect the delivery of DMT provided in equimolar amounts. DMT conjugated peptides adherence to peripheral exine could slowly release into the cytoplasm and scavenge ROS over time. The conformation and the length of the conjugated peptides are other factors affecting the mechanism of uptake. The smaller tetra-peptides (SS-31, SS-02) showed maximum activity, compared to larger conjugated peptides (DMT-cTP1, DMT-cTP3, DMT-mTP3, DMT-mTP4) (Table 1). Direct Translocation of a small hydrophilic peptide across a lipid membrane is possible, without having to overcome the energy barrier (Trabulo et al. 2010), whereas comparatively larger and hydrophobic conjugated peptides can easily be trapped within the cell membrane. Among the larger conjugated peptides, the hydrophilic (DMT-cTP1, DMT-mTP4)

produced more ELS compared to less hydrophilic (DMT-cTP3, DMT-mTP3) (Tables 1, 2). Therefore, we assume that the antioxidant efficacy of these conjugated peptides relies on size and hydrophilicity.

The cell produces a high amount of ROS under stress conditions, some of which contributes to signaling, and the excess cause damage. When microspore culture was incubated with ROS inhibitor (N-acetyl-L-cysteine, NAC), as a negative control, the fluorescence signal nearly disappeared (Fig. 3e). *NAC* is a widely used thiol-containing antioxidant that is a precursor of reduced glutathione, several reports showed that NAC protects against oxidative stress-induced cell death (Halasi and Gartel 2013; Qanungo et al. 2004). The results of confocal microscopy showed the difference in green fluorescence intensity in the presence and absence of ROS inducer and scavenger (Fig. 3c–f), which demonstrate the presence of ROS inside the cell can be mitigated using ROS inducer and scavenger.

When SS-31 treated microspores were observed in confocal microscopy, significantly less fluorescence intensity was observed (Fig. 3d). These results were further evaluated using flow cytometry, where SS-31 treated cells showed 40 % less florescence intensity as compared to control. This confirms the role of SS-31 in scavenging ROS produced in the cell (Fig. 3), likely in the mitochondria and possibly in the proplastid. Earlier, Żur et al. (2014) have observed an increased production of superoxide anion radicals in recalcitrant DH lines in comparison to responsive DH lines after low temperature cold treatment. They also absorbed an enhanced amount of SOD in the anther of responsive DH lines, which can protect the microspores from oxidative stress by dismutation of the superoxide radical to H_2O_2 thereby reducing the risk of formation of the toxic and highly reactive oxidant, hydroxyl radical (Gill and Tuteja 2010). Similarly, DMT labelled short peptides are capable of neutralizing ROS and can protect microspores from potentially damaging ROS (Zhao et al. 2005). It could be the reason for a higher viability of microspores treated with SS-31 in comparison to control. The increased ROS production, the decreased cell viability and caspase-3-like activity in control, compared to SS-31 and Ac-DEVD-CHO treatments, suggested interplay among ROS production, caspase activity and cell viability. This also suggested that in the first 48 h, the decrease in cell death in SS-31 treated samples compared to control was probably due to the damage caused by stress treatment and oxidative stress which was also correlated with higher ROS activity (Fig. 3g). In agreement with our work, it has been shown that the ROS increase was involved in the stress-induced PCD occurring in the early stages of barley embryogenic suspension cultures (Rodriguez-Serrano et al. 2012) and triticale microspore embryogenesis (Żur et al. 2014). This would further support early contribution of SS-31 in neutralizing excess ROS in mitochondria and cytoplasm, and minimize oxidative damage and cell death (Zhao et al. 2004). Our results also suggest that, ROS damage activates caspase-3-like activities which are linked to cell death.

Conclusions

In conclusion, we report that DMT conjugated peptides can be taken up by plant host cells and can target specific sites of ROS formation, protect cells and help to overcome oxidative stresses during the first 48 h of culture. The substituted antioxidants at an early stage of microspore culture can reduce microspore cell death and produce increased numbers of embryo like structures and green plants. SS-31 and SS-02 were the most efficient DMT conjugated peptides in mitigating oxidative stresses and cell death in plants, resulting in increased success of green haploid plant production.

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