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Programmed cell death induced by high levels of cytokinin in *Arabidopsis* cultured cells is mediated by the cytokinin receptor CRE1/AHK4

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Abstract

High levels of cytokinins (CKs) induce programmed cell death (PCD) both in animals and plant cells. High levels of the CK benzylaminopurine (BA) induce PCD in cultured cells of *Arabidopsis thaliana* by accelerating a senescence process characterized by DNA laddering and expression of a specific senescence marker. In this report, the question has been addressed whether members of the small family of *Arabidopsis* CK receptors (AHK2, AHK3, CRE1/AHK4) are required for BA-induced PCD. In this respect, suspension cell cultures were produced from selected receptor mutants. Cell growth and proliferation of all receptor mutant and wild-type cell cultures were similar, showing that the CK receptors are not required for these processes in cultured cells. The analysis of CK metabolites instead revealed differences between wild-type and receptor mutant lines, and indicated that all three receptors are redundantly involved in the regulation of the steady-state levels of isopentenyladenine- and *trans*-zeatin-type CKs. By contrast, the levels of *cis*-zeatin-type CKs were controlled mainly by AHK2 and AHK3. To study the role of CK receptors in the BA-induced PCD pathway, cultured cells were analysed for their behaviour in the presence of high levels of BA. The results show that *CRE1/AHK4*, the strongest expressed CK receptor gene of this family in cultured cells, is required for PCD, thus linking this process to the known CK signalling pathway.

Key words: *Arabidopsis* cultured cells, cytokinin, histidine kinase cytokinin receptors, programmed cell death.

Introduction

Cytokinins (CKs) play a crucial role in regulating the proliferation and differentiation of plant cells. They are involved in many aspects of plant growth and development, such as seed germination, de-etiolation, chloroplast differentiation, apical dominance, plant–pathogen interactions, flower and fruit development, and senescence (Sakakibara, 2006; Argueso *et al.*, 2009; Werner and Schmülling, 2009).

It has been demonstrated recently that high levels of CKs induce programmed cell death (PCD) in both animal and plant cells (Ishii *et al.*, 2002; Mlejnek and Prochazka, 2002; Carimi *et al.*, 2003), revealing an unexpected role for this

central plant hormone. When 6-benzylaminopurine (BA) was added at high doses to proliferating suspension cell cultures of several plant species (including *Arabidopsis thaliana*, *Daucus carota*, and *Medicago truncatula*), cell growth was reduced and cell death induced (Carimi *et al.*, 2004, 2005; Zottini *et al.*, 2006). The analysis of a number of hallmarks (DNA laddering, nuclear chromatin condensation, and the release of cytochrome *c* from mitochondria) revealed the programmed nature of the induced cell death (Carimi *et al.*, 2003). By characterizing PCD events, two observations of particular interest were made. The first was

that cell cultures treated at different times during a sub-culture cycle showed different sensitivities to BA. Since dividing cells were more responsive than resting cells, this suggested that some sort of competence was required to undergo PCD (Carimi *et al.*, 2003). The second observation was that high levels of BA induced PCD by accelerating a senescence-like process. When *Arabidopsis* cells were treated with high levels of BA during the exponential growth phase, the percentage of cell death rapidly increased and the appearance of DNA laddering was detected concomitantly with the expression of the senescence-specific marker *SAG12* (Carimi *et al.*, 2004).

The first CK receptor was identified in *Arabidopsis thaliana* 10 years ago (Inoue *et al.*, 2001; Suzuki *et al.*, 2001). Three CK receptor genes have been isolated since then, namely *AHK2, AHK3*, and *CRE1/AHK4*, all encoding histidine kinase (HK) sensors (see review by Heyl *et al.*, 2011). Single, double and triple receptor mutants have been isolated and the *ahk2 ahk3 cre1* triple mutant, in particular, showed a severe but not lethal phenotype (Nishimura *et al.*, 2004; Higuchi *et al.*, 2004; Riefler *et al.*, 2006). Analysis of these loss-of-function mutants revealed the implication of these receptor genes in regulating numerous aspects of plant growth and development, including root and shoot growth, leaf senescence, seed size, and germination (Nishimura *et al.*, 2004; Higuchi *et al.*, 2004; Riefler *et al.*, 2006).

In this study, the question whether PCD induced by high levels of BA in cultured cells depends on one or several of these CK receptors was approached. To this end, cultured cell lines from seedlings of different CK receptor mutants were produced and characterized. The analyses revealed cell growth parameters comparable with wild-type cell line, but differences in the response to high levels of BA. The results pinpointed a central role of CRE1/AHK4 in mediating the BA-induced PCD.

Materials and methods

Plant material, culture conditions, and treatments

The plants were grown in a phytotron at 22 °C under long-day conditions (16/8 h light/dark) and exposed to white light (~75 µE).

Seeds were surface-sterilized and vernalized at 4 °C for 3 d in the dark for RNA extraction from seedlings grown *in vitro*. Then, the seeds were exposed to white light and allowed to germinate and grow at 22 °C for 6 d on horizontal plates containing half-strength MS liquid medium, 0.1% sucrose, and 0.5 g l^{-1} MES. The pH of the media was adjusted to 6.0±0.1 with 0.5 M KOH before autoclaving at 121 °C for 20 min.

Cell lines from wild-type *Arabidopsis thaliana* ecotype Columbia (Col-0) and the CK receptor mutants *cre1-2, ahk2-5 ahk3-7*, and *ahk2-5 ahk3-7 cre1-2* (Riefler *et al.*, 2006) were generated from cotyledons of 12-d-old seedlings. Briefly, isolated cotyledons were incubated on modified Murashige and Skoog (1962) solid medium [0.8% (w/v) plant agar] [MSR2: 2.70 mM KH$_2$PO$_4$, 40 µM nicotinic acid, 33 µM thiamine hydrochloride, 60 µM pyridoxal hydrochloride, 0.8% (w/v) plant agar] supplemented with 0.5 g l^{-1} malt extract, 30 g l^{-1} sucrose, 9 µM BA, and 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) for 3 weeks in order to induce callus formation. Subsequently, callus produced from explants was transferred to liquid medium and a suspension cell culture was

produced. The pH of the solid and liquid media was adjusted to 5.7±0.1 with 0.5 M NaOH before autoclaving at 121 °C for 20 min. Cells were routinely subcultured every 7 d. The addition of BA was not required to maintain cell growth, but strongly reduced the formation of cell clumps in the culture. For subculture cycles, 1.5 ml of packed cell volume was placed in 250 ml Erlenmeyer flasks containing 50 ml of liquid medium. Cells were subcultured in fresh medium at 7 d intervals and maintained in a climate chamber on a horizontal rotary shaker (80 rpm) at 25±1 °C at a 16/8 h light/dark cycle. Three-day-old wild-type and mutant cells were incubated with 44 µM BA and collected 4 d later to determine the effect of BA.

Cell viability and analysis of nuclear morphology

Cell growth was determined by measuring the cell dry weight of the cell cultures at different times of the subculture cycle. To determine dry weight, cells were separated from the culture medium and cell debris using a vacuum filtration unit (Sartorius, Florence, Italy). The collected cells were dried overnight at 60 °C.

Cell death was determined by spectrophotometric measurements of cell uptake of Evan's blue, as described by Shigaki and Bhattacharyya (1999).

Nuclei were visualized by staining with 4′,6-diamidino-2-phenyl-indole (DAPI, Alexis Biochemicals, Florence, Italy) as described by Traas *et al.* (1992), with some modifications. An aliquot of 500 µl of suspension culture was added to an equal volume of fixation solution [4% (w/v) paraformaldehyde in PEM buffer (100 mM HEPES, pH 6.9, 10 mM EGTA, and 10 mM MgSO$_4$)]. After 30 min, cells were washed three times in PEM buffer and re-suspended in 500 µl of PEM buffer. An aliquot of 200 µl of fixed cells was then added to an equal volume of PEM buffer containing 0.2% (w/v) Triton X-100 and 1 µg ml^{-1} DAPI. Stained cells were laid on a glass slide treated with poly-L-Lys, and nuclei were visualized with a fluorescence microscope (Leica, Milan, Italy) with an excitation filter of 330–380 nm and a barrier filter of 410 nm (De Michele *et al.*, 2009).

Identification and quantification of endogenous cytokinins

Three-day-old cultured cells were harvested, frozen in liquid N$_2$, and stored at –80 °C. Three independent biological samples, each of ~1 g, were collected for each cell line. The procedure used for CK purification was a modification of the method described by Faiss *et al.* (1997). Deuterium-labelled CK internal standards (Olchemim Ltd., Czech Republic) were added, each at 1 pmol per sample, to check the recovery during purification and to validate the determination (Novák *et al.*, 2008). The samples were purified using a combined cation (SCX-cartridge) and anion (DEAE-Sephadex-C18-cartridge) exchanger and immunoaffinity chromatography (IAC) based on wide-range specific monoclonal antibodies against CKs (Novák *et al.*, 2003). The metabolic eluates from the IAC columns were evaporated to dryness, dissolved in 30 µl of the mobile phase, and finally analysed by ultra-performance liquid chromatograph-electrospray ionization tandem mass spectrometry. Quantification was obtained by multiple reaction monitoring of [M+H]$^+$ and the appropriate product ion. Optimal conditions, dwell time, cone voltage, and collision energy in the collision cell, corresponding to the exact diagnostic transition, were optimized for each CK for selective MRM experiments (Novák *et al.*, 2008). Quantification was performed by Masslynx software using a standard isotope dilution method (Novák *et al.*, 2003).

RNA isolation and cDNA synthesis

Cells and seedlings were harvested, frozen in liquid N$_2$, and stored at –80 °C. RNA was isolated with the TRIzol method, as described by Riefler *et al.* (2006). Then the total RNA was purified using an RNeasy kit, including DNase digestion (Quiagen, Hilden,

Germany). cDNA was synthesized by SuperscriptIII (Invitrogen, Karlsruhe, Germany) from 1 µg of purified RNA.

DNA primer

The quantitative real-time RT-PCR expression analysis of CK receptors genes was performed using the following primers: CRE1-F (GGCACTCAACAATCATCAAG) and CRE1-R (TCTTTCTCG-GCTTTTCTGAC) for the expression analysis of the *CRE1/AHK4* gene; AHK2-F (GAGCTTTTTGACATCGGG) and AHK2-R (TTCTCACTCAACCAGACGAG) for the expression analysis of the *AHK2* gene; AHK3-F (GTGACCAGGCCAAGAACTTA) and AHK3-R (CTTCCCTGTCCAAAGCAA) for the expression analysis of the *AHK3* gene; ARR4-F (CCGTTGACTATCTCGCCT) and ARR4-R (CGACGTCAACACGTCATC) for the expression analysis of the *ARR4* gene; ARR5-F (CTACTCGCAGCTAAAACGC) and ARR5-R (GCCGAAAGAATCAGGACA) for the expression analysis of the *ARR5* gene; ARR6-F (GAGCTCTCCGATG-CAAAT) and ARR6-R (GAAAAAGGCCATAGGGGT) for the expression analysis of the *ARR6* gene; and finally, EF-1α-F (TGAG-CACGCTCTTCTTGCTTTCA) and EF-1α-R (GGTGGTGGCA-TCCATCTTGTTACA) for the expression analysis of the *elongation factor-1α* (*EF-1 α*) gene.

RNA analysis

Quantitative real-time RT-PCR using FAST SYBR Green I technology was performed on an ABI PRISM 7500 sequence detection system (Applied Biosystems, Darmstadt, Germany) using the following cycling conditions: initial denaturation at 95 °C for 15 min, 40 cycles of 30 s at 95 °C, 15 s at 55 °C, and 10 s at 72 °C, followed by melt curve stage analysis to check for specificity of the amplification.

The reactions contained SYBR Green Master Mix (Applied Biosystems), 300 nM of gene-specific forward and reverse primers and 1 µl of the diluted cDNA in a 20 µl reaction. The negative controls contained 1 µl RNase free water instead of the cDNA. The primer efficiencies were calculated as $E=10^{-1/slope}$ on a standard curve generated using a 4-fold or a 2-fold dilution series over at least five dilution points of cDNA (Cortleven *et al.*, 2009). The expression analysis of CK receptor and *ARR* genes was performed by the Pfaffl method, using *EF-1α* as the reference gene (Pfaffl, 2001; Remans *et al.*, 2008).

Statistical analysis

All data are representative of at least three independent biological replicates. Values are expressed as mean ±SD. The statistical significance of differences was evaluated by Student's *t* test and one-way analysis of variance (ANOVA).

Results

Expression analysis of CK receptor genes in plants and cultured cells of wild-type Arabidopsis thaliana

In order to evaluate the relevance of CK receptors in mediating the BA effect on PCD, firstly, appropriate CK receptor mutants had to be selected for the production of cultured cell lines. To this end, the expression levels of the three CK receptor genes *AHK2*, *AHK3*, and *CRE1/AHK4* were evaluated by quantitative real-time RT-PCR analysis, both in wild-type *Arabidopsis* seedlings and a cultured cell line. In seedlings, the most strongly expressed gene was *AHK2*; *AHK3* was less expressed than *AHK2*, and *CRE1/AHK4* was expressed at an even lower level (Fig. 1). In
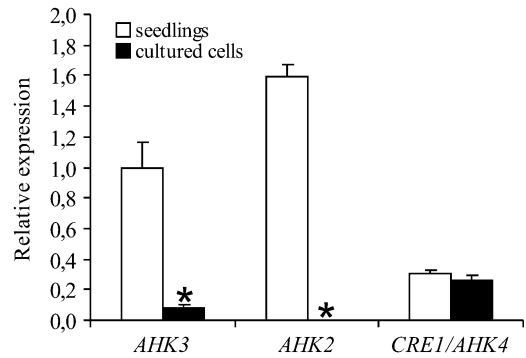

Fig. 1. Quantitative real-time RT-PCR expression analysis of CK receptor genes in *Arabidopsis* wild-type seedlings and cultured cells. The relative expression values for all genes are related to the expression level of *AHK3*, which was set to 1. Values represent mean ±SD of the RQ value of three experiments performed by using templates from three independent biological samples. Asterisks indicate expression levels that are significantly different from those found in seedlings as calculated by Student's *t* test (*P <0.01).

wild-type cultured cells, the expression levels of *AHK2* and *AHK3* receptor genes were lower than in seedlings, while *CRE1/AHK4* was expressed at approximately the same level. The most strongly expressed gene was *CRE1/AHK4*, while expression of *AHK2* was almost undetectable, and *AHK3* was expressed at a low level (Fig. 1). Taking the high expression of *CRE1/AHK4* as an indication for putative functional relevance, three mutants were selected to produce cultured cells: the single mutant *cre1-2* to analyse the behaviour of the cultured cells in the absence of CRE1/AHK4; the double mutant *ahk2-5 ahk3-7* to evaluate only the CRE1/AHK4 function; and the triple mutant *ahk2-5 ahk3-7 cre1-2* to evaluate BA effects in the absence of all three CK receptors. These cell lines are named in the following *cre1*, *ahk2 ahk3*, and *ahk2 ahk3 cre1*, respectively.

### Establishment and characterization of receptor mutant cell lines

Young seedlings of the selected mutants and wild-type *Arabidopsis* were used as starting material to induce de-differentiation and callus formation. For each of these lines, callus cultures were easily produced and, successively, suspension cell cultures were obtained by transferring callus cultures into liquid medium (Fig. 2A). After stable cell cultures were established, cell growth (Fig. 2B) and cell viability (Fig. 2C) were determined. No major differences in growth kinetics were noted among the four lines, and the maximum dry weight was reached at about the same time (Fig. 2B). The only notable difference observed in the triple mutant line was a greenish phenotype, corresponding indeed to a doubled chlorophyll content compared with the other three lines (data not shown), and a delay in entering the senescence phase (Fig. 2C). In the triple mutant cell population, the greenish phenotype corresponded to a lower level of cell death at 21 d after culture initiation (24.8±2%)
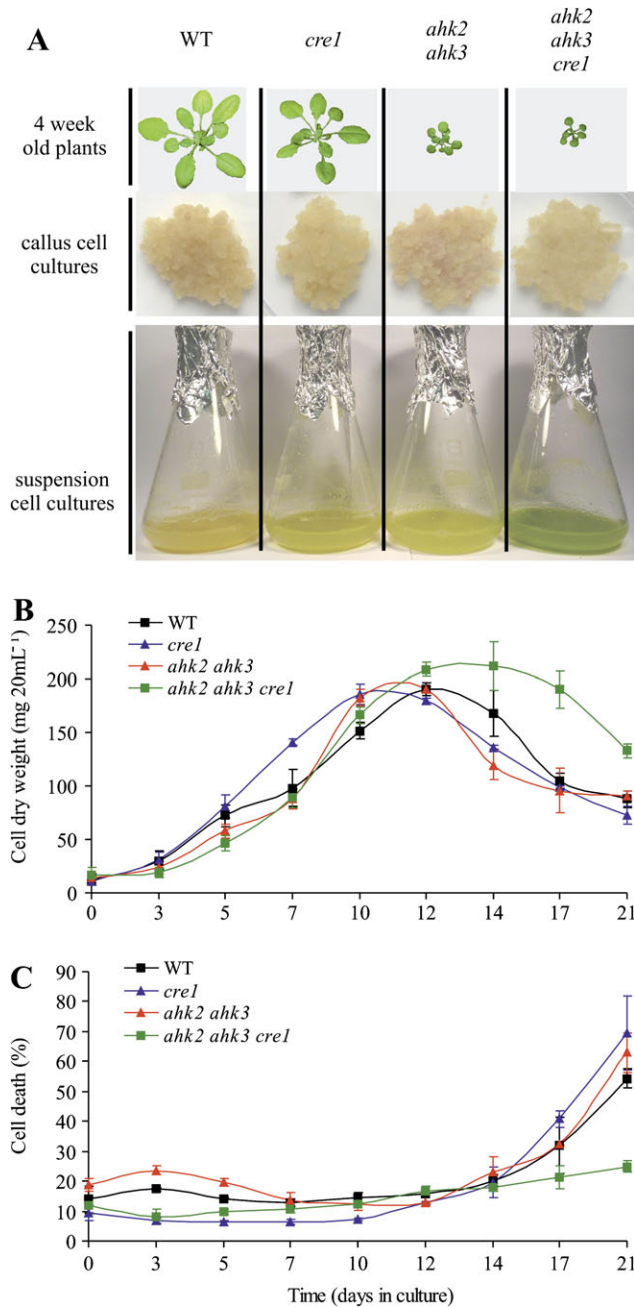
Fig. 2. *Arabidopsis* plants and cultured cells of wild-type, *cre1*, *ahk2 ahk3*, and *ahk2 ahk3 cre1* receptor mutants. From top to bottom: (A) Plants, callus cultures, suspension cell cultures; (B) cell dry weight at different times after culture initiation; (C) cell viability (Evan's blue staining) at different times after culture initiation. Cell dry weight and cell death were measured from 0–21 d after culture initiation. Values represent mean ±SD of three independent experiments.

when compared with the level of cell death measured in the other cell lines (in the range of 54-63%) (Fig. 2C).

The expression levels of the three CK receptor genes were measured in the mutant cell cultures and compared with their expression in the wild-type cell line (Fig. 3). The steady-state transcript levels of *AHK3* were similar in wild-type and *cre1* cell lines while the expression of *AHK2* was enhanced in the
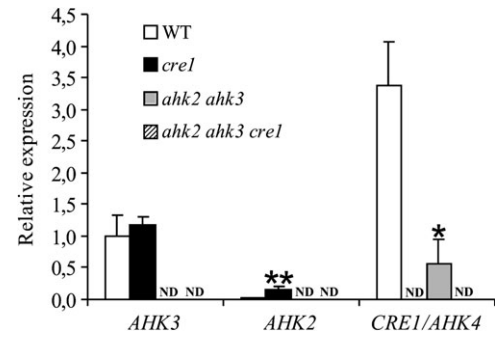
Fig. 3. Quantitative real-time RT-PCR expression analysis of CK receptor genes in wild-type, *cre1, ahk2 ahk3*, and *ahk2 ahk3 cre1* cultured cell lines. The relative expression values for all CK receptor genes are related to the expression level of *AHK3* (set to 1) in the wild-type cell line. Values represent mean ± SD of the RQ value of three experiments performed by using templates from three independent biological samples. ND, not detected. Asterisks indicate expression levels that are significantly different from those found in wild-type cell line as calculated by Student's *t* test ($*P <0.01$, $**P <0.05$).

cre1 mutant. Unexpectedly, the expression level of *CRE1/AHK4* was reduced in the *ahk2 ahk3* mutant. None of the receptor genes was expressed in the triple mutant.

Free and conjugated CKs in wild-type and mutant cell cultures

Because the concentrations of several CKs were increased in CK receptor mutant plants (Riefler *et al.*, 2006), the concentrations of free and conjugated CKs were determined in the different cell cultures (Table 1). Several differences were observed. The levels of most CK metabolites increased to a different extent in the CK receptor mutant lines. Among the iP-type CKs, the riboside iPR was most strongly enhanced, whereas only a moderate increase in iP and even a decrease in iP9G concentration was measured. The free base *t*Z and the conjugate *t*ZOG were detectable only in mutant lines and a significant increase in *t*ZR content was observed in the double and triple mutant cell lines. The concentrations of various *c*Z-type CKs, which are synthesized via a different pathway (Miyawaki *et al.*, 2006; Sakakibara, 2006), were also significantly higher in CK receptor mutant lines. In particular, the concentrations of the riboside *c*ZR and the nucleotide *c*ZR5'MP were strongly increased in the double and triple mutant lines. The strong increase of *c*Z-type metabolites was also found in the receptor mutant lines treated by BA as is described below (data not shown). Taken together, all three receptors seem to have a function in regulating the steady-state levels of iP-, *t*Z-type, and/or *c*Z-type CKs.

Effects of high levels of BA on *Arabidopsis* receptor mutant cell lines

Once the receptor mutant cell lines were established and characterized, experiments to detect the effects of high concentrations of BA were performed. Three-day-old

Table 1. Cytokinin content of *Arabidopsis* wild-type and receptor mutant cultured cells

One gram of 3-d-old *Arabidopsis* cultured cells per sample was collected, and three independent biological samples were taken for each genotype. Data shown are pmol g^{-1} fresh weight ±SD. *tZ*, trans-zeatin; *cZ*, cis-zeatin; iP, N^6-(Δ^2isopentenyl)adenine; tZOG, trans-zeatin *O*-glucoside; cZROG, c-zeatin riboside *O*-glucoside; tZR, trans-zeatin riboside; cZR, c-zeatin riboside; iPR, N^6-(Δ^2isopentenyl)adenosine; iP9G, N^6-(Δ^2isopentenyl)adenine 9-glucoside; and iPR5'MP, N^6-(Δ^2isopentenyl)adenosine 5'-monophospate. ND, not detectable. Bold letters mark concentrations of CKs in mutants that are significantly different from those of the wild type tested by ANOVA analysis. *, **, and *** correspond to *P*-values of 0.05>*P*>0.01, 0.01>*P*>0.001, and 0.001>*P*, respectively.

Line/CK metabolite	iP	iPR	iP9G	tZ	tZR
Wild-type	0.18±0.06	0.31±0.11	3.03±0.20	ND	0.04±0.01
cre1	**0.63±0.21***	**1.28±0.19****	**0.29±0.05*****	4.44±1.24	0.03±0.01
ahk2 ahk3	**0.68±0.28***	**1.72±0.62***	**0.18±0.02*****	3.92±1.51	**0.10±0.03***
ahk2 ahk3 cre1	**0.63±0.10****	**9.03±1.11*****	**0.36±0.04*****	1.12±0.31	**0.20±0.05****

	tZOG	cZ	cZR	cZROG	cZR5'MP
Wild-type	ND	0.08±0.02	5.97±0.73	1.74±0.49	5.21±1.71
cre1	0.66±0.15	0.15±0.04	**3.58±0.33****	1.06±0.29	3.04±1.10
ahk2 ahk3	1.15±0.38	**0.19±0.06***	**12.13±3.09***	2.46±0.80	**34.31±4.86*****
ahk2 ahk3 cre1	2.47±0.55	**0.33±0.04*****	**47.42±14.61****	**8.04±2.10***	**73.08±18.55****

proliferating cell cultures were incubated with and without 44 μM BA. Expression of known CK primary response genes, namely *ARR4*, *ARR5*, and *ARR6*, were tested to evaluate whether this treatment activated the CK signalling pathway (D'Agostino *et al.*, 2000). The results showed a clear induction of all three genes after 2 h BA treatment in wild-type cells although the induction levels for the three genes differed (Fig. 4). Differences in the cytokinin response of the reporter genes were also noted in the mutant lines. Low but reproducible *ARR* gene induction was found in those cell lines retaining one or two of the receptors while no induction was detected in the triple mutant. The weaker response of the mutant cell lines compared with the wild type may, in part, be explained by reduced expression levels of the receptor genes (e.g. of *CRE1/AHK4* in the *ahk2 ahk3* mutant; see Fig. 3) and/or a reduction of downstream components in the signalling chain. Notably, a very low expression level of *ARR* genes has also been reported for CK receptor mutant seedlings (Nishimura *et al.*, 2004).

The cell dry weight and cell death (Evan's blue staining) of 4-d-treated cells were measured to evaluate the effects of BA on growing cells. Treatment of wild-type cells with BA at the beginning of the exponential growth phase induced PCD: cell dry weight was significantly reduced (30%) (Fig. 5A) and the percentage of cell death doubled after 4 d of treatment (Fig. 5B). The double mutant cell line *ahk2 ahk3* was affected by BA treatment to a similar extent as the wild-type. By contrast, the same treatment did not affect cell growth and viability either in the cell line derived from the *cre1* mutant, or in the triple mutant cell line (Fig. 5A, B). To test whether the cell death was due to PCD, the nuclear morphology was investigated using DAPI staining and analysis by fluorescence microscopy (Fig. 5C, lower panel). A strong increase in the percentage of stretched nuclei (Fig. 5C, upper panel) was detected in wild-type and double mutant cultures, but not in single *cre1* and triple mutant cell lines. This confirmed the programmed nature of cell death induced by high levels of BA.
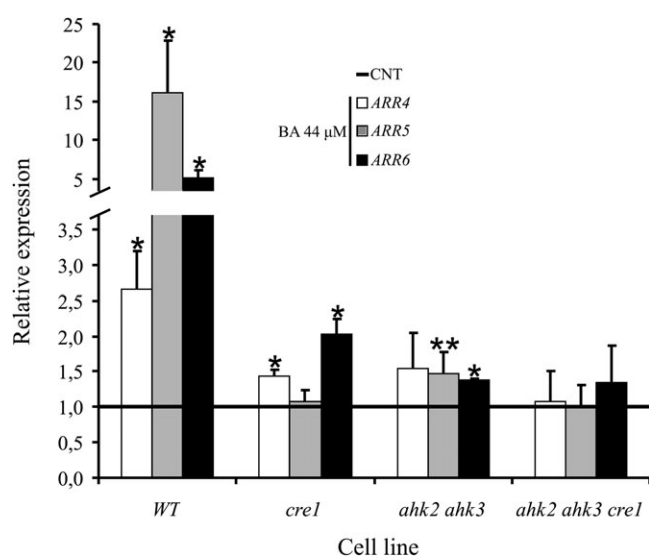
Fig. 4. Quantitative real-time RT-PCR expression analysis of *ARR4, ARR5,* and *ARR6* genes in wild-type, *cre1, ahk2 ahk3,* and *ahk2 ahk3 cre1* cultured cell lines after incubation with 44 μM BA for 2 h. The relative expression values of *ARR* genes are related to the expression level in untreated cells (set to 1). Values represent mean ±SD of the RQ value of three experiments performed by using templates from three independent biological samples. Asterisks indicate expression levels that are significantly different from those found in untreated cell lines as calculated by Student's *t* test (**P* <0.01, ***P* <0.05).

Discussion

It has been shown previously that high levels of CKs, and BA in particular, induced PCD in proliferating suspension cell cultures of several plant species including *Arabidopsis*. This PCD was shown by analysing senescence-associated markers to be an accelerated senescence process. In plants, the presence of high concentrations of BA induced more rapid leaf yellowing and precocious DNA fragmentation,
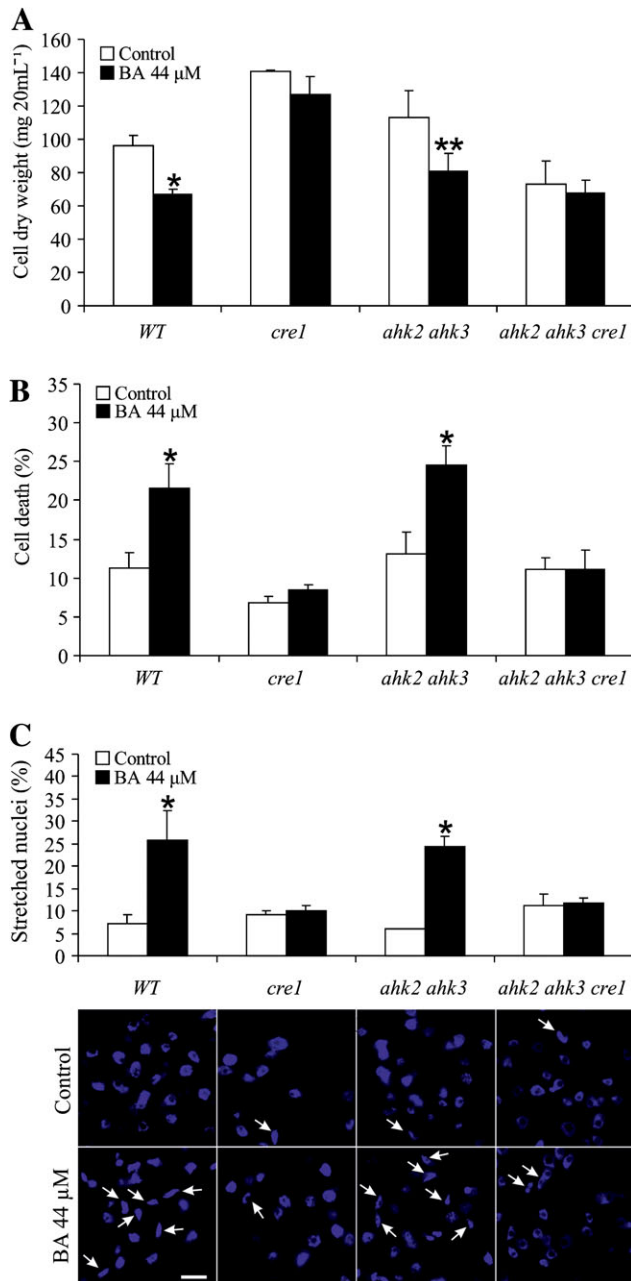
Fig. 5. Effects of BA treatment on PCD parameters of wild-type and CK receptor mutant cultured cells. *Arabidopsis* cells were treated 3 d after subculturing with 44 μM BA for 4 d. (A) Cell dry weight of *Arabidopsis* cultured cells measured 4 d after BA addition. (B) Cell death measured by Evan's blue staining 4 d after BA addition. (C) Lower panel: nuclei of treated and untreated cells, stained with DAPI; upper panel: percentage of stretched nuclei after BA addition; white arrows indicate stretched nuclei; bar=20 μm. Values represent mean ±SD of three independent experiments. Asterisks indicate values that are significantly different from those of untreated cells by Student's *t* test (*$P < 0.01$, **$P < 0.05$).

both in carrot and *Arabidopsis* (Carimi *et al.*, 2004). Recently a classification of PCD in plants mainly based on cell morphology has been proposed (van Doorn *et al.*, 2011; van Doorn, 2011), distinguishing between two major classes: 'autolytic' and 'non-autolytic'. The BA-induced

PCD seems to belong to the first one, being a slow process and showing similarities to the senescence process. However, detailed morphological analyses will precisely define to which PCD class the BA-process belongs.

In this report, the question as to whether PCD induced by high levels of CK depends on one or several members of the *Arabidopsis* CK receptor family has been addressed. To this purpose, cultured cell lines from selected receptor mutants were produced focusing on *CRE1/AHK4*, as it was the highest expressed CK receptor gene in cell cultures. The relatively high expression of *CRE1/AHK4* was not completely unexpected, as cell cultures are enriched in proliferating cells. Different expression analyses have previously shown that the *CRE1/AHK4* gene is particularly strongly expressed in proliferating tissue, including the root tip, the shoot apical meristem (SAM), and during nodule formation in *Medicago truncatula*. By contrast, the *AHK2* and *AHK3* genes are expressed more strongly in non-dividing leaf cells (Nishimura *et al.*, 2004; Frugier *et al.*, 2008; Gordon *et al.*, 2009; Stolz *et al.*, 2011).

The fact that all receptor mutant cell lines proliferated well and were comparable to the wild-type cell line showed that CK was not needed to induce cell division and the CK receptors were not involved in the control of cell cycle progression in these cultures. This observation was confirmed in another independent triple mutant cell line harbouring a different allele combination (*ahk2-2 ahk3-3 cre1-12*; Higuchi *et al.*, 2004; data not shown). This result is interesting as it is generally thought that CK is required for plant cell division. However, it is known that cytokinin-independent growing cells can be selected during the establishment of the cell culture (Binns and Meins, 1973). It may also be that a separate cell-autonomous CK response system may function in cultured cells and maintain cell division independently of CK receptors. Cell cycle phase-specific sharp peaks in the levels of CK were identified in cytokinin-autonomous tobacco BY-2 cell cultures and it was suggested that CK may act through modulation of the activity of cell cycle-regulating kinases (Redig *et al.*, 1996). In fact, CK inhibition of cyclin-dependent kinases is well known from mammalian cell cultures (Veselý *et al.*, 1994).

The analysis of the CK content showed a strong increase of different metabolites in the CK receptor mutant cell cultures. In general, an increase in the steady-state levels of iP- and *t*Z-type CKs was observed in all mutant cells, indicating a negative regulation of the synthesis pathway by its product through all three receptors. This confirms an earlier observation made in CK receptor mutant seedlings (Riefler *et al.*, 2006). Considering *c*Z-type CKs, an increase in *c*Z, *c*ZR, and *c*ZR5'MP levels was noted only in the *ahk2 ahk3* double mutant and triple receptor mutant cell lines and not in the *cre1* line. In *Arabidopsis*, *c*Z-type CKs are synthesized through a distinct pathway, the tRNA pathway, with two tRNA-IPT enzymes catalysing the initial step (Miyawaki *et al.*, 2006; Sakakibara, 2006). Our result indicates that biosynthesis of *c*Z-type CKs is also under the negative control of CK receptors, in this case mainly of only AHK2 and AHK3. This negative feedback control may

indicate a biological relevance of *cZ*-type CKs which is still debated (Dobrá *et al.*, 2010; Gajdošová *et al.*, 2011). Furthermore, the result shows that AHK2 and AHK3 are active in these cell cultures, despite the low expression levels of the corresponding genes.

BA treatment of the cell cultures induced PCD only in the presence of CRE1/AHK4. It caused a severe decrease of dry weight and cell viability in wild-type and in *ahk2 ahk3* mutant lines, but did not affect the growth and cell viability of *cre1* mutant and *ahk2 ahk3 cre1* mutant lines. The same result was obtained with a different receptor mutant allele combination (*ahk2-2 ahk3-3 cre1-12*; data not shown). The dependence on CRE1/AHK4 may also explain why high amounts of BA are required to induce PCD, as CRE1/AHK4 has only a low affinity to BA (Spíchal *et al.*, 2004; Romanov *et al.*, 2006). It is interesting to note that AHK2 and AHK3 were incapable of coupling the BA signal to the downstream response leading to cell death, although BA induced, in heterologous systems, a stronger cytokinin response through AHK2 and AHK3 than through CRE1/AHK4 (Spíchal *et al.*, 2004; Romanov *et al.*, 2006), and despite a similar capacity of all three receptors to interact with phosphotransmitter proteins acting immediately downstream in the signalling chain (Dortay *et al.*, 2006).

In this report, by using a gentic approach, the involvement of CRE1/AHK4 in causing PCD has been shown in cultured cells treated with high levels of BA. The reason for the specificity of the action of CRE1/AHK4 in this pathway needs to be explored further. Similarly, it will be interesting to identify additional components of this specific response and reveal in which context the pathway is activated *in planta*.

Acknowledgements

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