Quantification of endogenous aromatic cytokinins in Pinus radiata *embryonal masses after application of heat stress during initiation of somatic embryogenesis*

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Trees
Structure and Function

ISSN 0931-1890

Trees
DOI 10.1007/s00468-020-02047-x

Springer

Trees
https://doi.org/10.1007/s00468-020-02047-x

SHORT COMMUNICATION

Quantification of endogenous aromatic cytokinins in *Pinus radiata* embryonal masses after application of heat stress during initiation of somatic embryogenesis

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Received: 11 June 2020 / Accepted: 6 October 2020

Abstract

Key Message **Short-term exposure to high temperatures modulates the aromatic cytokinin profile of radiata pine embryonal masses when measured 8 weeks after initiation.**

Abstract Recent research has demonstrated that cytokinins could be involved in abiotic stress responses. However, little research has been published about the role of endogenous aromatic cytokinins during these processes. Therefore, in this study, different temperature and incubation periods (23 ºC, 8 weeks; 40 ºC, 4 h; 50 ºC, 30 min; 60 ºC, 5 min) were applied during induction of radiata pine somatic embryogenesis and the aromatic cytokinin content of the resulting embryonal masses was measured. Results indicated that temperature during induction had a significant effect on the profile of most of the aromatic cytokinins detected. All tested higher temperatures than control provoked a decline in some free bases, including N^6-benzyladenine, ***ortho***-Topolin and ***para***-Topolin. On the other hand, the values of N^6-benzyladenine nucleotides, ribosides and irreversible metabolites (9G), as well as ***para***-Topolin ribosides, increased in embryonal masses originating from 40 ºC treatment. These results suggest that despite not being traditionally considered part of the stress response machinery, aromatic cytokinins might be involved in heat-stress responses during early stages of somatic embryogenesis in radiata pine.

Keywords Embryogenic tissue · N^6-Benzyladenine · *Orto*-topolin · *Para*-topolin · Radiata pine · Temperature

Abbreviations

CK	Cytokinins
SE	Somatic embryogenesis
EM	Embryonal mass
ECL	Established cell line
BA	N^6-Benzyladenine
BAR	N^6-Benzyladenosine
BARMP	N^6-Benzyladenosine-5′monophosphate
BA7G	N^6-Benzyladenine-7-glucoside
BA9G	N^6-Benzyladenine-9-glucoside
oT	*ortho*-Topolin
oTR	*ortho*-Topolin riboside
oT7G	*ortho*-Topolin-7-glucoside
oT9G	*ortho*-Topolin-9-glucoside
mT	*meta*-Topolin
mTR	*meta*-Topolin riboside
mT7G	*meta*-Topolin-7-glucoside
mT9G	*meta*-Topolin-9-glucoside
pT	*para*-Topolin
pTR	*para*-Topolin riboside
pT7G	*para*-Topolin-7-glucoside
pT9G	*para*-Topolin-9-glucoside
K	Kinetin
KR	Kinetin riboside
K9G	Kinetin-9-glucoside

Communicated by Klimaszewska.

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Published online: 18 October 2020

Introduction

Cytokinins (CK) are one of the most important phytohormone groups controlling cell division, proliferation and differentiation in plants, and they are considered master regulators during plant growth and development (Wani et al. 2016). Despite not being traditionally classified as "stress-hormones", research carried out during the last decades has highlighted the importance of CK during numerous stress response, adaptation (Bielach et al. 2017) and hardening processes (De Diego et al. 2015).

Among abiotic stresses, drought and heat represent probably some of the most common constraints for plants. Apart of the widely studied function of CK during drought, CK may also take part in temperature sensing and heat signalling in *Arabidopsis* (Černý et al. 2014). They enhance heat-stress tolerance (Prerostova et al. 2020) and recovery (Escandón et al. 2016), they regulate photosynthesis and the antioxidant and osmotic defence against heat in tobacco (Dobra et al. 2010; Lubovská et al. 2014) and could potentially be involved in long-term physiological responses against heat in somatic embryogenesis (SE) of radiata pine (Moncaleán et al. 2018; Castander-Olarieta et al. 2020).

However, most of those experiments were centred on the study of isoprenoid CK. Little information is available about the role of endogenous aromatic CK in stress events. Natural aromatic CK are mainly constituted by N^6-benzyladenine (BA), kinetin (K), *meta*-Topolin (mT), *ortho*-Topolin (oT) and their derivatives, and differ both in terms of chemical structure and biological activity from their isoprenoid counterparts (Strnad 1997). There are several reports suggesting that some aromatic CK, such as kinetin (K), could mitigate heat and salinity stress (Chhabra et al. 2009; Ahanger et al. 2020), but most of the studies have evaluated their effect via external application, without their endogenous determination.

Therefore, in this study, we have tried to elucidate if endogenous aromatic CK could be involved in heat-stress responses in radiata pine. To this purpose, we have employed SE, a useful biotechnological approach which has long been applied as a model to study different developmental and biochemical processes in plants.

Materials and methods

The selection of plant material and the hormone analysis followed the procedure described in Castander-Olarieta et al. (2020). Briefly, green cones collected from 4 genetically different open pollinated trees were surface-sterilized with 70% (v/v) ethanol, split into quarters and all the seeds were extracted and surface-sterilized following Montalbán et al. (2012). Seed coats were removed and intact megagametophytes were excised out aseptically and placed horizontally onto EDM initiation medium (Walter et al. 2005) supplemented with 3.5 g l^{-1} gellan gum (Gelrite®; Becton Dickinson). At this point, the megagametophytes enclosing immature zygotic embryos were subjected to different temperature and incubation times based on results from previous studies (Moncaleán et al. 2018; Castander-Olarieta et al. 2019, 2020): 23 ºC (8 weeks, control), 40 ºC (4 h), 50 ºC (30 min) and 60 ºC (5 min). The culture medium was pre-warmed for 30 min before the start of the incubation period and at the end all the megagametophytes were kept at 23 ºC in darkness. Emerging embryonal masses (EM) were subcultured fortnightly to proliferation medium (same composition as initiation medium but 4.5 g l^{-1} gellan gum), and after 5 subculture periods, vigorously proliferating EM were frozen in liquid nitrogen and stored at − 80 ºC until aromatic CK analysis.

Extraction, purification and quantification of endogenous aromatic CK were carried out from three established cell lines (ECL) per induction treatment, comprising a total of 12 different samples. The following 20 aromatic CK types were analysed: BA, N^6-benzyladenosine (BAR), N^6-benzyladenosine-5′monophosphate (BARMP), N^6-benzyladenine-7-glucoside (BA7G), N^6-benzyladenine-9-glucoside (BA9G), oT, *ortho*-Topolin riboside (oTR), *ortho*-Topolin-7-glucoside (oT7G), *ortho*-Topolin-9-glucoside (oT9G), mT, *meta*-Topolin riboside (mTR), *meta*-Topolin-7-glucoside (mT7G), *meta*-Topolin-9-glucoside (mT9G), *para*-Topolin (pT), *para*-Topolin riboside (pTR), *para*-Topolin-7-glucoside (pT7G), *para*-Topolin-9-glucoside (pT9G), K, kinetin riboside (KR) and kinetin-9-glucoside (K9G).

Two replicates of 10 mg from each ECL were analysed following a slightly modified protocol described by Svačinová et al. (2012) using miniaturized purification (pipette tip solid-phase extraction). Samples were extracted in 1 ml of modified Bieleski with the addition of stable isotope-labelled internal standards (0.2 pmol for bases, ribosides and N9- and N7-glucosides; 0.5 pmol for CK nucleotides). After extraction, from each sample, three technical replicates of 300 µl were transferred onto Stage Tips and purified using C18, SDB-RPS, and Cation-SR sorbents (Empore™). Eluates were evaporated to dryness and dissolved in 30 µl of mobile phase.

Mass analysis was carried out using an Acquity UPLC® System (Waters, Milford, MA, USA), and a triple-quadrupole mass spectrometer Xevo™ TQ-S MS (Waters MS Technologies, Manchester, United Kingdom). All mass spectrometry data were processed using the MassLynxTM software with TargetLynx™ program (version 4.2. Waters, Milford,

MA, USA) and compounds were quantified by standard isotope dilution analysis.

The results from the hormone quantification were analysed using ANOVA. A Tukey's post hoc test ($\alpha = 0.05$) was used for multiple comparisons. When required, the ECL was included in the model as a random effect to improve the fit and analyse the effect of treatments more accurately. When the analysis of variance did not fulfil the normality hypothesis, a Kruskal–Wallis test was performed.

Results and discussion

Some ribosides (oTR, KR) and most of the N-glucosides (oT7G, oT9G, mT7G, mT9G, pT7G, pT9G, K9G) were under limit of detection. In contrast, the levels of BA were exceptionally high (Table 1). This fact reflected the presence of BA in the proliferation medium. Interestingly, the levels of K were relatively high (the third most abundant CK type), which correlates with recent research that highlights the role of K as an anti-stress agent and inducer of programmed cell death (Żur et al. 2015). In fact, in vitro culture represents an unusual combination of stress factors, and programmed cell is an integral component of SE (Castander-Olarieta et al. 2019).

Although aromatic CK are likely to be present in many plant species and hydroxylated forms of BA occur naturally, in conifers, they have only been detected in tissues cultivated in media containing BA (Cuesta et al. 2012; Montalbán et al. 2013). Particularly, during SE, despite the high concentration of aromatic CK during proliferation, no aromatic CK could be detected in BA-independent phases (Moncaleán et al. 2018; Vondrakova et al. 2018). These results suggest that the occurrence of aromatic CK has a strong dependency on exogenous BA, or it could be associated with early stages of SE.

In the case of BA, the two N-glucoside forms could be detected. However, the amount of BA9G was between one and two orders of magnitude bigger than the amount of BA7G (Table 1), suggesting that 9-glycosilation is the favoured deactivation pathway in *P. radiata*, as already observed in previous studies (Montalbán et al. 2013).

Despite this, the levels of N-glucosides were very low or no detectable for most of the CK types, which is consistent with previous studies in conifers (Vondrakova et al. 2018; Gautier et al. 2019; Castander-Olarieta et al. 2020). These data confirm that the CK N-glucosyltransferase pathway is almost insignificant during SE or not necessary under the described conditions, since an enhancement of N-glycosylation has been reported as a detoxification mechanism when plants are exposed to supra-optimal CK concentrations (Montalbán et al. 2013).

In line with this idea, the total levels of oT and pT were lower than those of mT (Table 1). Montalbán et al. (2013) observed that hydroxylation of BA at the *ortho*- and the *para*-position rather than in the *meta*-position could also serve as a regulation mechanism by the reduction of CK activity.

Analysing the results by functional groups, the most abundant forms were bases (active forms) in all aromatic CK detected, followed by ribosides (Table 1; Fig. 1). In the case of BA, the concentration of bases was considerably higher than the rest of forms, indicating that the BA taken up by the embryogenic culture is metabolically quite stable, as already observed in SE of other conifer species (Gautier et al. 2019). Nonetheless, all the other functional groups could also be detected, being the precursors (BARMP), the second most abundant, in contrast to

Table 1 Endogenous aromatic cytokinins (pmol g^{-1} FW) in *P. radiata* EM initiated under different induction treatments (23ºC, 8 weeks; 40 ºC, 4 h; 50 ºC, 30 min; 60 ºC, 5 min), followed by cultivation at control temperature of 23 ºC

Cytokinins (pmol/g FW)	Induction treatment (ºC)			
	23	40	50	60
BA	285.25 ± 12.77^{a}	187.93 ± 18.5^{b}	122.71 ± 11.14^{c}	181.27 ± 14.39^{b}
BAR	1.13 ± 0.17^{ab}	2.37 ± 0.41^{a}	1.01 ± 0.06^{b}	1.1 ± 0.12^{b}
BARMP	3.86 ± 0.56^{b}	18.89 ± 4.82^{a}	5.87 ± 0.28^{ab}	6.91 ± 1.2^{ab}
BA7G	0.011 ± 0.007^{a}	0.01 ± 0.002^{a}	0.007 ± 0.001^{b}	0.007 ± 0.001^{b}
BA9G	0.21 ± 0.03^{b}	0.69 ± 0.1^{a}	0.1 ± 0.023^{b}	0.14 ± 0.021^{b}
oT	0.26 ± 0.013^{a}	0.11 ± 0.007^{c}	0.09 ± 0.01^{c}	0.22 ± 0.034^{b}
mT	0.24 ± 0.022^{a}	0.23 ± 0.015^{a}	0.33 ± 0.1^{a}	0.27 ± 0.021^{a}
mTR	0.027 ± 0.002^{a}	0.029 ± 0.002^{a}	0.03 ± 0.003^{a}	0.03 ± 0.002^{a}
pT	0.075 ± 0.01^{b}	0.054 ± 0.008^{c}	0.05 ± 0.002^{c}	0.093 ± 0.011^{a}
pTR	0.023 ± 0.003^{b}	0.03 ± 0.003^{a}	0.019 ± 0.002^{b}	0.025 ± 0.003^{ab}
K	2.19 ± 0.26^{a}	2.16 ± 0.27^{a}	1.795 ± 0.3^{a}	2.86 ± 0.3^{a}

Three ECLs per treatment, 2 replicates per ECL and 3 technical replicates per sample were used. Data are presented as mean values ± SE. Significant differences within a row at $p < 0.05$ are indicated by different letters

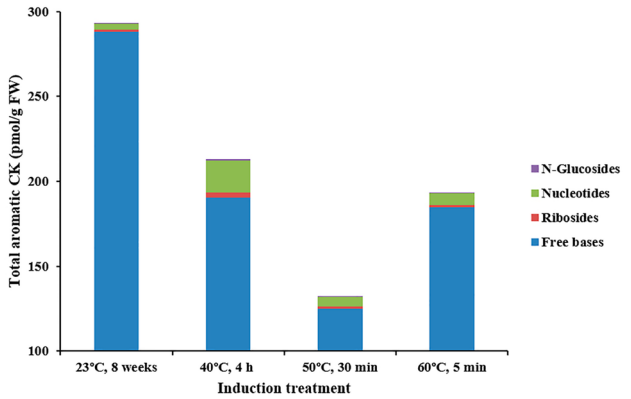

Fig. 1 Concentration of total aromatic CK in EM of *P. radiata* originated from different temperature and incubation periods (23 ºC, 8 weeks; 40 ºC, 4 h; 50 ºC, 30 min; 60 ºC, 5 min). The following aromatic CK derivatives were detected: free bases (BA, oT, mT, pT and K), ribosides (BAR, mTR, pTR), nucleotides (BAR5MP), and *N*-glucosides (BA7G, BA9G)

EM of Douglas-fir, where BAR was more abundant than BARMP (Gautier et al. 2019).

Considering the effect of temperature on the amount of aromatic CK, a significant decreasing tendency was observed for some free bases (BA, oT, pT), especially in EM originating from high temperatures and long exposures (40 ºC, 4 h; 50 ºC, 30 min) (Table 1, Fig. 1). Fluctuating and slightly reduced aromatic CK contents were also reported in thermo-inhibited *Tagetes minuta* achenes (Stirk et al. 2012), and other stresses, such as drought, have also been demonstrated to reduce the endogenous content of some aromatic CK (Ghafari et al. 2020).

This tendency was also observed for some isoprenoid bases in EM of *P. radiata* generated under the same conditions in Castander-Olarieta et al. (2020) and coincides with the initiation and proliferation rates observed during SE in Castander-Olarieta et al. (2019). The highest levels of BA, which have been detected under induction treatment of 23 ºC (285.25 ± 12.77 pmol g^{-1} FW), seem to correlate with the high proliferation rates ($10.6 \pm 2.121\%$) observed under the same treatment in the above-mentioned study, while the lowest BA contents in 50 ºC treatment (122.71 ± 11.14 pmol g^{-1} FW) correlate with the lowest proliferation rates ($4.1 \pm 1.26\%$) observed under the same treatment. Both proliferation rates and BA levels for 40ºC and 60ºC treatments showed intermediate values. High levels of active free bases are known to be essential during cell proliferation (Moncaleán et al. 2005), and under certain stress conditions, an increase of BA can trigger the synthesis of some osmoprotectants (Alvarez et al. 2008), whose function during stress has widely been documented (De Diego et al. 2015).

Although oT and pT are considered aromatic CK with low biological activity (Strnad 1997), there is increasing evidence that they could be involved in different cellular and biochemical processes, i.e. induction of cell differentiation and apoptosis in mammals (Ishii et al. 2003; Casati et al. 2011; Wang et al. 2019). The differences observed under high temperatures in this experiment support this idea (Table 1).

In parallel, 40 ºC treatment increased the levels of BAR, pTR, BARMP and BA9G (Table 1, Fig. 1). An enhancement of the main deactivation pathway (BA9G) could correlate with the lower amounts of active BA bases observed at 40 ºC. Surprisingly, this effect was not observed under 50 ºC and 60 ºC treatments. A simultaneous increase in BARMP could seem quite contradictory because of its precursor nature. However, as reported in Moncaleán et al. (2005), BARMP plays a key role in BA homeostasis, and its accumulation could be the result of BA imbalances under stress. In fact, despite being not significant, the levels of BARMP were also increased in 50 ºC and 60 ºC treatments (Table 1; Fig. 1). In any case, high levels of nucleotides and ribosides in expense of free bases have been related with low rates of cell proliferation and organogenic capacity in some pine species (Cuesta et al. 2012), which could also contribute to the low proliferation of EM observed in Castander-Olarieta et al. (2019) under high temperatures.

Conclusion

This study provides novel information about the possible involvement of endogenous aromatic CK during both SE and heat-stress responses. The presence of aromatic CK during SE in radiata pine seems to be exogenous BA-dependent, or at least more relevant during early stages of SE. As SE in radiata pine can only be induced in the presence of exogenous BA, a choice of another conifer species that does not require any cytokinin for SE induction would be preferable for such a study to confirm the results obtained. Despite this fact, endogenous aromatic CK appear to be highly influenced by the temperature during induction and could strongly influence the success of the process, reinforcing their function as stress-mediators. Further research is required to confirm their activity in different plant species and developmental stages.

Author contribution statement PM and IAM conceived and planned the experiments. ACO prepared all the plant material. CP, AP, IP, IP, ON and MS executed the hormonal analysis. ACO wrote the manuscript and all authors provided critical feedback and helped shape the research, analyses and manuscript.

Funding This research was funded by MINECO project (AGL2016-76143-C4-3R), CYTED (P117RT0522), DECO (Basque government) and MULTIFOREVER project, supported under the umbrella of ERA-NET Cofund ForestValue by ANR (FR), FNR (DE), MINCyT (AR), MINECO-AEI (ES), MMM (FI) and VINNOVA (SE). ForestValue has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No. 773324. This work was also supported by the Internal Grant Agency of Palacký University (IGA_PrF_2020_021) and from European Regional Development Fund-Project "Plants as a tool for sustainable global development" (No. CZ.02.1.01/0.0/0.0/16_019/0000827).

Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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