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Molecular and physiological mechanisms of stress signalling in plants.

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CHAPTER 2

***Arabidopsis thaliana* GLYCERALDEHYDE-3-PHOSPHATE**

ABSTRACT

The aim of this PhD research project was the understanding of some molecular and physiological mechanisms involved in stress signalling in plants. When plants are exposed to environmental growth conditions far from the ideals, plants are under stress. Under stress conditions, plant growth and crop yield could be severely affected. In general, adverse growth conditions are caused by plant pathogens (biotic stress) and not ideal environmental conditions (abiotic stress) such as heat, cold, drought, lack of nutrients, and heavy metals. Plants experiencing biotic and abiotic stresses activate signalling pathways that lead to changes in gene expression, thus, alteration of transcriptome, proteome and metabolome. All these processes are oriented to the acclimation of plants to the changed growth conditions.

During my PhD I approached the study of two stress signalling mechanisms, one occurring as consequence of high concentration of cytokinins (CKs), a physiopathological condition (biotic stress), and the other one as consequence of cadmium-induced oxidative stress (abiotic stress).

Concerning the first signalling pathway, I investigated the involvement of CK receptors in triggering the programmed cell death (PCD) signalling in *Arabidopsis thaliana* cell cultures treated with high concentrations of CKs. In order to do so, we generated cell culture lines from wild-type and three *Arabidopsis* mutants, knocked-out in genes coding for different CK receptors (*cre1-2*, *ahk2-5 ahk3-7* and *ahk2-5 ahk3-7 cre1-2*). Then, we characterized some physiological parameters of these cell cultures; in particular, we determined the

growth and death kinetics, and the CKs content. No evident differences in the growth and death kinetics of wild-type, single and double mutant cell culture lines were observed, except for a delay in increasing cell death in the late part of the triple mutant subculture cycle. Moreover, the levels of most CKs resulted increased to a different extent in the CK receptor mutant lines. Then, in order to define whether the CK-induced PCD mechanisms required the functionality of the CK receptors, wild-type and mutant cell cultures were assayed for their sensitivity to high concentrations of CKs. The results of these experiments revealed that the CRE1/AHK4 was the CK-receptor involved in triggering the PCD signals in *Arabidopsis* cell cultures exposed to high concentrations of CKs.

Concerning the second signalling pathway, the role of *Arabidopsis* cytosolic GAPDH (GAPC-1 and GAPC-2) in cadmium-induced oxidative stress signalling was investigated. To this aim, *Arabidopsis* seedlings were exposed to cadmium, and I demonstrated that the levels of both nitrogen oxide (NO) and hydrogen peroxide (H_2O_2) increased leading to the induction of an oxidative stress condition. Under these stress conditions the up-regulation of both *GAPC-1* and *GAPC-2* occurred. Given that, the more affected gene was *GAPC-1*, I produced *Arabidopsis* transgenic lines expressing GAPC-1 transcriptional and translational reporters. The exposure of these transgenic lines to cadmium revealed that GAPC-1 is affected by oxidative stress both at the transcriptional and post-transcriptional levels. Moreover, *in vitro* analyses revealed that NO and H_2O_2 negatively affected the GAPDH catalytic activity in a time- and concentration-dependent manner. These results confirmed that the oxidative stress condition induced by the accumulation of NO and H_2O_2 can affect the catalytic activity of the GAPC-1 enzyme, activating a signal cascade that enhances its expression and then its

accumulation. Furthermore, reducing the redox state with reduced glutathione restored the catalytic activity of the NO-inactivated recombinant GAPC-1, and also reduced the *GAPC-1* promoter activity and GAPC-1 protein level *in vivo*. On the other hand, decreasing the glutathione pool *in vivo* (i.e. more oxidizing condition) led to the increase of *GAPC-1* promoter activity and GAPC-1 protein level. Finally, prolonged oxidative stress conditions led to the GAPC-1 nuclear accumulation. All together these results demonstrate that GAPC-1 is an oxidative stress sensor, and its nuclear relocalization could be a key step in the oxidative stress signalling.

The study of how plants perceive stress conditions, the mechanisms by which plants transmit stress signals, and finally the acclimation to adverse growth conditions could be important to understand how to reduce the negative effects of biotic and abiotic stresses on plant growth and ultimately crop yield.

RIASSUNTO

Lo scopo di questa tesi di dottorato è stato lo studio dei meccanismi molecolari e fisiologici coinvolti nella trasduzione dei segnali di stress nelle piante. Quando le piante sono esposte a condizioni ambientali di crescita non ideali, si trovano in condizioni di stress. In queste condizioni, la crescita delle piante e la resa delle colture d'interesse agronomico sono di solito fortemente ridotte. In generale, le condizioni di crescita sfavorevoli sono causate da agenti patogeni (stress biotici) e da condizioni ambientali non ideali (stress abiotici) come caldo, freddo, siccità, carenza di nutrienti e metalli pesanti. Le piante esposte a stress biotici e abiotici attivano vie di segnalazione che inducono il cambiamento dell'espressione genica, quindi, l'alterazione del trascrittoma, proteoma e metaboloma. Tutti questi processi sono orientati all'acclimatazione delle piante alle condizioni di crescita avverse.

Durante il mio dottorato di ricerca ho affrontato lo studio di due meccanismi di segnalazione dello stress, uno che si verifica in seguito ad esposizione ad elevate concentrazioni di citochinine (CKs), una condizione fisiopatologica (stress biotici), e l'altro in risposta a stress ossidativo indotto da alte concentrazioni di cadmio (stress abiotici).

Per quanto riguarda il primo meccanismo di segnalazione, ho studiato il ruolo dei recettori citochininici nella via di segnalazione e induzione della morte cellulare programmata (PCD) in colture cellulari di *Arabidopsis thaliana* trattate con alte concentrazioni di CKs. Per far ciò, abbiamo generato colture cellulari da una linea wild-type e da tre linee mutanti (knock-out) di *Arabidopsis*, in geni che

codificano per diversi recettori citochininici (*cre1-2*, *ahk2-5 ahk3-7* e *ahk2-5 ahk3-7-cre1-2*). In seguito, abbiamo caratterizzato alcuni parametri fisiologici di queste colture cellulari, in particolare, abbiamo determinato la crescita e la cinetica di morte e il contenuto di CKs. Non sono state riscontrate differenze evidenti nelle cinetiche di crescita e morte tra cellule wild-type, e linee cellulari mutanti, ad eccezione di un ritardo nella morte cellulare nelle ultime fasi del ciclo di coltura del triplo mutante. Inoltre, sono stati misurati livelli più elevati di CKs nelle linee cellulari mutanti. Infine, per verificare se i recettori citochininici fossero coinvolti nell'induzione della PCD in seguito al trattamento con alte concentrazioni di CK, è stata analizzata la risposta delle colture cellulari wild-type e mutanti ad alte concentrazioni di CKs. I risultati di questi esperimenti hanno rivelato che il recettore CRE1/AHK4 sia quello coinvolto nel meccanismo di segnalazione e induzione della PCD in colture cellulari di *Arabidopsis* esposte ad alte concentrazioni di CK.

Per quanto riguarda il secondo meccanismo di segnalazione, ho studiato il ruolo delle GAPDH citosoliche (GAPC-1 e GAPC-2) di *Arabidopsis* nelle vie di segnalazione indotte da stress ossidativo in presenza di alte concentrazioni di cadmio. A tal fine, sono state trattate piantine di *Arabidopsis* con alte concentrazioni di cadmio e i risultati hanno mostrato che tale trattamento induceva un aumento dei livelli di ossido nitrico (NO) e perossido d'idrogeno (H_2O_2), e tale aumento causava l'induzione di una condizione di stress ossidativo. In queste condizioni di stress si è osservato un aumento dell'espressione di entrambi i geni GAPC. Essendo *GAPC-1* il gene che mostrava l'aumento di espressione maggiore, ho prodotto delle linee transgeniche di *Arabidopsis* che esprimevano stabilmente geni *reporter* trascrizionali e traduzionali per *GAPC-1*.

L'esposizione di queste linee transgeniche al cadmio ha rivelato che GAPC-1 è influenzata dallo stress ossidativo sia a livello trascrizionale che post-trascrizionale. Inoltre, dalle analisi *in vitro* si è osservato che NO e H_2O_2 influenzavano negativamente l'attività catalitica GAPDH in modo tempo- e concentrazione-dipendente. Questi risultati hanno confermato che le condizioni di stress ossidativo indotte da un aumento delle concentrazioni di NO e di H_2O_2 possono influenzare l'attività catalitica di GAPC-1, attivando una via di segnalazione che porta a un aumento della sua espressione e quindi il suo accumulo. Riducendo lo stato redox con il glutatione ridotto, l'attività catalitica della GAPC-1 ricombinante inattivata dal trattamento con NO si ripristinava. Inoltre, la presenza di glutatione ridotto riduceva sia l'attività promotoriale di *GAPC-1* che i livelli di GAPC-1 *in vivo*. Peraltro, riducendo il contenuto di glutatione *in vivo* (quindi imponendo una condizione più ossidante) si è osservato l'aumento dell'attività promotoriale di *GAPC-1* ed un aumento dei livelli di GAPC-1. Infine, prolungate condizioni di stress ossidativo hanno portato all'accumulo di GAPC-1 all'interno del nucleo. Questi risultati dimostrano che GAPC-1 è un sensore di stress ossidativo, e la sua rilocalizzazione nucleare potrebbe essere un passaggio chiave per la segnalazione dello stress ossidativo.

Lo studio di come le piante percepiscano le condizioni di stress, i meccanismi con cui le piante trasmettono segnali di stress, ed infine lo studio dei meccanismi di acclimatazione a condizioni di crescita sfavorevoli potrebbe aiutare ad intervenire per ridurre gli effetti negativi di stress biotici e abiotici sulla crescita delle piante e sulla resa delle colture di interesse agronomico.

CHAPTER 1

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

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 in cultured cells of *Arabidopsis thaliana* PCD by accelerating a senescence process characterised 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. In 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.

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 & Schmülling, 2009).

Recently, it has been demonstrated that high levels of CKs induce programmed cell death (PCD) both in animal and plant cells (Ishii et al., 2002; Mlejnek & 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; Carimi et al., 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 characterising PCD events, two observations of particular interest were made. The first was that cell cultures treated at different times during a subculture 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 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* ten 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 characterised. The analyses revealed cell growth parameters comparable to wild-type cell lines, 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.

Material and methods

Plant material, culture conditions and treatments

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

Seeds were surface-sterilized and vernalized at 4°C for three days 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 six days on horizontal plates containing half-strength MS liquid medium, 0.1% sucrose and 0.5 g/L 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-day-old seedlings. Briefly, isolated cotyledons were incubated on modified Murashige & Skoog (1962) solid medium (0.8% (w/v) plant agar) (MSR2: 2.70 mM KH_2PO_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 in liquid medium and a suspension cell culture produced. The pH of the solid and liquid media was adjusted to 5.7 ± 0.1 with 0.5M NaOH before autoclaving at 121°C for 20 min. Cells were routinely subcultured every 7 days. 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 seven-day intervals and maintained in a climate chamber on a horizontal rotary shaker (80 rpm) at 25 ± 1°C at a 16 h light/8 h dark cycle. Three-day-old wild-type and mutant cells were incubated with 44 µM BA and collected four days 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 & Bhattacharyya (1999).

Nuclei were visualized by staining with 4',6-diamidino-2-phenylindole (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 resuspended 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 to 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 (TCTTTCTCGGCTTTTCTGAC) 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 (GAGCTCTCCGATGCAAAT) and ARR6-R (GAAAAAGGCCATAGGGGT) for the expression analysis of the *ARR6* gene; and finally, EF-1α-F (TGAGCACGCTCTTCTTGCTTTCA) and EF-1α-R (GGTGGTGGCATCCATCTTGTTACA) 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 four- or two-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 3 independent biological replicates. Values are expressed as mean ± S.D. 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 *AHK*2, and *CRE1/AHK4* was expressed at an even lower level (Fig. 1). In 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.

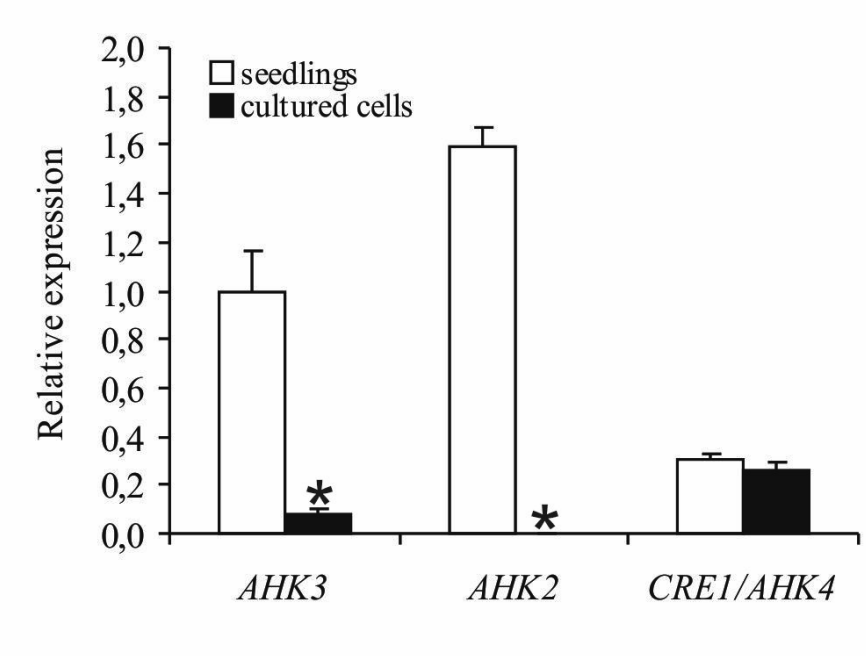

Figure 1: Quantitative real-time RT-PCR expression analysis of CK receptor genes in *Arabidopsis* wild-type seedlings and cultured cells. The relative expression values for all genes are related to the expression level of *AHK3*, which was set to 1. Values represent mean ± SD of RQ value of three experiments 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 (* $p<0.01$).

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 to 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 days after culture initiation (24.8% ± 2%) when compared to 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 to 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 *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.

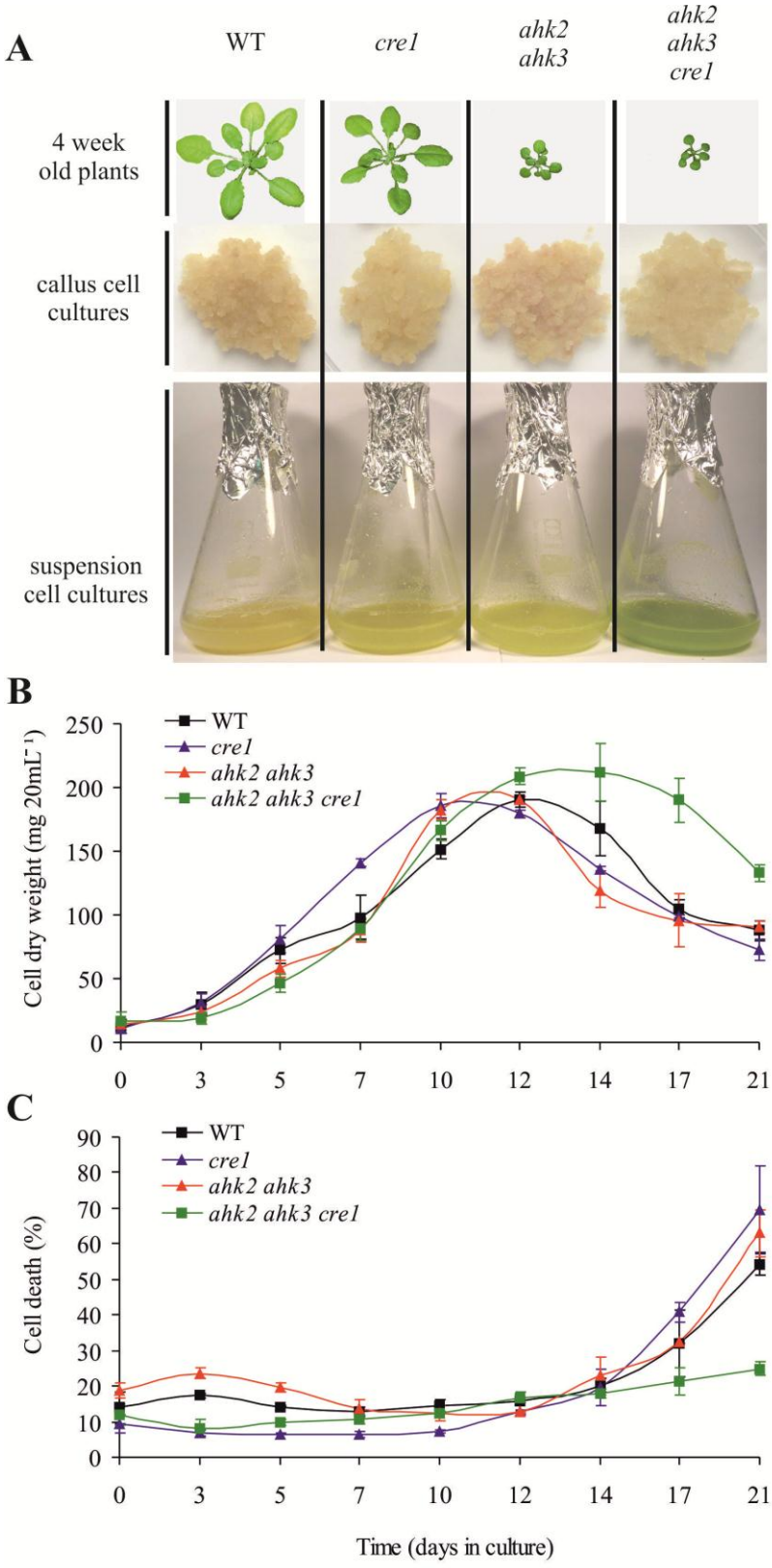

Figure 2: *Arabidopsis* plants and cultured cells of wild-type, *cre1*, *ahk2 ahk3*, and *ahk2 ahk3 cre1* receptor mutants. From top to bottom: (A) Plants, callus cultures, suspension cell cultures. (B) Cell dry weight at different times after culture initiation. (C) Cell viability (Evan's blue staining) at different times after culture initiation. Cell dry weight and cell death were measured from 0 to 21 days after culture initiation. Values represent mean ± SD of three independent experiments.

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), we determined the concentrations of free and conjugated CKs in the different cell cultures (Table 1). Several differences were observed. The levels of most CK metabolites resulted 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 tZ and the conjugate tZOG were detectable only in mutant lines and a significant increase in tZR content was observed in the double and triple mutant cell lines. The concentrations of various cZ-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 cZR and the nucleotide cZR5'MP were strongly increased in the double and triple mutant lines. The strong increase of cZ-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-, tZ-type and/or cZ-type CKs.

Line / CK metabolite	iP	iPR	iP9G	tZ	tZR
Wild-type	0.18 ± 0.06	0.31 ± 0.11	3.03 ± 0.20	N.D.	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****
Line / CK metabolite	tZOG	cZ	cZR	cZROG	cZR5'MP
Wild-type	N.D.	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****

Table 1: Cytokinin content of *Arabidopsis* wild-type and receptor mutant cultured cells. One gram of three-day-old *Arabidopsis* cultured cells per sample was collected, and three independent biological samples were taken for each genotype. Data shown are pmol/g fresh weight ± SD. *t*Z, *trans*-zeatin; cZ, *cis*-zeatin; iP, N^6-(Δ²isopentenyl)adenine; *t*ZOG, *trans*-zeatin *O*-glucoside; *c*ZROG, *c*-zeatin riboside *O*-glucoside; *t*ZR, *trans*-zeatin riboside; *c*ZR, *c*-zeatin riboside; iPR, N^6-(Δ²isopentenyl)adenosine; iP9G, N^6-(Δ²isopentenyl)adenine 9-glucoside; and iPR5'MP, N^6-(Δ²isopentenyl)adenosine 5'-monophospate. N.D., not detectable. Bold letters mark concentrations of CKs in mutants that are significantly different from those of 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.

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

Once the receptor mutant cell lines were established and characterised, experiments to detect the effects of high concentrations of BA were performed. Three-day-old 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 as compared to wild type may be in part 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 te al., 2004)

The cell dry weight and cell death (Evan's blue staining) of 4-day-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 four days of treatment (Fig. 5B). The double mutant cell line *ahk2 ahk3* was affected by BA treatment to a similar extent as the wild-type. On the contrary, 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, we investigated the nuclear morphology 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.

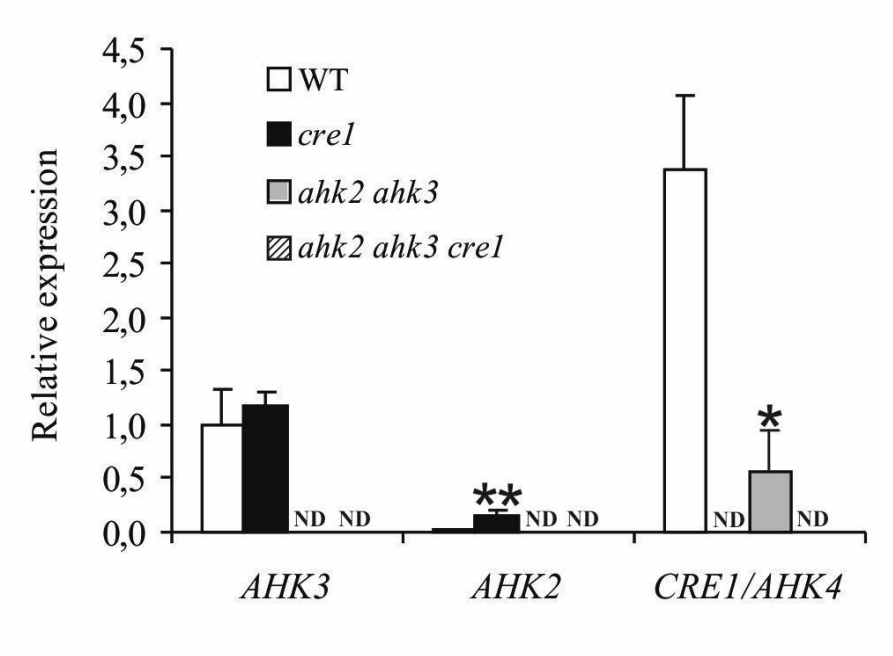

Figure 3: Quantitative real-time RT-PCR expression analysis of CK receptor genes in wild-type, *cre1*, *ahk2 ahk3* and *ahk2 ahk3 cre1* cultured cell lines. The relative expression values for all CK receptor genes are related to the expression level of *AHK3* (set to 1) in the wild-type cell line. Values represent mean ± SD of 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 (* $p < 0.01$, ** $p < 0.05$).

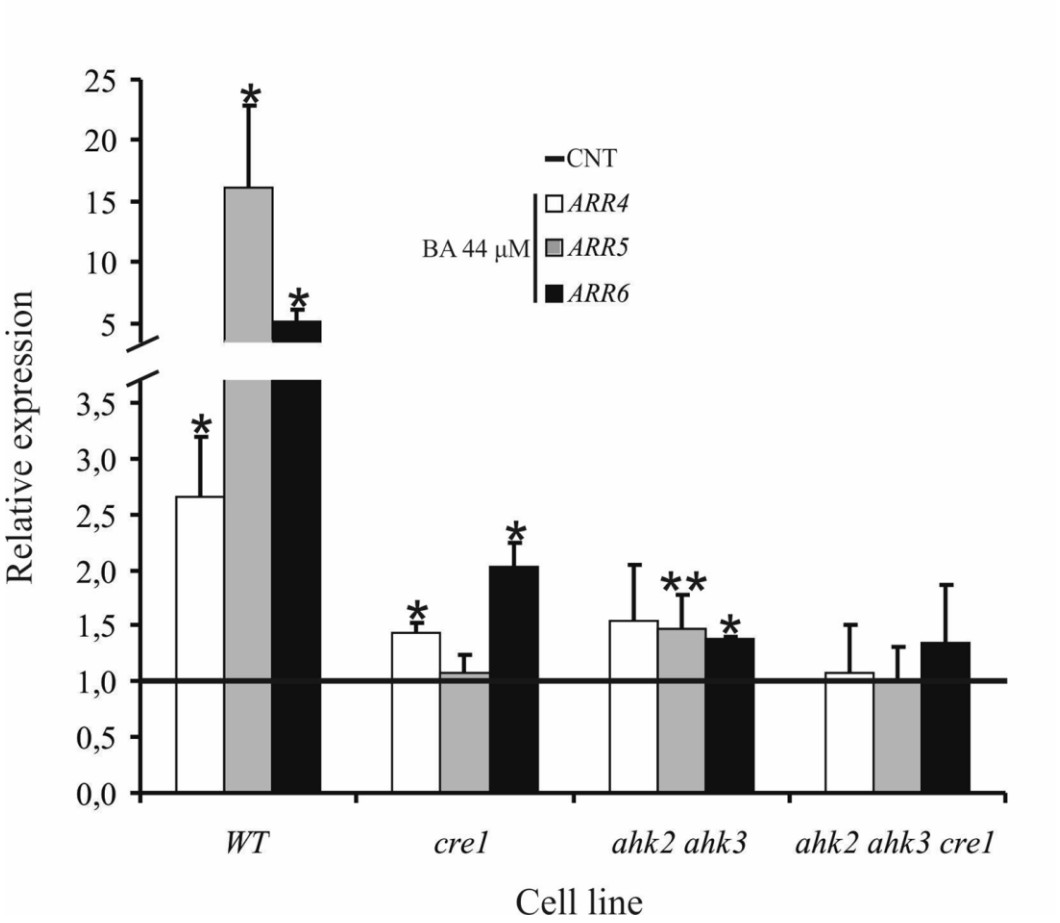

Figure 4: Quantitative real-time RT-PCR expression analysis of *ARR4, ARR5* and *ARR6* genes in wild-type, *cre1, ahk2 ahk3* and *ahk2 ahk3 cre1* cultured cell lines after incubation with 44 μM BA for 2 h. The relative expression values of *ARR* genes are related to the expression level in untreated cells (set to 1). Values represent mean ± SD of 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 (* $p < 0.01$, ** $p < 0.05$).

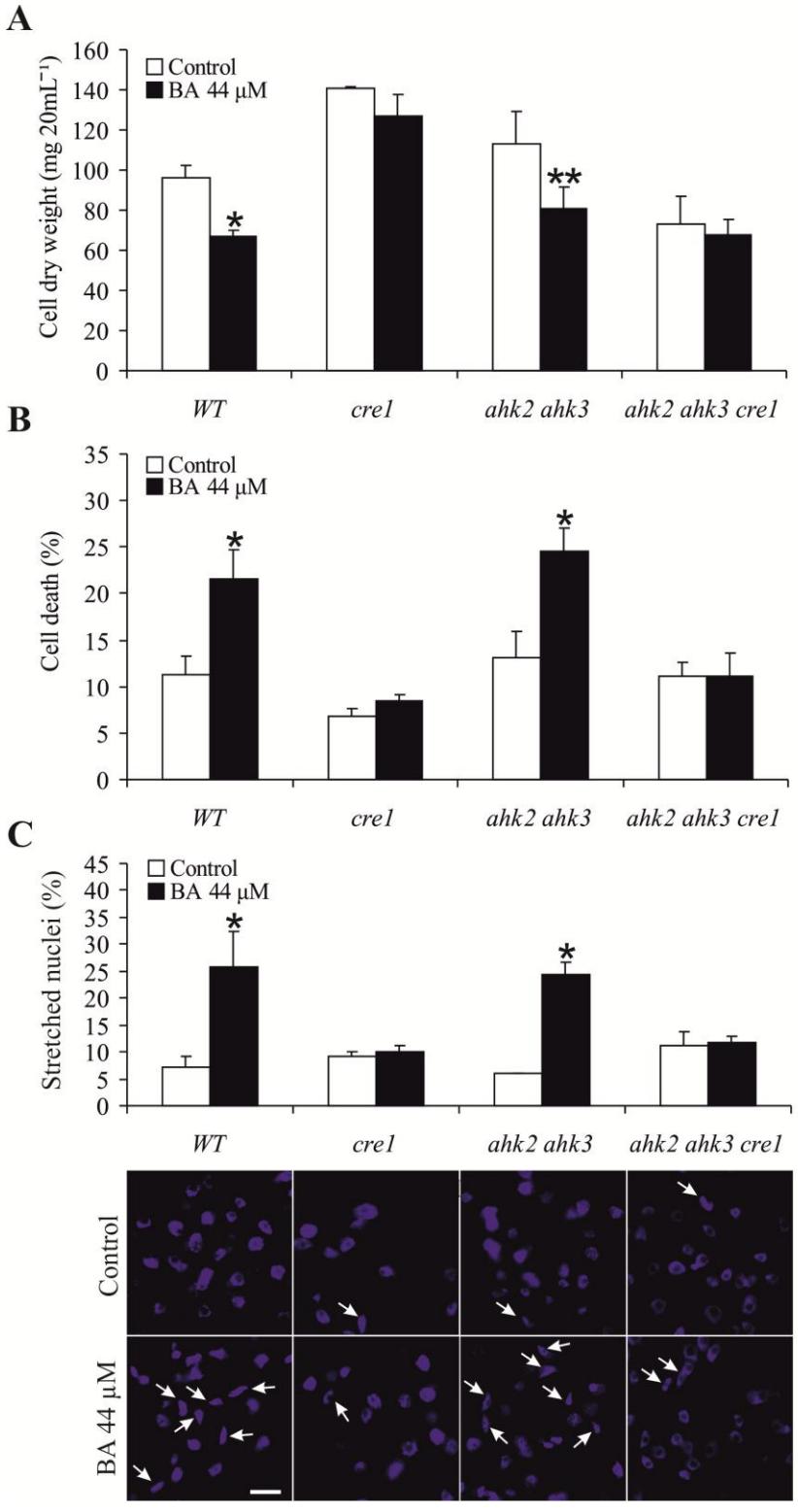

Figure 5: Effects of BA treatment on PCD parameters of wild-type and CK receptor mutant cultured cells. *Arabidopsis* cells were treated three days after subculturing with 44 µM BA for four days. (A) Cell dry weight of *Arabidopsis* cultured cells measured four days after BA addition. (B) Cell death measured by Evan's blue staining four days 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 (* $p < 0.01$, ** $p < 0.05$).

Discussion

Previously, it has been shown that high levels of CKs, in particular BA, 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, 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 focussing 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.* In 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 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 & Meins, 1973). It may also be that a separate cell-autonomous CK response system may function in cultured cells and maintain cell division independent 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 (e.g. 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 of the steady-state levels of iP- and tZ-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 cZ-type CKs, an increase in cZ, cZR and cZR5'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*, cZ-type CKs are synthesized through a distinct pathway, the tRNA pathway, with two

tRNA-IPT enzymes catalyzing the initial step (Miyawaki et al., 2006; Sakakibara, 2006). Our result indicates that biosynthesis of cZ-type CKs is also under 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*.

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CHAPTER 2

Arabidopsis thaliana GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE as an oxidative stress sensor

Abstract

NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a conserved enzyme among all living organisms involved in the glycolytic pathway. It has been widely demonstrated that in mammalian cells, besides its role in glycolysis, it is involved in different cell mechanisms. In particular, it has been reported that under oxidative stress conditions, GAPDH is subjected to oxidative post-translational modifications. In *Arabidopsis* plants, it has been demonstrated that, the homologous cytosolic enzymes GAPC-1 and GAPC-2, are targets of reactive oxygen species. In order to study whether in plant GAPC enzymes are involved in oxidative stress signalling, we investigated the role of these two enzymes analysing *Arabidopsis* knock out mutants for both *GAPC-1* and *GAPC-2* genes. We treated *Arabidopsis* seedlings with cadmium, a well-known oxidative stress inducer, and we studied the effect of this abiotic stress on GAPC enzymes. The analyses revealed that GAPC-1 was more sensitive to cadmium-induced oxidative stress, being up-regulated both at the transcriptional and post-transcriptional level. It has been also observed that modulation of the redox cell state by altering glutathione steady state level, led to alteration of the *GAPC-1* expression and GAPC-1 level. Moreover, as described in mammalian model, we observed that under oxidative stress conditions the protein is inactivated and translocated into the nucleus through a mechanism in which is probably involved the catalytic cysteine. All together our results suggest that GAPC-1 is highly sensitive to the cell redox state making this enzyme a key-actor in oxidative stress sensing in plants.

Introduction

Plants are exposed to several biotic and abiotic stresses, among the latter drought, salinity, extreme temperatures, anoxia, excess light, xenobiotics and heavy metals dramatically affect plant growth and development as well as crop yield (Jaspers and Kangasjärvi, 2010; Urano et al., 2010; Kosová et al., 2011). In response to abiotic stresses, plants have developed two main resistance mechanisms to overcome negative effects: avoidance, a mechanism by which plants escape stress exposure, and tolerance, see also acclimation, by which plants actively modify their physiology to improve their fitness (Yamaguchi-Shinozaki and Shinozaki, 2006; Kosová et al., 2011). Plant acclimation to abiotic stress conditions requires changes in gene expression, thus in plant transcriptome, proteome and metabolome profiles (Urano et al., 2010; Kosová et al., 2011). To make this possible, plants have to activate stress signalling pathways that link the perception of the stress signal to stress-responsive gene expression (Yamaguchi-Shinozaki and Shinozaki, 2006). Among different possible actors involved in the abiotic stress signalling pathways, reactive oxygen species (ROS) and reactive nitrogen species (RNS) play important roles. ROS and RNS are normally produced during plant growth and development, but, their production increases during stress conditions (Lamattina et al., 2003; Apel and Hirt, 2004; Møller et al., 2007; Miller et al., 2008; Wilson et al., 2008; Molassiotis and Fotopoulos, 2011; Astier et al., 2011). In plants, under optimal growth conditions, the levels of these molecules are tightly regulated by several mechanisms and participate in the control of various processes such as plant growth and development (Besson-Bard et al., 2008; Wilson et al., 2008; Tsukagoshi et al., 2010; Mittler et al., 2011). When plants are exposed to different stresses the production rate of these

molecules can overcome their scavenging rate, leading to the accumulation of high level of ROS and RNS with the establishment of oxidative stress conditions inducing oxidative damages, with eventually plant cell death (Valderrama et al., 2007; Corpas et al., 2008; Miller et al., 2008; Molassiotis and Fotopoulos, 2011). Among all ROS and RNS, hydrogen peroxide (H_2O_2) and nitrogen oxide (NO) have been largely investigated since they have been reported to induce cell death (Delledonne et al., 2001). But they are also implicated in redox signalling due to their "ability" to produce post-translational modifications of target proteins. In fact, some cysteine (Cys) residues present in target proteins can be subjected to H_2O_2 and NO oxidation leading to the formation of sulfenic, sulfinic, and then sulfonic acid, glutathionylation, disulfide bond and nitrosylation (Thomas et al., 1995; Thomas and Mallis, 2001; Lindermayr et al., 2005; Hancock et al., 2006; Holtgrefe et al., 2008; Astier et al., 2011; Molassiotis and Fotopoulos, 2011). Sulfinic and sulfonic oxidative modifications are irreversibly oxidized forms in cells, while the other oxidized forms could be reduced by several redox active enzymes such as glutaredoxins (GRXs), that act as mediators for reversible electron transfer between the reducing molecule glutathione redox buffer and oxidized cysteines of target proteins (Thomas and Mallis, 2001; Lemaire, 2004; Meyer, 2008; Zaffagnini et al., 2008). Glutathione is a tripeptide composed of Glu, Cys and Gly, and thanks to the central Cys can be reversibly converted from the reduced form (GSH) to the oxidized form (GSSG) (Meyer et al., 2007). The presence of the glutathione redox buffer, along with redox active enzymes and redox-sensitive proteins is fundamental for an efficient redox signalling (Meyer, 2008). In fact, the redox-dependent post-translational modifications of reactive cysteines of target proteins, followed by changes of structural, catalytic or

regulatory functions are very important in redox-dependent cellular signalling, thus putatively important in abiotic stress response and acclimation (Padgett and Whorton, 1998; Meyer and Hell, 2005).

In recent years, several papers have reported that H_2O_2, NO-donor and abiotic stresses affect the redox state of reactive cysteines as well as transcriptome and proteome profiles of plants (Sweetlove et al., 2002; Chen et al., 2003; Polverari et al., 2003; Parani et al., 2004; Lindermayr et al., 2005; Herbette et al., 2006; Roth et al., 2006; Sarry et al., 2006; Astier et al., 2011). In almost all of these papers, the *Arabidopsis thaliana* cytosolic glyceraldehyde-3-phosphate dehydrogenases (GAPDH) have been found to be regulated both at transcripts and proteins levels. Moreover, GAPDHs possesses two cysteine residues (Cys 155 and Cys 159 in *Arabidopsis*), with one of the two absolutely required for the catalytic activity (Holtgrefe et al., 2008). Interestingly, the catalytic cystein (Cys 155 in *Arabidopsis*) has been reported to undergo oxidative modifications in different organisms (Almeida et al., 2007; Holtgrefe et al., 2008; Astier et al., 2011; Tristan et al., 2011).

GAPDH is a conserved enzyme among all living organisms being a key enzyme of the glycolytic process. Besides its role in basic metabolism it has been demonstrated that participates in several non-metabolic cell pathways (Hancock et al., 2005; Almeida et al., 2007; Nakajima et al., 2007; Baek et al., 2008; Holtgrefe et al., 2008; Morigasaki et al., 2008; Wawer et al., 2010; Tristan et al., 2011). In particular, it has been reported that under oxidative stress conditions, GAPDH is subjected to post-translational modifications that affect its catalytic activity in plant, animal and yeast models (Lindermayr et al., 2005; Almeida et al., 2007; Romero-Puertas et al., 2008; Tristan et al., 2011). In animal and yeast, under

oxidative stress conditions, the inactivated GAPDH is involved in cell death pathways and oxidative stress signalling (Almeida et al., 2007; Tristan et al., 2011). It has been demonstrated that in rat cells exposed to stress conditions, the NO produced nitrosylation of the GAPDH catalytic cysteine leading to the binding with Siah1 (an E3 ubiquitin ligase), whose nuclear localization signal drives the GAPDH-Siah1 complex into the nucleus (Hara et al., 2005). The GAPDH-Siah1 binding requires the nitrosylation of the GAPDH catalytic cysteine, and is dependent on GAPDH lysine (Lys) 225; moreover, this binding reduces the rapid turnover of Siah1 (Hara et al., 2005). These events enhance the Siah1 E3 ubiquitin ligase activity in the nucleus leading to apoptosis. This suggests the involvement of GAPDH in molecular mechanisms of cytotoxicity (Hara et al., 2005). More recently, it has been also proposed that GAPDH can mediate trans-nitrosylation of nuclear proteins (Kornberg et al., 2010).

To study the role played by the *Arabidopsis* GAPDHs, in oxidative stress conditions, the attention was focused on the two cytosolic isoforms GAPC-1 (At3g04120) and GAPC-2 (At1g13440). In this work, for the first time, we studied the involvement of *Arabidopsis thaliana* GAPC-1 and GAPC-2 enzymes in oxidative stress signalling by performing *in vivo* analyses. Our experiments revealed that *GAPC* genes expression is affected by oxidative stress, and that the *GAPC-1* gene is more sensitive than *GAPC-2*. Then, through the generation of transgenic plants expressing transcriptional and translational GAPC-1 reporters it has been possible to demonstrate that oxidative stress, induced by cadmium treatment, affects GAPC-1 expression both at the transcriptional and post-transcriptional levels. Moreover, we demonstrated that under oxidative stress conditions, as already described for rat GAPDH (Hara et al., 2005), the enzyme is

translocated into the nucleus by a mechanism in which is probably involved the catalytic Cys 155.

Material and methods

Plant material and growth conditions

All the *Arabidopsis thaliana* plants used in this study were of the Columbia ecotype. Plants were grown on Jiffy Pot (http://www.jiffypot.com/) 16/8 h cycles of white light (~75 μE) at 22°C. Seedlings were grown *in vitro* for the selection of transformants and experiments. Seeds were surface-sterilized and, after cold treatment for 2-3 days, were exposed to 16/8 h cycles of white light (~75 μE) in growth chambers. Seeds were grown at 23°C on vertical plates containing half-strength MS medium (Murashige and Skoog, 1962) including 0.8% agar (Duchefa). The medium was enriched with 0.1% sucrose and 0.05% 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. Seeds of the transgenic roGFP2 *Arabidopsis* line were kindly provided by Markus Schwarzländer (Meyer et al., 2007). The transgenic cHyPer *Arabidopsis* plants were generated and reported in a previous study (Costa et al., 2010). Seeds of *gapc1* and *gapc2 Arabidopsis* T-DNA insertional mutant lines were from the SALK Institute (SALK_010839 and SALK_016539 respectively).

Genotyping of insertional gapc-1 *and* gapc-2 *mutants*

The wild-type *GAPC-1* (At3g04120), *GAPC-2* (At1g13440) and T-DNA insertion *gapc1* and *gapc2* alleles were identified using PCR with the following primers: LP-GAPC1 (5'-CCGCACATCTGTTAATGAATTTC-3'), RP-GAPC1 (5'-CTCAGAAGACTGTTGATGGGC-3') and LP-GAPC2 (5'-GGTTAGGACTGAGGGTCCTTG-3'), RP-GAPC2 (5'-

GGCATCAGGTACATAATCATGG-3') to identify, respectively, *GAPC-1* and *GAPC-2* wild-type alleles; LBa1-SALK (5'-TGGTTCACGTAGTGGGCCATCG-3') with RP-GAPC1 (5'-CTCAGAAGACTGTTGATGGGC-3') or RP-GAPC2 (5'-GGCATCAGGTACATAATCATGG-3') to identify, respectively, *gapc-1* and *gapc-2* mutated alleles.

Enzyme analyses

For the analyses of CATs activities, roots pooled from 400 7-day-old *Arabidopsis* seedlings were homogenized in 50 mM Tris–HCl pH 7.4, 0.05% cysteine at 4°C.

After centrifugation at 16100 g for 20 min at 4°C, the protein content was quantified according to the method of Bradford and successively used for zymograms. For the analysis of CAT activity, 10 µg of total protein extracts were loaded and separated in native polyacrylamide gels, and gels were then assayed as reported previously by Zimmermann et al. (2006). For the analysis of CAT activity three independent protein extractions were performed.

GAPDH activity was monitored spectrophotometrically at 340 nm and 25 °C, in a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 3 mM 3-phosphoglycerate, 1 mM EDTA, 5 units ml^{-1} of 3-phosphoglycerate kinase (from rabbit muscle, Sigma), 2 mM ATP, 0.2 mM NADH. For calculations, an ε340 for NADH of 6.23 mM^{-1} was used.

Analysis of cell viability by Evan's blue staining

Arabidopsis seedlings were stained for 10 min in 0.05% (w/v) Evan's blue solution, and then rinsed with water for five times. The stained seedlings were then observed under a Leica DMR optical microscope (Leica, Milan, Italy).

Detection of NO Production

For detection of NO production seedlings were incubated in the cell-permeable fluorescent probe DAF-FM diacetate (4-amino-5-methylamino-2',7'-difluorofluorescein diacetate, Alexis Biochemicals, Florence, Italy) at a concentration of 5 μM in incubation buffer (50 mM KCl and 10 mM MES-KOH, pH 7.2) for 15 min. Seedlings were then washed three times with fresh buffer and examined by confocal microscope as previously reported (Zottini et al., 2007).

RNA isolation and cDNA synthesis

Seedlings were harvested, frozen in liquid N_2 and stored at -80°C. The frozen samples were powdered in liquid N_2, and RNA was isolated with the TRIzol method. Then, the total RNA was purified using an RNeasy kit, including DNase digestion (Quiagen, Milan, Italy). cDNA was synthesized by Improm-II™ Reverse Transcriptase (Promega, Milan, Italy) from 1 μg of purified RNA.

Quantitative real-time RT-PCR primer

The quantitative real-time RT-PCR expression analysis of *GAPC-1* and *GAPC-2* genes was performed using the following primers: GAPC1-F (GAGGTGATGGGAGTTTGTAGAC) and GAPC1-R (TACGTCATCATCAACGGG) for the expression analysis of the *GAPC-1* gene;

GAPC2-F (TGCGCAGTCATGAGAGTT) and GAPC2-R (AGTTGCCAGTTGGGTTTG) for the expression analysis of the *GAPC-2* gene; and finally, EF-1α-F (TGAGCACGCTCTTCTTGCTTTCA) and EF-1α-R (GGTGGTGGCATCCATCTTGTTACA) 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, Monza, Italy) using the following cycling conditions: initial denaturation at 95°C for 10 min, 40 cycles of 15 s at 95°C, 1 min at 60°C, followed by melt curve stage analysis to check for specificity of the amplification. The reactions contained Power SYBR Green PCR 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 four- or two-fold dilution series over at least five dilution points of cDNA. The expression analysis of *GAPC* genes was performed by the Pfaffl method, using *EF-1α* as the reference gene (Pfaffl, 2001; Remans et al., 2008).

Genetic materials

The *Arabidopsis GAPC-1* coding sequence was amplified by PCR from *Arabidopsis* cDNA using the following primers where the *NcoI* sites were introduced: For 5'- CATGCCATGGCTGACAAGAAGATTAGG-3' and Rev 5'-

CATGCCATGGCGGAGGCCTTTGACATGTGGACGATCAA-3'. The *Arabidopsis GAPC-1* promoter sequence (-633 bp to -1 bp) was amplified by PCR from *Arabidopsis* genomic DNA using the following primers where the *EcoRI* site was introduced: For 5'-CATGGAATTCCGAGTTTTTGATAGGGACTTTTGCT-3' and Rev 5'-CATGGAATTCTGTAGAATCGAAAACGAGAGTTAGA-3'.

DNA constructs

For the expression of the *β-glucoronidase* transcriptional reporter and the *GAPC1-YFP* translational reporter genes, the pGreen0029 (Hellens et al., 2000) binary vector was used.

In order to isolate the *GAPC-1* promoter we amplified by PCR 633 bp upstream of the *GAPC-1* ATG start codon. The amplicon was digested with *EcoRI* and ligated into a modified pGreen0029 binary vector upstream of the β-glucoronidase (GUS) coding sequence, fused with the *nos* terminator (Valerio et al., 2011). The obtained vector was named *pGAPC-1-GUS.*

In order to obtain the pGreen0029-*p35S::GAPC1-YFP* binary vector, the *GAPC-1* coding sequence was amplified by PCR using as template cDNA obtained from the retro-transcription of total RNA extracted by 4-week old *Arabidopsis* leaves. The *GAPC-1* amplicon was digested with the *NcoI* enzyme and ligated upstream of the *YFP* coding sequence in the pGreen-*p35S::YFP* plasmid (Valerio et al., 2011). The pGreen0029-*pGAPC1::GAPC1-YFP* binary vector was constructed introducing the *GAPC-1* promoter sequence upstream of the *GAPC-1-YFP* coding sequence by replacing the *35S* promoter using the *EcoRI* restrction sites.

To study the role of specific GAPC-1 residues, several transgenic *Arabidopsis* line carrying the *pGAPC1::GAPC1-YFP* expression cassette mutated in the *GAPC-1* sequence were produced. The introduction of specific mutations in the *GAPC-1* sequence was carried out as described by Asada et al. (2000) with some modifications. The mutagenesis was performed by PCR reaction using 0.5 µg of pGreen0029-*pGAPC1::GAPC1-YFP* binary vector as template in a reaction volume of 25 µL containing 0.5 U Phusion® High-Fidelity DNA Polymerase (Finnzymes, Milan, Italy), 0.2 mM dNTPs and 0.2 µM of mutation introducing forward primers. To introduce the mutations C155S, C159S, K230A and the double mutations C155S-C159S, the following primers were used: 5' - CATTGTCTCCAACGCTAGCTCCACCACTAACTGCCTTGCTC - 3', 5' - CGCTAGCTGCACCACTAACTCCCTTGCTCCCCTTGCCAAGG - 3', 5' - CTCCAACGCTAGCTCCACCACTAACTCCCTTGCTCCCCTTG - 3' and 5' - GCTTCCAGCTCTTAACGGAGCATTGACTGGAATGTCTTTCC - 3' respectively. The PCR reaction was performed using the following cycling conditions: initial denaturation at 95°C for 3 min, 18 cycles of 15 s at 95°C, 1 min of annealing at 61°C, 5 min of extension at 72°C and a final step of 10 min at 72°C. Then, the PCR product was treated with *DpnI* endonuclease for 2 hours at 37°C. Finally, 2 µL of digested DNA was transformed into *Escherichia coli* XL1 Blue supercompetent cells.

Agrobacterium tumefaciens strains

For the use of pGreenII–derived binary vectors, the *A. tumefaciens* GV3101-pSoup strain was used (Hellens et al., 2000). Competent cells of *A. tumefaciens* GV3101 strain were prepared as described by Main et al. (1995), the

binary vectors were introduced by "freeze-thaw" method. 1μg of plasmid DNA was added to the competent cells, frozen in liquid nitrogen for 5 min and schoked at 37°C for 5 min. The bacterial culture was incubated at 28°C for 3 hours with gentle shaking in 1mL YEP medium (10 gL^{-1} bacto-trypton, 10 gL^{-1} yeast extract, 5 gL^{-1} NaCl; pH 7.0) and then spread out on a YEP agar plate containing the appropriate antibiotic selection (gentamycin 50 mgL^{-1}, rifampicin 50 mgL^{-1}, kanamycin 50 mgL^{-1} and tetracyclin 5 mgL^{-1}).

Transgenic plants

Transgenic *Arabidopsis* plants were generated by floral-dip method (Clough and Bent, 1998) using transformed *Agrobacterium tumefaciens* strains GV3101. Transformed seedlings were selected on medium supplemented with 50 μg mL^{-1} kanamycin. For each construct, different *Arabidopsis* independent transgenic lines were isolated. None of the transgenic lines selected, with the different constructs, showed phenotypic differences or abnormalities in our standard growth conditions.

GUS staining and β-glucoronidase activity assay

Histochemical analyses of the transcriptional reporter GUS, and measurement of β-glucoronidase enzymatic activity were performed as described by Jefferson et al. (1987).

For the histochemical analyses samples were fixed by 30 min vacuum infiltration in the fixation solution (50 mM phosphate buffer pH 7.2, 1.5% (v/v) formaldehyde, 0.5% (w/v) Triton X-100), then the samples were washed three times with the 50 mM phosphate buffer pH 7.2 solution and vacuum infiltrated for

15 min in the reaction solution (50 mM phosphate buffer pH 7.2, 1 mM X-gluc, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$-3H2O, 0.5% (w/v) Triton X-100), and then incubated at 37°C overnight and 20 min for qualitative semi-quantitative analyses, respectively. Samples were cleared by washing them in 3:1 (v/v) methanol/acetic acid solution.

For β-glucoronidase enzymatic activity assay, 300 roots of 7-day-old seedlings treated up to 72 hours were homogenized in 2 mL of GUS extraction buffer (50 mM NaH_2PO_4 pH 7.0, 0.1 % (w/v) Sodium Lauryl sarcosine, 20 % (v/v) methanol, 10 mM β-mercaptoethanol, 10 mM EDTA). The homogenate was centrifuged at 2000 rpm for 2 min at 4°C to eliminate debris. The supernatant was centrifuged at 14000 rpm for 15 min at 4°C, and the supernatant collected. Protein concentration was determined by the Bradford method, using the Bio-Rad protein assay (Bio-Rad, Segrate, Italy).

An appropriate amount of sample was incubated in the reaction solution (GUS extraction buffer, 2 mM 4-Methylumbelliferyl-β-D-glucuronide trihydrate) 1 hour at 37° C. Aliquots were collected and mixed with stop reaction buffer (0,2 M Na_2CO_3) at the start of the reaction and every 15 minutes. Fluorescence of samples collected was measured with excitation at 365 nm, emission at 455 nm on a Luminescence Spectrometer LS 55 (PerkinElmer, Monza, Italy).

Detection of GAPC1-YFP synthesis by immune-blot analysis

For protein extraction, 600 seedling roots were harvested and homogenized in 250 µL of protein extraction buffer (0.320 M sucrose, 50 mM Tris pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM dithiothreitol (DTT) 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5% PVPP. The

homogenate was centrifuged at 2000 rpm for 2 min at 4°C to eliminate debris. The supernatant was centrifuged at 10200 rpm for 20 min at 4°C, and the supernatant collected. Protein concentration was determined by the Bradford method, using the Bio-Rad protein assay (Bio-Rad, Segrate, Italy). Extracted proteins (10 μg) were separated by 12% (w/v) SDS–PAGE, transferred to a nitrocellulose membrane (Sartorius) and analysed with antibodies raised against GFP (Invitrogen, Monza, Italy). Equal loading and transfer of proteins were checked by staining the membranes with Ponceau. Densitometric analyses of the blots were performed with a digital imaging analysis system.

Confocal microscopy analyses

Confocal microscopy analyses were performed using a Nikon PCM2000 (Bio-Rad, Germany) and an inverted SP5II (Leica, Milan, Italy) laser scanning confocal imaging systems. For DAF-FM detection, excitation was at 488 nm and emission between 515/530 nm. For HyPer and YFP detection, excitation was at 488 nm and emission between 530/560 nm. To extract quantitative data, pixel values were measured over root regions, which were located manually on confocal images and calculated using IMAGEJ BUNDLE software (http://rsb.info.nih.gov/ij/). For NO, H_2O_2 and GAPC1-YFP detection, 5 roots from 3 independent experiments were observed. Then quantitative fluorescence values of root pictures were determined by analyzing pixel intensity. Data were expressed as (fluorescence intensity of treated root)/(fluorescence intensity of control root) ratios. For roGFP2 ratiometric analysis, the probe was excited first at 488 nm and then at 405 nm and, for both excitation wavelengths roGFP2 fluorescence was collected with a bandpass filter of 505-530 nm and ratiometric

analysis of fluorescence images was performed as described by Meyer et al., (2007).

Cloning, expression and purification of recombinant GAPC-1

The *Arabidopsis GAPC-1* coding sequence was amplified by PCR from *Arabidopsis* cDNA and was cloned into pET-28a(+) (Novagen) using the two following primers: 5'-TCCATATGGCTGACAAGAAGATTAGC-3', and 5'-GCGGATCCTTAGGCCTTTGACATGTCGACG-3' (containing *NdeI* and *BamHI* restriction sites). In the pET28/*GAPC-1* construct, the cDNA sequence for *GAPC-1* was in frame with a His tag and a cleavable thrombin site.

Protein expression was induced at exponential phase by adding 100 µM isopropyl-β-D-thiogalactopyranoside for 16 h at 30 °C. Then, the cultures were centrifuged and the resulting pellets were resuspended in 20 mM Tris-HCl buffer, pH 7.9. Cell lysis was performed by sonication (5 x 1 min with intervals of 1 min) and soluble and insoluble fractions were separated by centrifugation for 30 min at 30,000 x g. The soluble part was then applied onto a Ni^2 HiTrap chelating resin (His-Bind Resin, Novagen) and protein purification was performed according to the manufacturer's instructions.

The molecular mass and purity of the protein were analyzed by SDS-PAGE and Coomassie Blue staining after desalting on PD-10 columns equilibrated with 50 mM potassium phosphate buffer, pH 7.5. The protein concentration was determined spectrophotometrically using a molar extinction coefficient at 280 nm of 40.910 $M^{-1}cm^{-1}$. The resulting homogeneous protein was stored at –20 °C. For some sample proteins the N-terminal His-tag was removed by thrombin protease according to (Sparla et al., 1999). Excised His-tag was

eliminated by metal affinity chromatography and the resulting proteins were desalted and stored as just described.

Recombinant GAPC-1 activity assay

GAPC-1 activity was monitored spectrophotometrically at 340 nm and 25°C, in a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM MgCl2, 3 mM 3-phosphoglycerate, 5 units ml^{-1} of 3-phosphoglycerate kinase, 2 mM ATP and 0.2 mM NADH.

Before any treatments, GAPC-1 was incubated with 10 mM DTT for 30 min at RT for reduction. Thereafter, DTT was removed by desalting on NAP-5 columns equilibrated with 50 mM Bis-Tris buffer, pH 7.0.

Protoplasts isolation

Protoplasts were isolated as described by Yoo et al. (2007) with some modifications. Leaves of 3-4 weeks old Arabidopsis plants were dissected in 0.5-1 mm strips, placed in a Petri dish containing the enzymatic solution (1.25% cellulase R10 (Yakult Pharmaceutical, Japan), 0.3% macerozyme R10 (Yakult Pharmaceutical, Japan), 0.4 M mannitol, 20 mM KCl, 20 mM MES pH 5.7, 10 mM $CaCl_2$, 0.1% BSA; filter sterilized) and vacuum-infiltrated for 30min. The digestion was carried out for 3h, and then the solution containing protoplasts was filtered with 50 µm nylon mesh sieve and centrifuged in 10 mL polystyrene tubes at 100 xg for 5 min to pellet the protoplasts. The pelleted protoplasts were washed twice in W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.8; filter sterilized) and incubated on ice for 30 min. Then protoplasts were collected by centrifuging at 100 xg for 1 min and the pellet was resuspended in an

appropriate volume of MMg solution (0.4 M mannitol, 15 mM $MgCl_2$) in order to obtain approximately $2x10^4$ protoplasts in 0.1 mL of MMg.

Protoplasts transfection assay

Protoplasts were transfected as described by Yoo et al. (2007) with some modifications. In a 2 mL eppendorf tube 1 µg of plasmid DNA was added to $2x10^4$ protoplasts and gently mixed with an equal volume of a polyethylene-glycol (PEG) solution (40% (w/v) PEG4000 (Fluka), 0.1 M $CaCl_2$, 0.2 M mannitol). The solution was incubated for 20 min in the dark at room temperature. After incubation, 2 volumes of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.8; filter sterilized) were added to the tube to wash out the PEG. The protoplasts were pelleted by 1 min centrifugation at 100xg and then resuspended in 1 mL of W5 solution. The protoplasts were incubated at 20°C in the dark for at least 16 hours before the microscopy analysis.

Generation of Arabidopsis transgenic lines expressing transgenes in* gapc-1 *genetic background

The transgenic *Arabidopsis gapc-1* mutant plants stable transformed with *pGAPC1::GAPC1-YFP* and *pGAPC1::GAPC1(C155S)-YFP* were obtained by crossing their mature flowers: ovary of *pGAPC1::GAPC1-YFP* and *pGAPC1::GAPC1(C155S)-YFP* were impollinated by brushing them with stamens of fully mature flowers from *Arabidopsis gapc-1* mutant plants. Seeds obtained were sown and seedlings were selected by using PCR analyses.

Statistical analysis

All the data are representative of at least 3 independent biological replicates. Values are expressed as mean ± S.D. The statistical significance of differences was evaluated by Student's *t* test.

Results and discussion

Isolation and characterization of Arabidopsis thaliana gapc-1 *and* gapc-2 *homozygous mutants*

Arabidopsis cytosolic enzyme GAPDHs, called GAPC-1 (At3g04120, 1017 bp) and GAPC-2 (At1g13440, 1017 bp), are involved in the sixth step of the glycolytic pathway, catalyzing the conversion of glyceraldehyde-3-P to 1,3-bisphosphoglycerate (Rius et al., 2008). Besides their involvement in glycolysis, there are increasingly evidences of their involvement in abiotic and oxidative stress signaling (Yang et al., 1993; Hancock et al., 2005; Baek et al., 2008; Holtgrefe et al., 2008).

In order to better understand the role of these two enzymes in *Arabidopsis* plants, the knock out homozygous mutants for both *GAPC-1* and *GAPC-2* genes were isolated and characterized. In order to obtain *GAPC* mutants, T-DNA insertion mutant lines of both *GAPC-1* and *GAPC-2* genes were selected from the library of T-DNA insertion mutations of the Salk Institute (O'Malley et al., 2007), and the seeds of *gapc-1* (SALK_010839, Kanamycin^R) and *gapc-2* (SALK_016539, Kanamycin^R) *Arabidopsis* insertion lines provided by the NASC stock centre. The *GAPC-1* gene is composed of nine exons and eight introns, and the *gapc-1* insertion line presents the T-DNA insertion in the ninth exon (Rius et al., 2008) (Fig. 1A). The *GAPC-2* gene is composed of eleven exons and ten introns, and the *gapc-2* T-DNA insertion is located in the sixth exon (Fig. 1A). The provided seeds were from heterozygous plants, therefore, to select homozygous lines PCR screenings were performed on both insertional lines. By analyzing the genomic DNA of several plants from both mutant lines,

homozygous lines carrying the T-DNA insertions in *GAPC-1* and *GAPC-2* genes were isolated (Fig. 1B). In order to check if the T-DNA insertions led indeed to the absence of *GAPC-1* and *GAPC-2* transcripts, quantitative real-time RT-PCR analyses were performed (Fig. 1C). The analyses revealed that *GAPC-1* and *GAPC-2* transcripts were absent in *gapc-1* and *gapc-2* mutants, respectively. The same analyses revealed that in *gapc-1* and *gapc-2* mutants, the *GAPC-2* and *GAPC-1* genes, respectively, were expressed at the same extent as in wild-type. Moreover, it was observed that in wild-type background the *GAPC-2* gene was expressed ~38-fold more than *GAPC-1* (data not shown) showing a different expression level of the two GAPC isoforms.

It was also tried to isolate a homozygous double mutant line by crossing *gapc-1* and *gapc-2* homozygous single mutants. Out of 60 plants coming from self-pollination of T1 generation, no homozygous double mutants were isolated. To confirm that the homozygous double mutant was not vital, a *gapc-1-/- gapc-2+/-* plant coming from T2 generation, was let to self-pollinate. By genotyping 16 plants coming from this self-pollination no homozygous double mutant plants were isolated. These results suggest that one GAPC enzyme is necessary to develop a vital plant.

Having demonstrated that the homozygous mutants were fully impaired in mutated genes expression, the question whether the GAPDH catalytic activity was negatively affected in mutant backgrounds was approached. To this end, the GAPDH catalytic activity of 7-day-old wild-type, *gapc-1*, and *gapc-2* seedlings was measured (Fig. 1D). In both mutant backgrounds, the absence of one GAPC enzyme led to a significant decrease (~50%) of the catalytic activity. Data from quantitative real-time RT-PCR and GAPDH catalytic activity analyses suggested

that, in mutant backgrounds, no compensation effects at the transcriptional level occured. Instead, at the post-translational level some sort of regulation probably occurred, because the catalytic activity in the two mutant backgrounds was at the same level but the *GAPC-1* gene was ~38-fold less expressed than *GAPC-2*.

The *gapc-1* mutant was already well characterised (Rius et al., 2008), but only the phenotypes of mature plants were analyzed. In order to complete the phenotypic characterization, the phenotypes of both *gapc-1* and *gapc-2* mutants were analyzed in the first growth stage (i.e. seedlings growth). Under *in vitro* growth conditions, the only notable difference was observed at the level of root growth. In fact, 7-day-old seedlings from *gapc-1* and *gapc-2* lines had longer and shorter root, respectively, when compared with wild-type (Fig. 1E). In particular, roots of *gapc-1* seedlings were 13% longer (15 mm), and roots of *gapc-2* seedlings were 5% shorter (12.5 mm) than wild-type (13 mm). Recently it has been reported that H_2O_2 and $O_2^{\cdot -}$ are involved in the control of *Arabidopsis* root growth (Tsukagoshi et al., 2010). In particular, the authors stated that in root tips of wild-type plants, $O_2^{\cdot -}$ accumulates in the meristematic zone maintaining cell proliferation. Whereas, H_2O_2 accumulates in the elongation zone, where it is required to drive cellular differentiation. When the $O_2^{\cdot -}$/ H_2O_2 ratio achieves a determinate extent, cells stop to proliferate and start to elongate. To understand whether the phenotype observed in the mutant lines was due to an alteration of $O_2^{\cdot -}$/ H_2O_2 ratio, the catalytic activity of catalases was assessed. The attention was focused on these enzymes because they are involved in H_2O_2 catabolism (Nyathi and Baker, 2006), and the up-regulation or down-regulation of their activity could led to variation of $O_2^{\cdot -}$/ H_2O_2 ratio. The catalase activity levels were determined by performing a zymogram assay (Zimmermann et al., 2006) using protein extracts

from wild-type and *gapc* mutant roots. The assay revealed that in *gapc* mutants the catalase activity was affected when compared to the wild-type line (Fig. 1F). In particular, the *gapc1* line showed a higher catalase activity, and *gapc-2* showed a lower catalase activity when compared to wild-type. This result could suggest that in *gapc-1* mutant the H_2O_2 level is lower than in wild-type as leading to longer roots, instead, in *gapc-2* mutant the H_2O_2 level is higher than in wild-type as leading to shorter roots.

The *gapc-1* and *gapc-2* characterization revealed that GAPC isoforms have a redundant catalytic function and, at least one of the two is necessary to develop viable plants. Moreover, it has been demonstrated that in mutant backgrounds the root growth is affected by the $O_2^{\cdot-}$/ H_2O_2 ratio alteration. This last result suggests that GAPC enzymes could be involved in the control of ROS levels, as previously suggested by Hancock et al. (2005) and Baek et al. (2008).

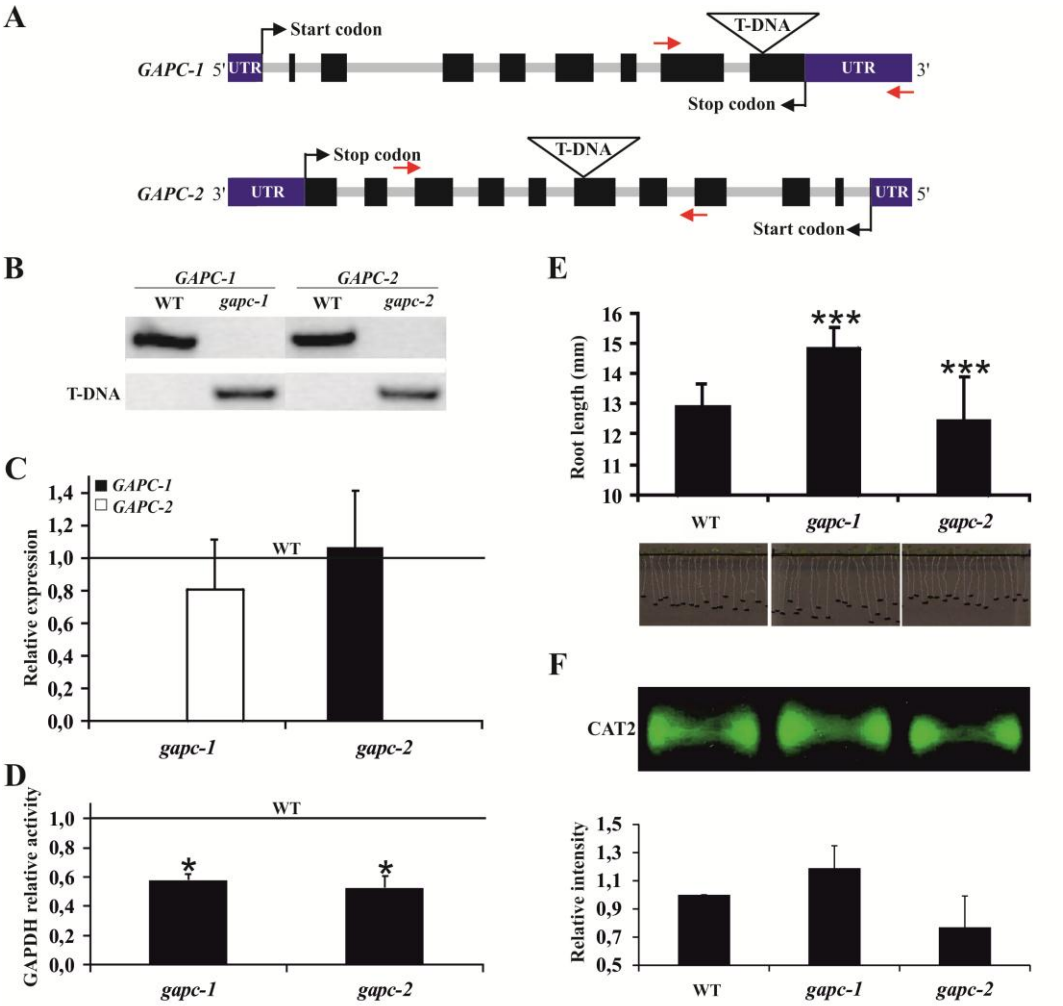

Figure 1: Isolation and characterization of *Arabidopsis gapc-1* and *gapc-2* mutants. From top to bottom: (A) Schemes of *GAPC-1* and *GAPC-2* gene structures of *gapc-1* (SALK_010839) and *gapc-2* (SALK_016539) insertional mutants. The *GAPC-1* gene is composed of nine exons and eight introns, *GAPC-2* gene is composed of eleven exons and ten introns. The T-DNA insertions indicated by white triangles are localized in the ninth and sixth exons, respectively. Red arrows indicate the position of the primers used to screen homozygous mutants. (B) PCR analysis of *GAPC-1* and *GAPC-2* genes performed on genomic DNA from wild-type and homozygous *gapc-1* and *gapc-2* mutants. The upper bands correspond to the amplification of wild-type alleles, and lower band of mutated alleles. (C) Quantitative real-time RT-PCR expression analysis of *GAPC-1* and *GAPC-2* genes in *Arabidopsis* wild-type and homozygous *gapc-1* and *gapc-2* mutants seedlings. The relative expression values are related to the expression levels of the same genes in wild-type background, which were set to 1. Values represent mean ± SD of RQ value of three experiments performed by using templates from three independent biological samples. (D) GAPDH catalytic activity analysis performed in total protein extracts from 7-day-old *Arabidopsis* seedlings. The relative activity values of *gapc* mutants are related to the activity in wild-type background, which was set to 1. Values represent mean ± SD of relative activity values of three experiments performed by using protein extracts from three independent biological samples. Asterisks indicate activity levels that are significantly different from those found in wild-type as calculated by Student's *t* test (* $p < 0.001$). (E) Root length of 7-day-old *Arabidopsis* seedlings. Values represent mean ± SD of root length of at least 300 seedlings. Asterisks indicate root length that are significantly different from those found in wild-type as calculated by Student's *t* test (*** $p < 0.05$). (F) Catalase activity analysis. Zymogram performed using protein extracts from roots of 7-day-old *Arabidopsis* seedlings. The experiment was repeated four times showing always the same activity pattern. In the graph are presented the relative band intensity values normalized to wild-type.

Effects of high concentration of $CdCl_2$ on roots of wild-type Arabidopsis

It has been widely demonstrated in animal and yeast that GAPDH is involved in oxidative stress signalling pathways (Almeida et al., 2007; Tristan et al., 2011). As a consequence, the question whether, GAPC enzymes were involved in oxidative stress signalling also in plants was addressed. In plants, oxidative stress conditions occur very frequently in response to biotic and abiotic stresses (Bestwick et al., 1997; Jaspers and Kangasjärvi, 2010; Molassiotis and Fotopoulos, 2011). Under these stress conditions, plants produce Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) as signalling molecules. The accumulation of these molecules leads to oxidative stress conditions (Bestwick et al., 1997; Corpas et al., 2008; Molassiotis and Fotopoulos, 2011). Among abiotic stresses, exposure to heavy-metals, and, in particular, to cadmium leads to the accumulation of NO and H_2O_2 (Cho and Seo, 2005; Besson-Bard et al., 2009; De Michele et al., 2009; Cuypers et al., 2011). Thus, to study the possible effects of oxidative stress on GAPC-1 and GAPC-2 enzymes, *Arabidopsis* seedlings were exposed to cadmium-induced oxidative stress conditions. In literature, a wide range of cadmium concentrations and experimental conditions have been described to induce production of NO and H_2O_2 (Cho and Seo, 2005; Besson-Bard et al., 2009; Tamás et al., 2010). However, in most of the studies plants were exposed to very high (millimolar) cadmium concentrations that do not mimic the situation of cadmium-polluted soils (Sanità di Toppi and Gabbrielli, 1999; Olmos et al., 2003; Garnier et al., 2006, Tamás et al., 2010). Under the latter experimental conditions, ROS burst occurred within a few hours as well as cell death (Olmos et al., 2003; Cho and Seo, 2005; Garnier et al., 2006; Tamás et al., 2010). In order to find out the best experimental conditions that better reproduce

the situation of cadmium-polluted soils, 7-day-old wild-type *Arabidopsis* seedlings were exposed to different $CdCl_2$ concentrations, 100, 200 and 400 µM, up to 72 hours (Fig. 2). Given that root is the organ which cadmium is absorbed through (Dal Corso et al., 2010), the effects of cadmium treatments were monitored on root tips. The experiment revealed that all tested cadmium concentrations led to stress related responses just 24 hours after treatment. The first observed effect was the induction of root hairs along the elongation zone of root tips. The development of root hairs was more evident in 100 and 200 µM cadmium treatments. 48 hours later, the differentiation zone of treated roots changed to a brownish phenotype as already described by Suzuki (2005). 72 hours after treatments, a strong morphological change of root tips was observed in presence of 100 µM cadmium, while higher cadmium concentrations did not induce any morphological changes. The morphological change was due to cell enlargement at the level of the differentiation zone. The causes of this morphological change were never addressed before in *Arabidopsis* plant. We tested the hypothesis that cell enlargement could depend by to an alteration of hormones balance. Given that auxins are involved in cell expansion regulation (Perrot-Rechenmann, 2010), we treated *Arabidopsis* transgenic line expressing the *GFP* reporter gene under the control of the synthetic auxin DR5 promoter (Ulmasov et al., 1997) with 100 µM cadmium for 48 hours (Fig. 3). Confocal microscope analyses revealed a different *DR5* promoter activity pattern between treated and untreated roots. In particular, under control condition the promoter activity was detectable only in the meristematic region of root tips, while, in cadmium treated roots the promoter activity was detected in the differentiation and elongation zones, exactly where cell enlargement occurred, suggesting the

occurrence of an auxin accumulation. We can, therefore, speculate that this cell enlargement is directly linked to an altered auxin concentration/distribution. Interestingly, a similar situation was described by Fernández-Marcos et al. (2011) where the authors reported that NO treatment affects meristem size by decreasing cell-division and promoting cell differentiation as a consequence of reduced auxin transport due to high levels of NO. Moreover, by monitoring cell death by Evan's blue staining, it has been observed that 72 hours after treatment, 200 and 400 µM cadmium induced cell death close to the brownish zone. These evidences indicate that 100 µM cadmium treatment is a suitable experimental condition, since induced a prolonged stress condition without affecting root viability. Given that, the following experiments were performed treating 7-day-old wild-type *Arabidopsis* seedlings with 100 µM $CdCl_2$ up to 72 hours.

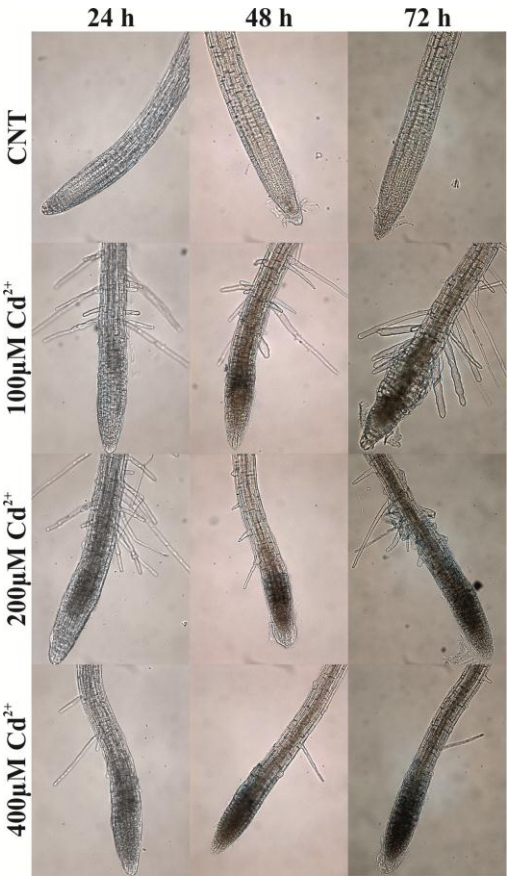

Figure 2: Evan's blue staining of *Arabidopsis* roots exposed to $CdCl_2$. 7-day-old wild-type *Arabidopsis* seedlings were treated with 100, 200 and 400 µM $CdCl_2$ from 24 to 72 hours, and then stained with Evan's blue. Roots were observed by an optical microscope. Pictures are representative of at least 50 roots from 3 independent experiments.

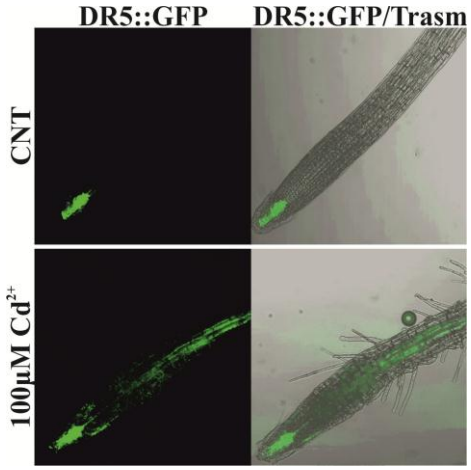

Figure 3: Pattern of *DR5::GFP* expression in *Arabidopsis* roots exposed to $CdCl_2$. 7-day-old *Arabidopsis DR5::GFP* transgenic seedlings were treated with 100 µM $CdCl_2$ for 48 hours. Roots were analysed by means of confocal microscope. Pictures are representative of at least 30 roots from 3 independent experiments.

100 µM $CdCl_2$ induces the accumulation of NO and H_2O_2 in **Arabidopsis** *root tips*

It has been demonstrated that, NO and H_2O_2 accumulated in cadmium-treated plants and cell cultures (Cho and Seo, 2005; Besson-Bard et al., 2009; Tamás et al., 2010; De Michele et al., 2009). Their accumulation induces oxidative stress conditions that in turn affect plant development and cell cultures growth (Cho and Seo, 2005; Suzuki, 2005; De Michele et al., 2009).

In order to investigate if 100 µM $CdCl_2$ was able to induce the synthesis and accumulation of NO and H_2O_2, we monitored their level in *Arabidopsis* root tips treated from 3 to 72 hours. The NO was analyzed by using DAF-FM diacetate, a membrane-permeable NO-sensitive indicator, whereas for the monitoring of H_2O_2 accumulation, transgenic *Arabidopsis* seedlings expressing the genetically encoded H_2O_2 sensor cHyPer (Costa et al., 2010) were used. Cadmium treatment up to 12 hours triggered a ~30% decrease of NO levels when compared to control, while the H_2O_2 levels were weakly affected 3 hours after treatment (Fig. 4A). After 24 hours, cadmium induced the accumulation of both NO and H_2O_2. NO level reached the maximum accumulation (+ 42%) 24 hours after treatment, then the level decreased (+ 18%) up to 72 hours. Instead, H_2O_2 started to accumulate (+ 33%) 24 hours after treatment reaching the maximum accumulation (+ 52%) up to 72 hours.

Our results demonstrate that 24 hours of 100 µM $CdCl_2$ treatment induces the accumulation of both NO and H_2O_2 in the root tip of *Arabidopsis* seedlings and this brought us to test whether such ROS accumulation was sufficient to affect the redox potential of the plant cells (Molassiotis and Fotopoulos, 2011). One of the key molecules involved in the control of intracellular redox potential is

the tripeptide glutathione (Meyer et al., 2007), a molecule that can be reversibly converted from the reduced form (GSH) to the oxidized one (GSSG). It is important to remember that the glutathione redox potential is dependent on its total concentration and its degree of oxidation (Meyer and Hell, 2005). Indeed, by determining the glutathione redox potential, it is possible to monitor variation of cell redox potential. For monitoring cell redox potential *in vivo,* Meyer et al., (2007) developed a redox-sensitive GFP called roGFP2. By using *Arabidopsis* transgenic plants expressing this genetically encoded redox sensitive probe, it is possible to monitor variation of cytoplasmic cell redox potential *in vivo*. Thus, in order to check the effect of cadmium treatment on root tip redox potential, *Arabidopsis* transgenic plants expressing the redox sensitive probe roGFP2 were treated for 24 hours with 100 μM $CdCl_2$ (Fig. 4B). The analyses revealed that cadmium led to a significant increase in the 405/488 nm probe ratio reflecting an increase in the probe oxidation status. Hence, the cytoplasmic redox potential in the cells of the root tip was indeed more oxidized than in the untreated cells, showing an induction of oxidative stress conditions after cadmium treatment.

In summary, these results showed that 100 μM $CdCl_2$ treatment induces the accumulation of both NO and H_2O_2 in *Arabidopsis* root tips 24 hours after exposure, and that their accumulation leads to an oxidative stress condition due to a decrease in the GSH/GSSG ratio.

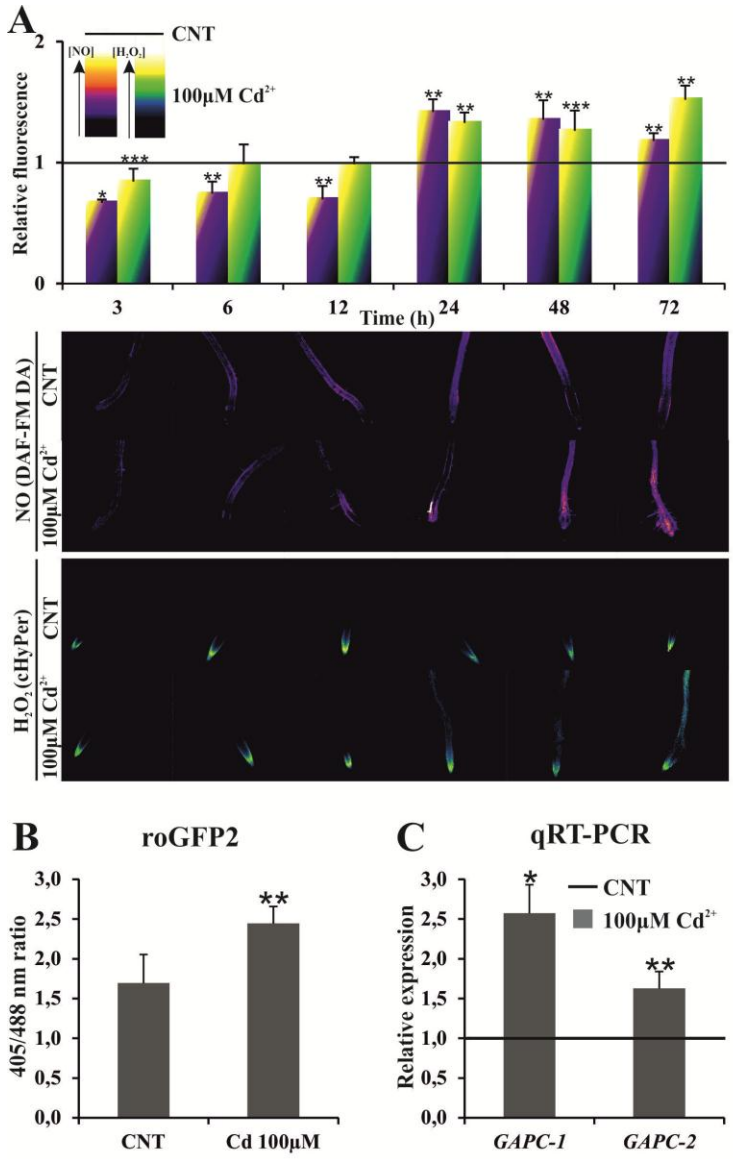

Figure 4: NO and H_2O_2 production, and alteration of redox state and *GAPC* genes expression in *Arabidopsis* seedlings exposed to $CdCl_2$. A) 7-day-old *Arabidopsis* wild-type and cHyPer transgenic seedlings were treated with 100 µM $CdCl_2$ from 3 to 72 hours. For NO detection, treated and untreated wild-type seedlings were stained with DAF-FM DA, then, roots were observed by a confocal microscope. For H_2O_2 detection, treated and untreated cHyPer transgenic seedlings were observed by a confocal microscope. Pictures are representative of at least 18 roots from 3 independent experiments, and values reported in the graph represent mean ± SD of relative fluorescence values of 18 roots from 3 independent experiments. The relative fluorescence values are related to the fluorescence level in untreated roots (set to 1). Asterisks indicate fluorescence levels that are significantly different from those found in untreated roots as calculated by Student's *t* test (* $p< 0.001$, ** $p< 0.01$, *** $p<0.05$). (B) 405/488 ratio values of cytosolic roGFP2 probe in 7-day-old *Arabidopsis* roGFP2 transgenic seedlings treated with 100 µM $CdCl_2$ for 24 hours. Values represent mean ± SD of 405/488 ratio values of 18 roots from 3 independent experiments. Asterisks indicate 405/488 ratio values that are significantly different from those found in untreated roots as calculated by Student's *t* test (** $p< 0.01$). (C) Quantitative real-time RT-PCR expression analysis of *GAPC-1* and *GAPC-2* genes in 7-day-old *Arabidopsis* wild-type seedlings treated with 100 µM $CdCl_2$ for 24 hours. The relative expression values of *GAPC* genes are related to the expression levels in untreated roots (set to 1). Values represent mean ± SD of 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 seedlings as calculated by Student's *t* test (* $p< 0.001$, ** $p< 0.01$).

Expression analysis of* GAPC-1 *and* GAPC-2 *genes in* Arabidopsis *wild-type plants exposed to cadmium-induced oxidative stress

Showing that 100 µM $CdCl_2$ treatment induces the accumulation of NO and H_2O_2, and that it has as consequence the induction of oxidative stress, we turned to analyse possible downstream effects. It was previously reported by Roth and co-workers (2006) that, in *Arabidopsis* roots, a 24 hours cadmium treatment, leads to an increase of the GAPC-2 protein when compared with control. At the same time Sarry and co-workers (2006) reported that the treatment of wild-type *Arabidopsis* cell cultures with 200 µM $CdCl_2$ for 24 hours led to a strong increase of GAPC-1 enzyme abundance. We therefore decided to investigate if, under our experimental conditions, the cadmium treatment could affect the expression of the two *GAPC* genes. To do so, quantitative real-time RT-PCR experiments were performed (Fig. 4C). The analyses revealed that 24 hours cadmium treatment induced the up-regulation of both *GAPC-1* and *GAPC-2* genes. Specifically, the expression of the *GAPC-1* isoform was ~2.5 fold, whereas only ~1.5 fold was for the *GAPC-2.* Being GAPC-1 the most sensitive one, further analyses were performed on this isoform.

Production of* Arabidopsis *transgenic plants expressing the* pGAPC1::GUS *transcriptional reporter and the* pGAPC1::GAPC1-YFP *translational reporter

In order to have reliable tools to study the effects of cadmium-induced oxidative stress on the expression and function of GAPC-1, two different *Arabidopsis* transgenic lines were generated. In the first one, the transcriptional reporter harboring the β-glucuronidase gene under control of the *GAPC-1*

promoter sequence (-633 to -1 from the ATG start codon) was introduced; the second transgenic line was obtained by the introduction of the chimeric *GAPC1-YFP* gene still under the control of the *GAPC-1* promoter (Fig. 5). The *Arabidopsis* transgenic plants were produced by using the floral dip method (Clough and Bent, 1998). For each expression cassette, several independent transgenic plants were isolated, all selected lines showing no changes in growth and morphology when compared to wild-type plants. Considering the 12 independently isolated lines transformed with the *pGAPC1::GUS* expression cassette, 11 of them showed an identical expression pattern (Fig. 6A). Considering plants transformed with the *pGAPC1::GAPC1-YFP* expression cassette, 17 independent transgenic lines were isolated and all of them showed a similar expression pattern, based on the YFP fluorescence analyses. To be sure that the fluorescent signal was dependent to the chimeric GAPC1-YFP protein, the protein extracts of three independent lines were analyzed by immune-blot analysis. (Fig. 6B). These lines showed small differences in GAPC1-YFP content, and free YFP was not detected, meaning that the chimeric protein was not degraded. Thus, the fluorescence observed by confocal microscope analyses was due to the GAPC1-YFP chimeric protein. The outcome of these analyses is that it was stated to use *pGAPC1::GAPC1-YFP17-2* line to study the effect of cadmium-induced oxidative stress on GAPC-1 protein.

Moreover, transgenic lines expressing the GAPC1-YFP chimeric protein under the control of *p35S* constitutive promoter were generated. Confocal microscope analyses of these lines revealed a very high expression of the transgene, leading to the formation of protein clusters. These results were confirmed by the immuno-blot analysis (Fig. 6B). In fact, the immuno-detection

analysis revealed a very high accumulation of the chimeric protein when compared with the *pGAPC1::GAPC1-YFP* transgenic lines. Then, this line was used to check whether the fusion protein maintained the enzymatic activity. In order to check this, the GAPDH catalytic activity of a *p35S::GAPC1-YFP* transgenic line was measured. The analysis revealed a dramatic increase of GAPDH catalytic activity in the transgenic line (Fig. 7), confirming that the catalytic activity of the enzymatic component is not affected by the fusion of YFP at its C-terminus end.

In previous papers, the study of the subcellular localization of GAPC proteins was performed by using *Arabidopsis* protoplast transformed with expression cassettes in which the expression of *GAPC* genes was under the control of *p35S* constitutive promoter (Holtgrefe et al., 2008; Wawer et al., 2010). In our hands, it was not possible, by using *p35S::GAPC1-YFP* transgenic lines, to define a clear subcellular localization of the chimera by confocal microscope analyses due to the formations of protein clusters (data not shown). For these reason, further experiments were carried out using transgenic lines transformed with expression cassettes in which the expression of translational reporter genes was controlled by the *GAPC-1* endogenous promoter.

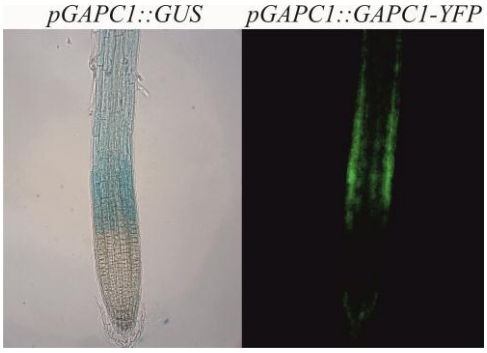

Figure 5: Picture of root tips of 7-day-old *Arabidopsis* transgenic seedlings stable transformed with *pGAPC1::GUS* and *pGAPC1::GAPC1-YFP* expression cassettes.

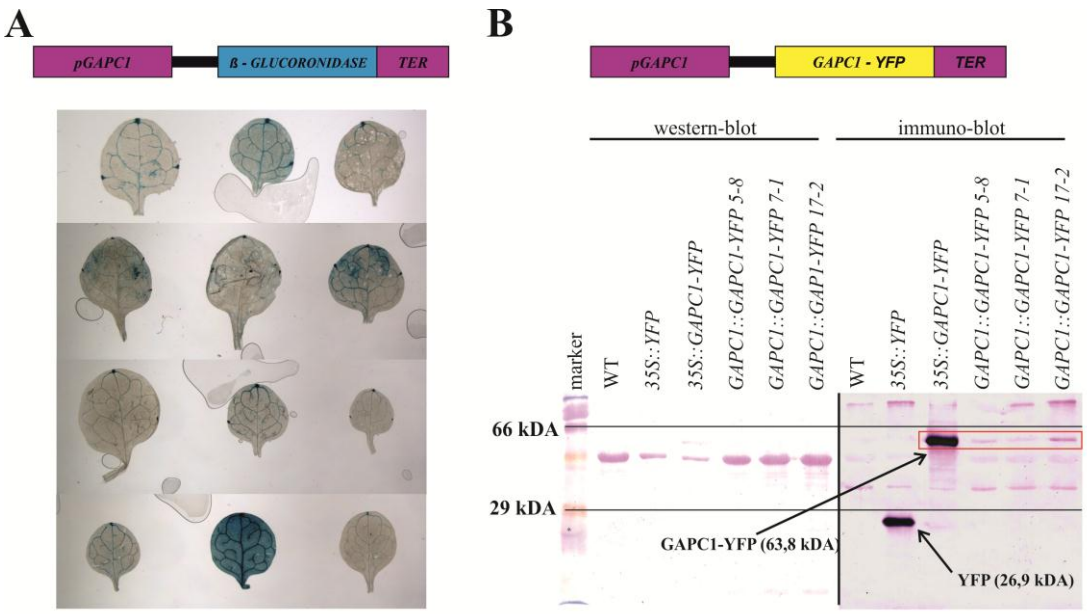

Figure 6: Validation of the *pGAPC1::GUS* and *pGAPC1::GAPC1-YFP* transgenic lines produced. (A) From top to bottom: structure of *pGAPC1::GUS* expression cassette, and histochemical analysis of GUS activity in cotyledons from *pGAPC1::GUS* transgenic seedlings. Each cotyledon comes from an independent transgenic line. (B) From top to bottom: structure of *pGAPC1::GAPC1-YFP* expression cassette, western- and immune-blot analyses using a GFP antibody. The analyses were performed using protein extracts from 7-day-old *Arabidopsis* seedlings of wild-type, *35S::YFP*, *35S::GAPC1-YFP*, and three independent *pGAPC1::GAPC1-YFP* transgenic lines. Black arrows indicate the antibody detection of free-YFP and GAPC1-YFP expressed under the control of the 35S constitutive promoter. The red square also indicates the antibody detection of GAPC1-YFP expressed under the control of the *GAPC-1* promoter in three independent transgenic lines.

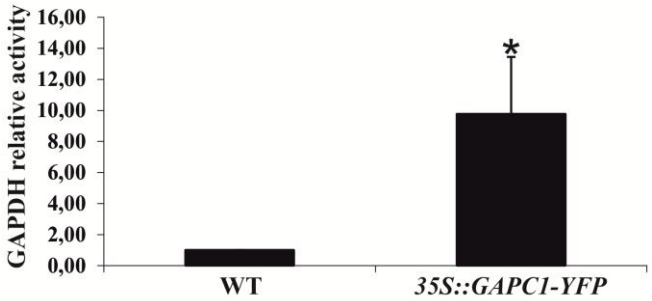

Figure 7: GAPDH catalytic activity analysis performed in total protein extracts from 7-day-old *Arabidopsis* wild-type and *35S::GAPC1-YFP* transgenic seedlings. The relative activity value of *35::GAPC-1-YFP* transgenic plants are related to the activity in wild-type background, which was set to 1. Values represent mean ± SD of relative activity values of three experiments by using protein extracts from three independent biological samples. Asterisks indicate activity levels that are significantly different from those found in wild-type as calculated by Student's *t* test (* p< 0.001).

Characterization of the* GAPC-1 *promoter activity pattern

In a previous paper (Yang et al., 1993) the authors reported the effects of some environmental stress on the expression of *GAPA, GAPB,* and *GAPC-1* genes. Yang and co-workers described that heat shock, anaerobiosis, and carbon source supply affected the steady-state mRNA level of the *GAPC-1*. Instead, the same treatments did not affect the steady-state mRNA levels of the plastidial isoforms *GAPA* and *GAPB.* Moreover, in order to identify the cis-acting regulatory elements of *GAPC-1*, they generated transgenic tobacco plants transformed with the β-glucuronidase gene fused to the 820-bp *GAPC-1* 5'-flanking DNA fragment of *Arabidopsis thaliana.* Then, they used these plants to study the activity of the 820-bp *GAPC-1* 5'-flanking DNA fragment under stress conditions, and to determine the *GAPC-1* promoter activity pattern they analyzed the GUS activity in several tobacco tissues. These analyses revealed that *GAPC-1* promoter was active in leaves, stems and roots, but the activity extent within each organ was different, showing that cells with high respiration rate, low photosynthetic activity, and xylem cells of all organs exhibit high level of *GAPC-1* expression. Moreover, by using the same tobacco transgenic plants, the authors also confirmed that heat shock and anaerobic treatment affected the *GAPC-1* promoter activity. The data coming from Yang et al., (1993) are quite unexpected, in fact, *GAPC-1* gene and the codified enzyme were considered housekeeping, and for this, the gene expression considered to be constitutive, and not affected by treatments.

We investigated the *GAPC-1* expression pattern trough the interrogation of the available microarray databases by using the eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) and the results are reported in

Figure 8. The analyses revealed that *GAPC-1* expression in *Arabidopsis* plants is more or less constitutive in all tissues, except in siliques and shoot meristem where the lowest and the highest expression levels, were respectively observed. Having generated the *Arabidopsis pGAPC1::GUS* lines, we decided to verify if our selected promoter was indeed functional in the same tissues where the *GAPC-1* was detected by microarray analyses. Hence the *GAPC-1* promoter activity was analyzed during growth and development of *Arabidopsis* plants. In particular the GUS histochemical assay was performed in seedlings (Fig. 9A) and bolting plants (Fig. 9B). The images show that GUS activity was detected in almost all tissues of both seedlings and bolting plants. In seedlings, up to 14 days after the germination, the highest GUS activity was detected in cotyledons, young leaves, hydatodes, apical meristem, crown, root tip, and in xylem cells. Instead a weaker activity was detected in stems and some root regions of the elongation zone. In bolting plants a strong GUS activity was detected in young leaves, cauline leaves, senescent leaves, petals, pistils, anthers, hydatodes, in the abscisic zone of siliques, and in xylem cells of leaves. A weaker activity was observed in fully expanded leaves and forming siliques. No activity was detected in stems and mature siliques.

The results here provided demonstrate that the 633 bp sequence cloned from 5' UTR region of *GAPC*-1 gene contains all the "required information" to drive *GAPC-1* expression, since GUS activity pattern is consistent with the microarray analyses (Fig. 8). Moreover, the characterization of promoter activity performed in this work shows that *GAPC-1* gene is not constitutively expressed as one would expect for a housekeeping gene. In fact, a very high *GAPC-1* expression was detected in growing tissues like shoot meristem and root tip,

instead, in mature tissues, like fully expanded leaves, mature siliques and stems the GUS activity was ranging from low to undetectable.

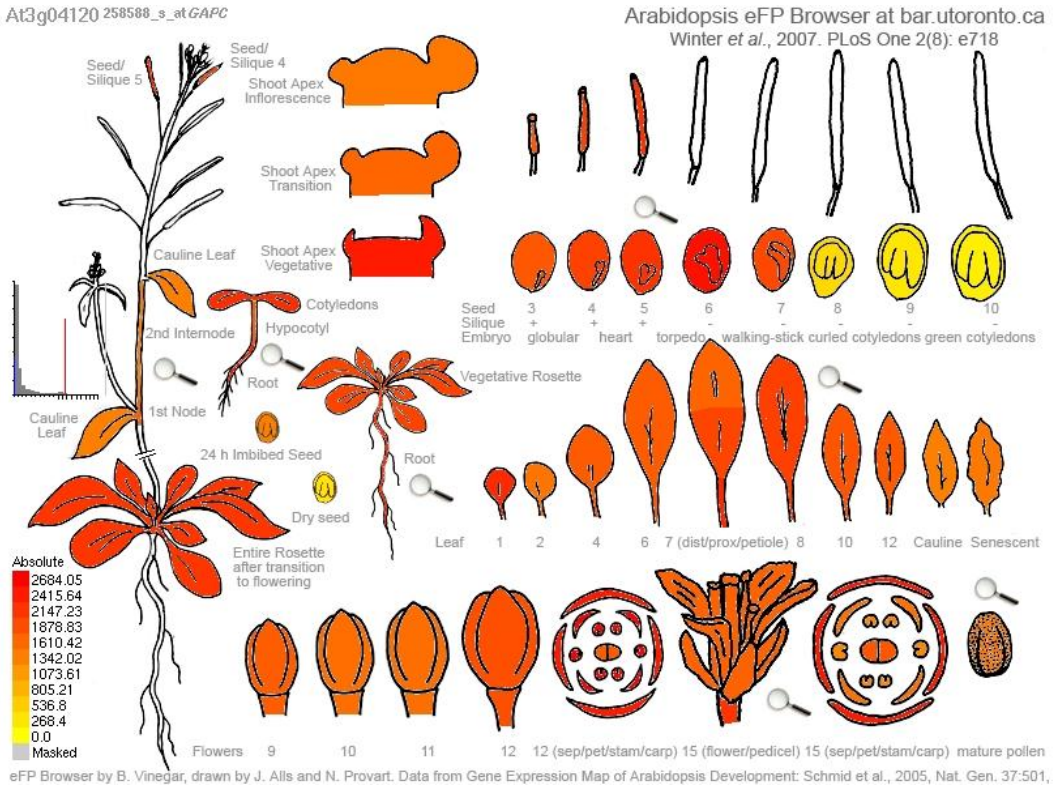

Figure 8: *GAPC-1* expression pattern through the interrogation of the available microarray databases, by using the eFP Browser.

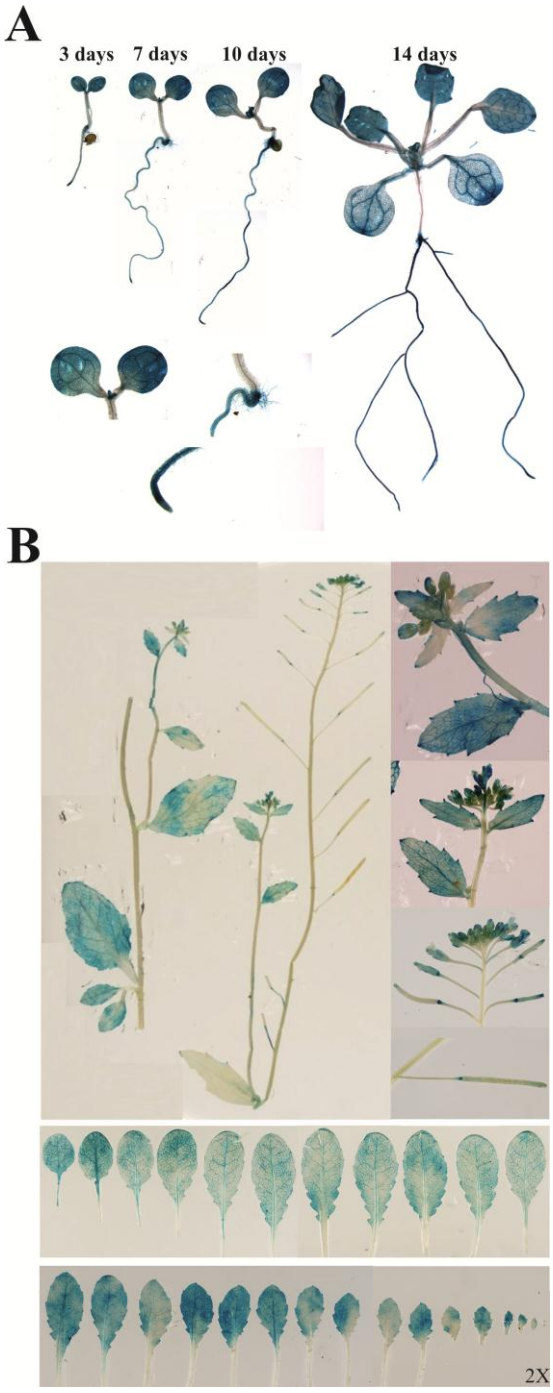

Figure 9: Characterization of *GAPC-1* promoter activity pattern. Histochemical analysis of GUS activity in *pGAPC1::GUS* transgenic plants. (A) *GAPC-1* promoter activity pattern in 3-,7-,10-, and 14-day-old seedlings. (B) *GAPC-1* promoter activity pattern in 4-week-old bolting plant.

Effect of cadmium-induced oxidative stress on* GAPC-1 *promoter activity and GAPC1-YFP chimeric protein

As already shown above, 100 µM $CdCl_2$ treatment induces the accumulation of both NO and H_2O_2, leading to oxidative stress in root tips. By performing quantitative real-time RT-PCR we demonstrated under these experimental conditions, the cadmium treatment could affect the expression of the *GAPC* genes, in particular *GAPC-1* expression was more affected than *GAPC-2*. For this reason, it was decided to study more in detail the effect of cadmium-induced oxidative stress on *GAPC-1* promoter activity and GAPC-1 protein accumulation/distribution. In order to do so, 7-day-old *pGAPC1::GUS* and *pGAPC1::GAPC1-YFP Arabidopsis* seedlings were exposed to 100 µM $CdCl_2$ from 3 to 72 hours (Fig. 10).

Figure 10A shows, by both histochemical and quantitative GUS analyses, that in seedlings treated with 100 µM cadmium, the *GAPC-1* promoter activity was clearly enhanced after 24 hours, remaining high for the successive 24 hours. The promoter activity was then reduced after 72 hours of treatment. Interestingly, our quantitative analyses demonstrated that after 6 hours of cadmium treatment a slight decrease in the promoter activity occurred, without affecting the protein accumulation (see below). The histochemical analyses revealed that the increased promoter activity observed after 24 hours of treatment occurred mainly in the cells present in the differentiation zone of the root tip, where the accumulation of NO was also reported (Fig. 4). On the other hands, 48 and 72 hours after treatment the promoter activity was also detected in the cells of the elongation zone where the accumulation of H_2O_2 was observed (Fig. 4). These results are consistent with

quantitative real-time RT-PCR analysis, showing *GAPC-1* mRNA levels (Fig. 4C) and *GAPC-1* promoter activity increased after 24 hours of cadmium treatment.

By treating *pGAPC1::GAPC1-YFP Arabidopsis* transgenic seedlings as described above, we studied the effects of cadmium-induced oxidative stress on the GAPC1-YFP protein (Fig. 10B). The confocal microscope analyses revealed that the treatment did not affect the protein level up to 12 hours. Instead, from 24 to 72 hours after treatment the protein level dramatically increased, reaching the maximum accumulation (~7 fold respect the control) after 72 hours. As previously described, 24 hours after treatment the GUS activity increase was localized in the differentiation zone of the root tip, at the same manner the chimeric protein accumulated where the GUS activity increased, suggesting that the increase of GAPC-1 protein is due to the enhancement of its expression. On the contrary, after 72 hours, GUS activity dropped, while the chimeric protein was still accumulating, suggesting that under oxidative stress conditions a post-translational regulation could occur.

These and previous results demonstrate that cadmium-induced oxidative stress conditions affect both *GAPC-1* expression and GAPC-1 level, turnover or activity.

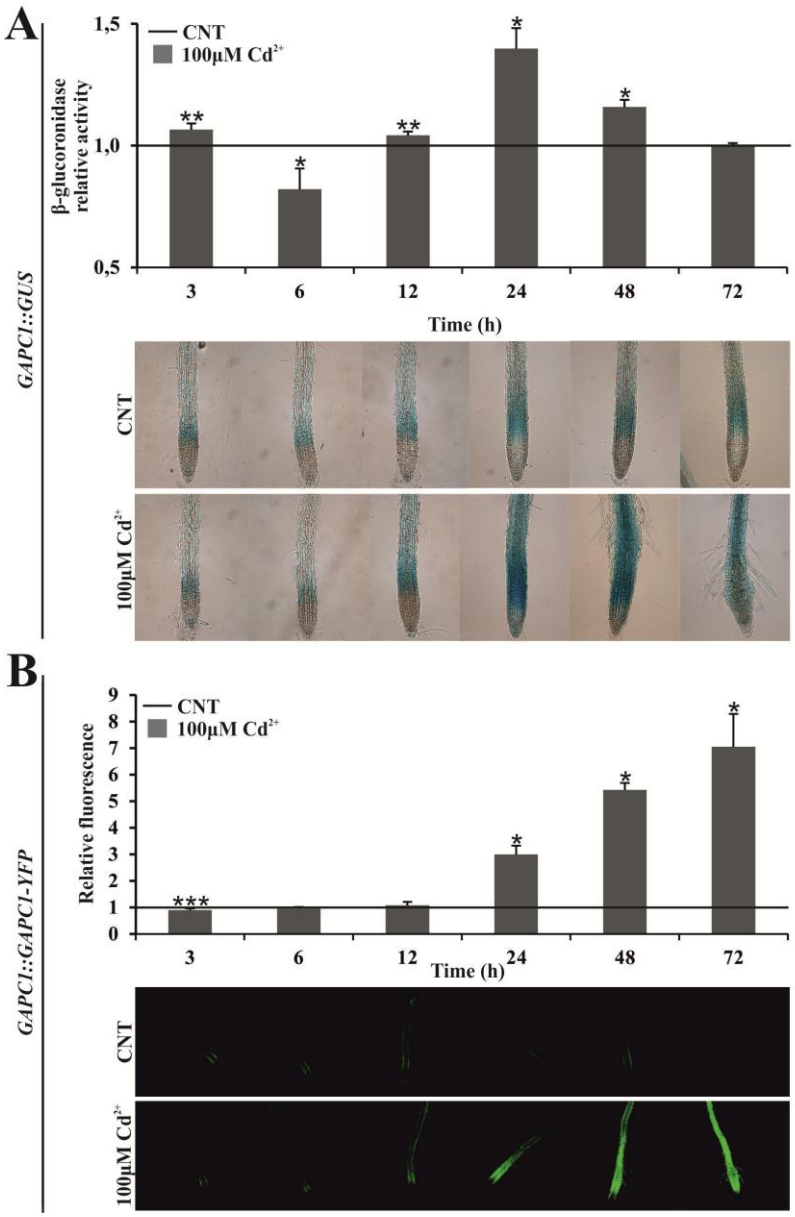

Figure 10: Effects of cadmium treatment on *GAPC-1* promoter activity and GAPC1-YFP level. A) Lower panel: 7-day-old *Arabidopsis pGAPC1::GUS* transgenic seedlings were treated with 100 µM $CdCl_2$ from 3 to 72 hours. Then, the histochemical analysis of GUS activity was performed and roots were observed by an optical microscope. Pictures are representative of at least 30 roots from 3 independent experiments. Upper panel: relative quantification of β-glucoronidase activity. 7-day-old *Arabidopsis pGAPC1::GUS* transgenic seedlings were treated with 100 µM $CdCl_2$ from 3 to 72 hours. The β-glucoronidase activity assay was performed using protein extracts from 600 roots from 3 independent experiments. Values reported in the graph represent mean ± SD of relative activity values of three independent technical replicates. The relative activity values of β-glucoronidase are related to the activity in untreated roots (set to 1). Asterisks indicate β-glucoronidase activity levels that are significantly different from those found in untreated roots as calculated by Student's *t* test (* $p < 0.001$, ** $p < 0.01$). (B) 7-day-old *Arabidopsis pGAPC1::GAPC1-YFP* transgenic seedlings were treated with 100 µM $CdCl_2$ from 3 to 72 hours. Treated and untreated transgenic seedlings were observed by a confocal microscope. Pictures are representative of at least 18 roots from 3 independent experiments, and values reported in the graph represent mean ± SD of relative fluorescence values of 18 roots from 3 independent experiments. The relative fluorescence values are related to the fluorescence level in untreated roots (set to 1). Asterisks indicate fluorescence levels that are significantly different from those found in untreated roots as calculated by Student's *t* test (* $p < 0.001$, *** $p<0.05$).

Effect of cadmium-induced oxidative stress on GAPC-1 and GAPC-2 catalytic activity

Since we observed that cadmium treatment induced the up-regulation of *GAPC-1* expression followed by a dramatic accumulation of the GAPC1-YFP chimeric protein, we also tested the total GAPDH catalytic activity. To this end the total protein extract from 7-day-old wild-type, *gapc-1* and *gapc-2* seedlings, exposed to cadmium for 72 hours, were used to perform the GAPDH catalytic activity assay (Fig. 11). The analyses revealed that the cadmium-induced oxidative stress did not affect the total GAPDH catalytic activity in the wild-type. The same result was observed by analyzing the GAPDH catalytic activity of treated *gapc-1* seedlings; in this case, the assay was more informative because in the *gapc-1* mutant, only the GAPC-2 isoform is present. Instead, in the *gapc-2* mutant, where only the GAPC-1 isoform is present, the treatment induced a detectable GAPDH catalytic activity increase that correlates with the increase of GAPC-1 accumulation. Since the total GAPDH catalytic activity increase, was detected when GAPC-1 was only present and not in the wild type background, it suggests that part of the GAPC-1 protein accumulated after 72 hours could be present in an inactivated form, and just a small part is in an active form. Interestingly, similar results were reported by Fourrat et al. (2007). They demonstrated that treating *Tetrahymena pyriformis* with the NO-donor SNP or H_2O_2 led to a dramatic increase of GAPDH protein level without affecting the total enzymatic activity. From these experiments we can conclude that even from a catalytic point of view the GAPC-1 isoform is more sensitive to oxidative stress, and it is subjected to a stronger regulation than GAPC-2 isoform.

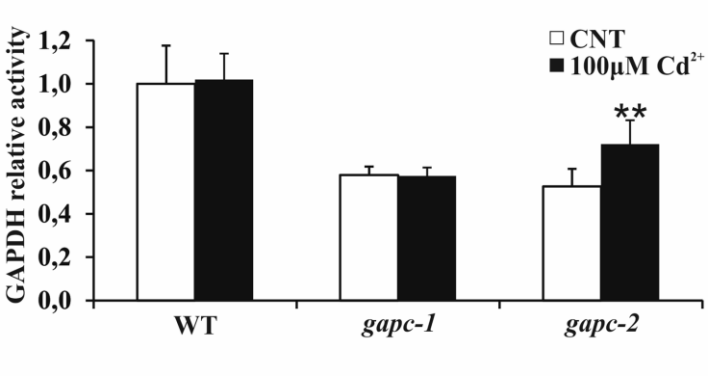

Figure 11: GAPDH catalytic activity analyses performed in total protein extracts from 7-day-old *Arabidopsis* wild-type, *gapc-1* and *gapc-2* mutant seedlings treated with 100 µM $CdCl_2$ for 72 hours. The relative activity values of untreated mutant seedlings and wild-type treated seedlings are related to the activity in untreated wild-type seedlings, which was set to 1. Values represent mean ± SD of relative activity values of three experiments performed by using protein extracts from three independent biological samples. Asterisks indicate activity levels that are significantly different from those found in untreated seedlings as calculated by Student's *t* test (* $p < 0.01$).

Effect of NO and H_2O_2 on recombinant GAPC-1 catalytic activity

We previously reported that cadmium treatment induces the up-regulation of *GAPC-1* expression, followed by the accumulation (~7 fold respect the control) of the GAPC1-YFP chimeric protein where the treatment induced the accumulation of both NO and H_2O_2. Moreover, under these experimental conditions we observed just a weak increase in GAPDH catalytic activity in *gapc-2* treated seedlings. Then, the question whether NO and H_2O_2 can directly affect GAPDH catalytic activity was approached. To this end, we produced a recombinant GAPC-1 in *E.coli* and we tested the effect of NO and H_2O_2 on its catalytic activity (Fig. 12A-B). By treating the enzyme with DEA-NONOate, a NO donor, and H_2O_2, it was observed that these two molecules negatively affected the GAPC-1 catalytic activity in a time- and concentration-dependent manner. We also observed that the GAPDH catalytic activity of NO-inactivated GAPC-1 could be restored by adding reducing molecules like DTT (10 mM) (Fig. 12C), while

was not possible to restore H_2O_2-inactivated GAPC-1 (Fig. 12D). Moreover, by treating NO-inactivated GAPC-1 with GSH (0,5 mM), a molecule involved in the regulation of redox state of cells (Meyer et al., 2007), the catalytic activity was restored (Fig. 12E). These results strongly suggest that *in vivo* the catalytic activity of GAPC-1 can be affected by the presence of NO and H_2O_2, as well as by the GSH/GSSG pool. In a possible scenario, we can therefore speculate that in the cell the GAPC-1 activity is strictly dependent by the competition between oxidant (NO and H_2O_2) and antioxidant molecule pools (GSH/GSSG).

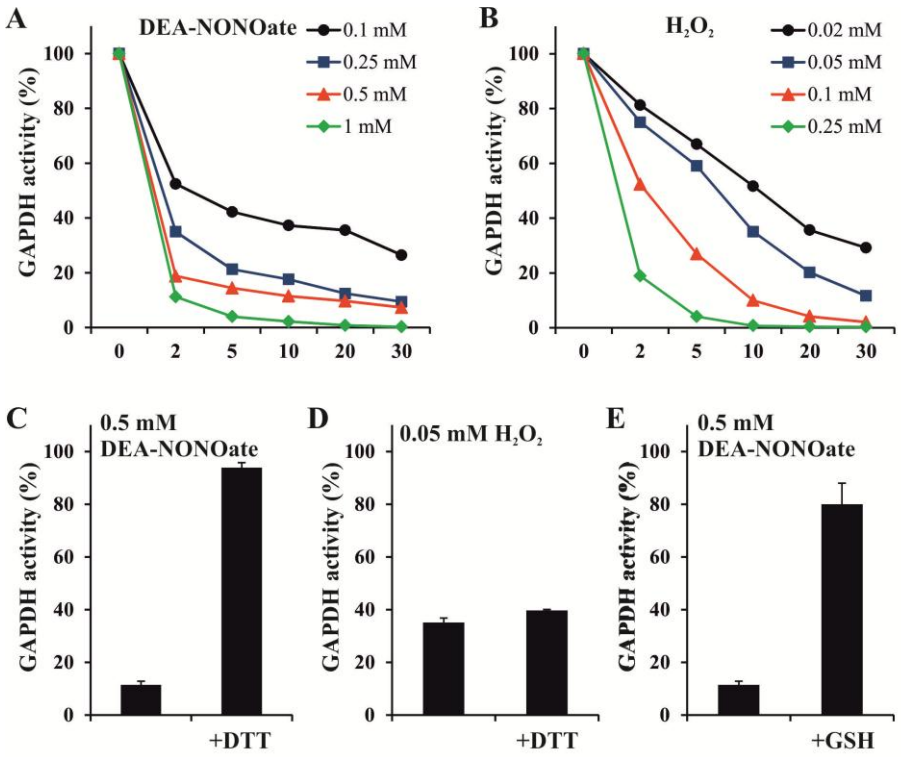

Figure 12: Effect of NO, H_2O_2, DTT and GSH on recombinant GAPC-1 catalytic activity. (A) Time- and concentration-dependent inactivation of recombinant GAPC-1 by the NO-donor DEA-NONOate. (B) Time- and concentration-dependent inactivation of recombinant GAPC-1 by H_2O_2. (C) Effect of 20 mM DTT on recombinant GAPC-1 exposed to 0.5 mM DEA-NONOate for 10 min. (D) Effect of 20 mM DTT on recombinant GAPC-1 exposed to 0.05 mM H_2O_2 for 10 min. (E) Effect of 0.5 mM GSH on recombinant GAPC-1 exposed to 0.5 mM DEA-NONOate for 10 min.

NO and H_2O_2 scavengers slightly reduced cadmium-induced GAPC-1 *expression and GAPC1-YFP accumulation*

Since it has been observed that NO and H_2O_2 could directly affect the catalytic activity of the recombinant GAPC-1 enzyme, we studied the scavenging effects of these molecules on *GAPC-1* promoter activity and GAPC1-YFP chimeric protein levels. In order to scavenge NO and H_2O_2, cPTIO and KI, were used, respectively (Besson-Bard et al., 2009; Tsukagoshi et al., 2010). Seven-day-old *pGAPC1::GUS Arabidopsis* transgenic seedlings were treated with 100 µM cPTIO and 1 mM KI for 24 hours (Fig. 13). The treatments with these molecules led to a slight reduction of *GAPC-1* promoter activity when compared with control. Instead, when the scavengers were added to cadmium treated seedlings *GAPC-1* promoter activity reduction was not observed. These results suggest that the scavenging of NO and H_2O_2 under physiological condition is sufficient to reduce the promoter activity. While, in cadmium treated seedlings, the increased amount of NO and H_2O_2 accumulated seemed to overcome the scavenging effects of cPTIO and KI.

In order to study the scavenging effects of 100 µM cPTIO and 1 mM KI on GAPC1-YFP chimeric protein levels, 7-day-old *pGAPC1::GAPC1-YFP Arabidopsis* transgenic seedlings were treated with 100 µM cPTIO and 1 mM KI from 24 up to 72 hours (Fig. 14). The cPTIO treatment did not affect the protein level in the first 24 hours, but after 24 and 72 hours induced a doubling of the GAPC1-YFP chimeric protein levels. The KI treatment, instead, did not affect the protein level in the first 48 hours, but after 72 hours caused a very small reduction of protein level. When the scavengers were added to cadmium treated seedlings, some reductions of GAPC1-YFP chimeric protein levels were observed.

Particularly, in 24 hours cadmium-treated seedlings, the addition of cPTIO led to a ~20% reduction of GAPC1-YFP fluorescence as well as 48 hours after treatment. Instead, KI addition in cadmium-treated seedlings led to a ~20% reduction of GAPC1-YFP fluorescence 48 hours after treatment.

These experiments reveal that scavenging NO and H_2O_2 *in vivo* did not dramatically affect the expression and post-transcriptional regulation of GAPC-1. Moreover, we indeed observed that the addition of KI to cadmium treated seedlings inhibited the development of root hairs in the differentiation zone. This latter result suggests that KI effectively scavenged H_2O_2, because the lacking of this molecule inhibited the ROS-dependent polar growth of root hairs (Cárdenas, 2009).

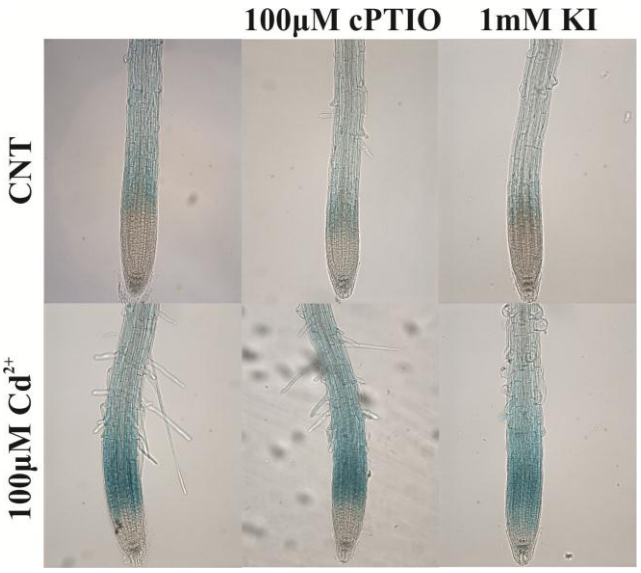

Figure 13: Effects of scavenging NO and H_2O_2 on *GAPC-1* promoter activity in untreated and cadmium treated roots. 7-day-old *Arabidopsis pGAPC1::GUS* transgenic seedlings were treated with 100 µM $CdCl_2$, 100 µM CPTIO, 1 mM KI, 100 µM $CdCl_2$ + 100 µM CPTIO and 100 µM $CdCl_2$ + 1 mM KI for 24 hours. Then, the histochemical analysis of GUS activity was performed, and roots were observed by an optical microscope. Pictures are representative of at least 30 roots from 3 independent experiments.

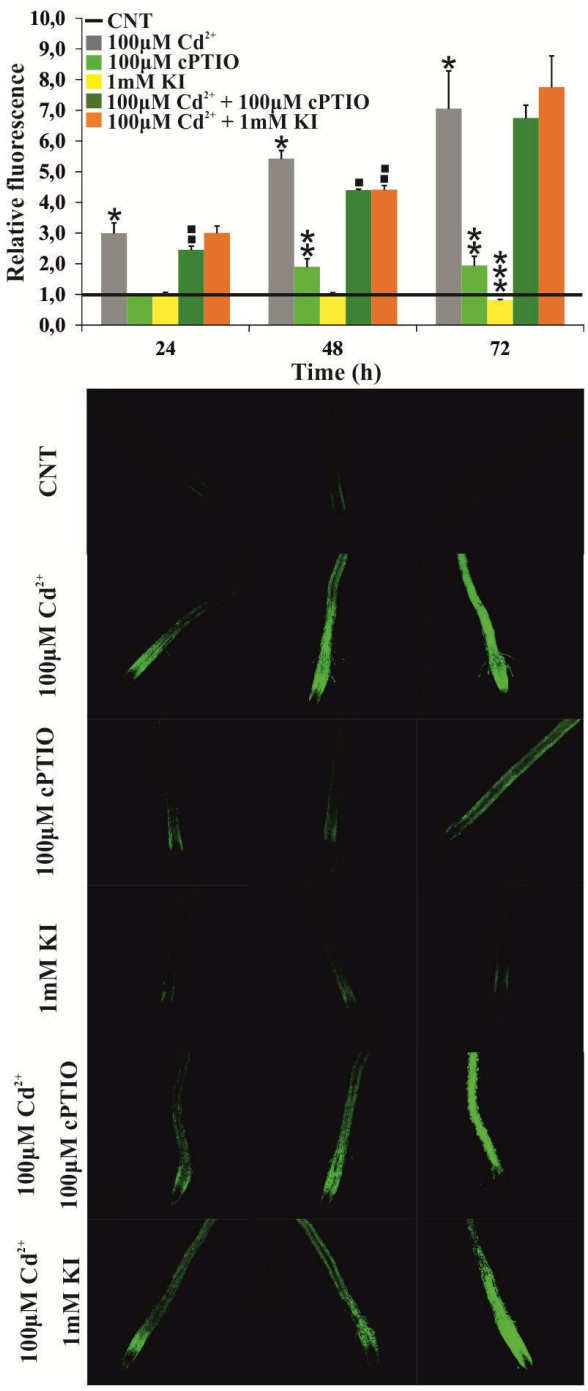

Figure 14: Effects of scavenging NO and H_2O_2 on GAPC1-YFP level in untreated and cadmium treated roots. 7-day-old *Arabidopsis pGAPC1::GAPC1-YFP* transgenic seedlings were treated with 100 µM $CdCl_2$, 100 µM CPTIO, 1 mM KI, 100 µM $CdCl_2$ + 100 µM CPTIO and 100 µM $CdCl_2$ + 1 mM KI from 3 to 72 hours. Treated and untreated transgenic seedlings were observed by a confocal microscope. Pictures are representative of at least 18 roots from 3 independent experiments, and values reported in the graph represent mean ± SD of relative fluorescence values of 18 roots from 3 independent experiments. The relative fluorescence values are related to the fluorescence levels in untreated roots (set to 1). Asterisks indicate fluorescence levels that are significantly different from those found in untreated roots as calculated by Student's *t* test (* $p< 0.001$, ** $p< 0.01$, *** $p<0.05$). Squares indicate fluorescence levels that are significantly different from those found in cadmium treated roots as calculated by Student's *t* test (■ $p< 0.001$, ■■ $p< 0.01$).

Altering the glutathione pool affects both GAPC-1 *promoter activity and GAPC1-YFP chimeric protein levels*

Previously, it was shown that GSH can restore the GAPDH catalytic activity of NO-inactivated GAPC-1, furthermore, we demonstrate that 24 hours cadmium treatment affects the cell redox potential by reducing the GSH/GSSG ratio. In order to study the effects of altering the GSH pool on *GAPC-1* promoter activity, 7-day-old *pGAPC1::GUS Arabidopsis* transgenic seedlings were treated with 1 mM GSH and 1 mM BSO, an inhibitor of glutathione biosynthesis (Griffith, 1982), for 24 hours (Fig. 15). Altering the glutathione homeostasis (i.e. redox state of cells) dramatically affected the activity of *GAPC-1* promoter. The reducing of GSH pool by BSO-inhibition of its synthesis, led to a strong increase of *GAPC-1* promoter activity, while increasing the level of GSH by adding 1 mM GSH caused a reduction of *GAPC-1* promoter activity when compared with untreated roots. These results showed that the *GAPC-1* promoter activity is highly regulated by the glutathione pool state. Moreover, we tested the effects of altering the glutathione pool homeostasis on 24 hours cadmium-treated seedlings. The inhibition of glutathione synthesis in cadmium-treated roots led to even stronger increase of *GAPC-1* promoter activity when compared with cadmium-treated roots. Instead, increasing the GSH pool in cadmium-treated roots prevented the increase of promoter activity when compared with cadmium treatment.

To test the effects of altering the GSH pool on GAPC-1 protein, *pGAPC1::GAPC1-YFP Arabidopsis* transgenic seedlings were treated as described in the previous paragraph (Fig. 16). Reducing GSH pool by treating with BSO led to an increase of the protein accumulation (~70% more than control) at 24 hours after treatment, and this level was maintained (~30% more)

up 72 hours after. The increase of GSH pool, instead, caused a reduction (~70% respect to the control) of GAPC1-YFP levels at 24 hours after treatment, and at 48 and 72 hours the GAPC1-YFP levels were even lower (~30% respect to the control). When the glutathione homeostasis of cadmium-treated seedlings was affected by BSO treatment, the GAPC1-YFP accumulation was dramatically increased if compared with cadmium treatment. In fact, the addition of BSO caused a 45% increase of protein level in 24 and 48 hours cadmium-treated seedlings. Instead, at 72 hours, the GAPC1-YFP level in seedlings treated with both BSO and cadmium was lower than in seedlings treated only with cadmium. The latter result is explained by the fact that the viability of BSO and cadmium-treated roots for 72 hours was too much affected. Increasing GSH pool in cadmium-treated seedlings reduced the cadmium-induced accumulation of GAPC1-YFP protein during the 72 hours treatment. More precisely, after 24, 48, and 72 hours, reductions of 55, 40, and 35%, were respectively detected.

All together, these and previous results demonstrate that the expression and post-transcriptional regulation of GAPC-1 are more affected by the redox state of glutathione pool than by free NO and H_2O_2 levels, suggesting that GAPC-1 could directly perceives the glutathione redox potential, i.e. the cell redox potential.

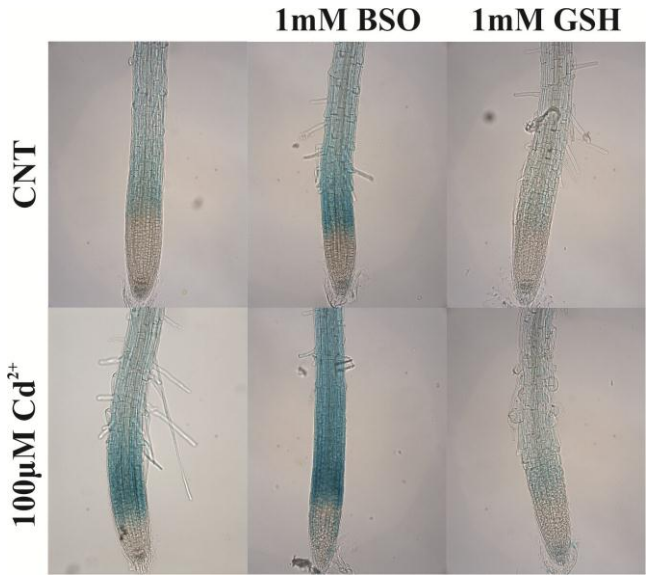

Figure 15: Effects of altering glutathione pool on *GAPC-1* promoter activity in untreated and cadmium treated roots. 7-day-old *Arabidopsis pGAPC1::GUS* transgenic seedlings were treated with 100 µM $CdCl_2$, 1 mM BSO, 1 mM GSH, 100 µM $CdCl_2$ + 1 mM BSO and 100 µM $CdCl_2$ + 1 mM GSH for 24 hours. Then, the histochemical analysis of GUS activity was performed, and roots were observed by an optical microscope. Pictures are representative of at least 30 roots from 3 independent experiments.

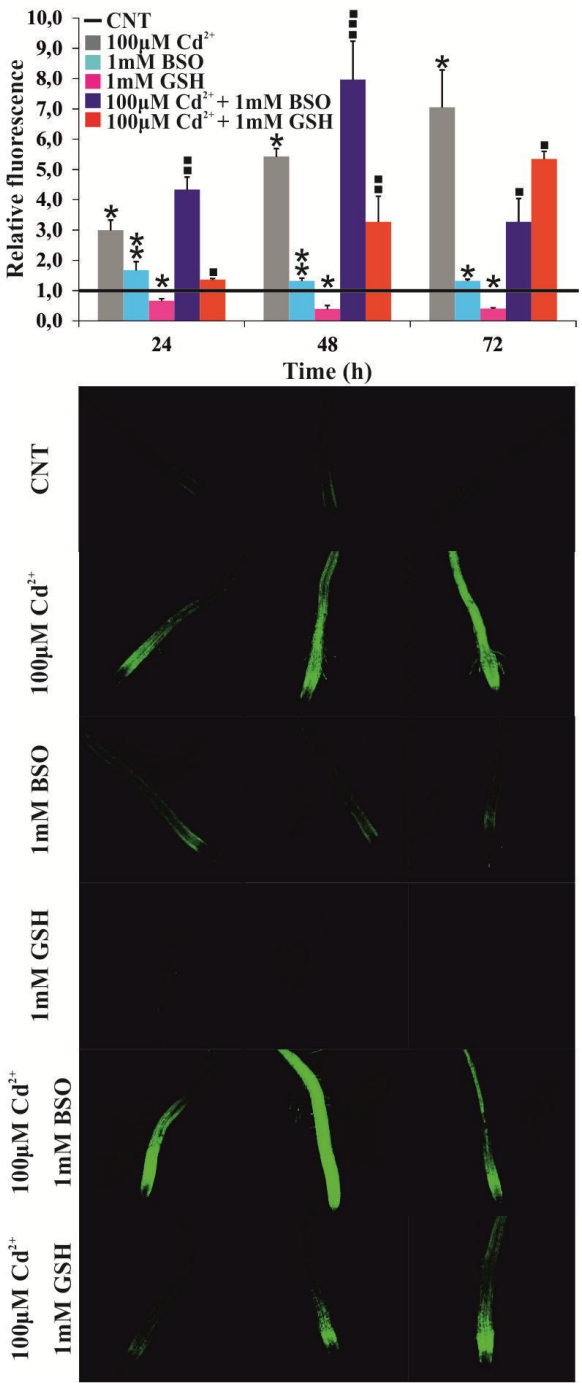

Figure 16: Effects of altering glutathione pool on GAPC1-YFP level in untreated and cadmium treated roots. 7-day-old *Arabidopsis pGAPC1::GAPC1-YFP* transgenic seedlings were treated with 100 µM $CdCl_2$, 1 mM BSO, 1 mM GSH, 100 µM $CdCl_2$ + 1 mM BSO and 100 µM $CdCl_2$ + 1 mM GSH from 3 to 72 hours. Treated and untreated transgenic seedlings were observed by a confocal microscope. Pictures are representative of at least 18 roots from 3 independent experiments, and values reported in the graph represent mean ± SD of relative fluorescence values of 18 roots from 3 independent experiments. The relative fluorescence values are related to the fluorescence level in untreated roots (set to 1). Asterisks indicate fluorescence levels that are significantly different from those found in untreated roots as calculated by Student's *t* test (* $p< 0.001$, ** $p< 0.01$). Squares indicate fluorescence levels that are significantly different from those found in cadmium treated roots as calculated by Student's *t* test (■ $p< 0.001$, ■■ $p< 0.01$, ■■■ $p< 0.05$).

Subcellular localization of GAPC1-YFP chimeric protein

Since in the paper of Hara et al. (2005) it is shown that under oxidative stress conditions the nitrosylated GAPDH is translocated into the nucleus by an ubiquitin ligase, the question whether also in plants under oxidative stress conditions the GAPC-1 protein was translocated into the nucleus was addressed. In order to check this, the elongation zone of root tips of untreated and 72 hours cadmium-challenged *pGAPC1::GAPC1-YFP Arabidopsis* transgenic seedlings was analysed (Fig. 17A). The attention was focussed on this root tip zone because the most affected by cadmium-treatment, in fact, the cells of this zone showed morphological changes and a strong increase of NO levels after treatment. In the same zone, where high levels of NO were detected and the cells showed morphological changes, an evident nuclear accumulation of the GAPC1-YFP chimeric protein was detected. Then, by quantifying the number of cells in which the protein was accumulated into nuclei, a ~5 fold increase of nucleated cells in cadmium-treated roots was detected (Fig. 17B).

In the previous paragraphs, it was demonstrated that by scavenging NO and H_2O_2, or altering the GSH pool it was possible to modulate the transcription regulation of *GAPC-1* gene and the GAPC1-YFP accumulation. We then tested whether these molecules could reduce or prevent also the GAPC1-YFP nuclear accumulation. In order to do so, 7-day-old *pGAPC1::GAPC1-YFP Arabidopsis* transgenic seedlings were treated with cadmium, 100 µM cPTIO, 1 mM KI, 1 mM BSO, and 1 mM GSH for 48 hours, then subcellular localization of GAPC1-YFP was analysed (Fig. 18). By treating transgenic seedlings with cPTIO, KI, BSO, and GSH no differences respect to the control were observed as well as when seedlings treated with cadmium and cPTIO/GSH were compared to seedlings

treated only with cadmium. Instead, when seedlings treated with cadmium and KI were analyzed, a reduction of cell morphological changes and no nuclear accumulation of GAPC1-YFP were observed. Finally, when seedlings treated with cadmium and BSO were analyzed, it was observed that no morphological changes occurred, but, nuclear accumulation of GAPC1-YFP increased. The reason why root tips treated with cadmium and BSO did not undergo morphological changes probably was that combined treatments reduced significantly root viability.

The results shown here suggest some similarities with the model proposed by Hara et al. (2005). In fact, in accordance with their model, we observed the nuclear accumulation of the GAPC-1 protein when an oxidative stress was imposed. The hypothesis that the increased NO production could induce a nitrosylation of the catalytic cysteine of GAPC-1 leading to its nuclear accumulation seems therefore to be the case also in plant cells.

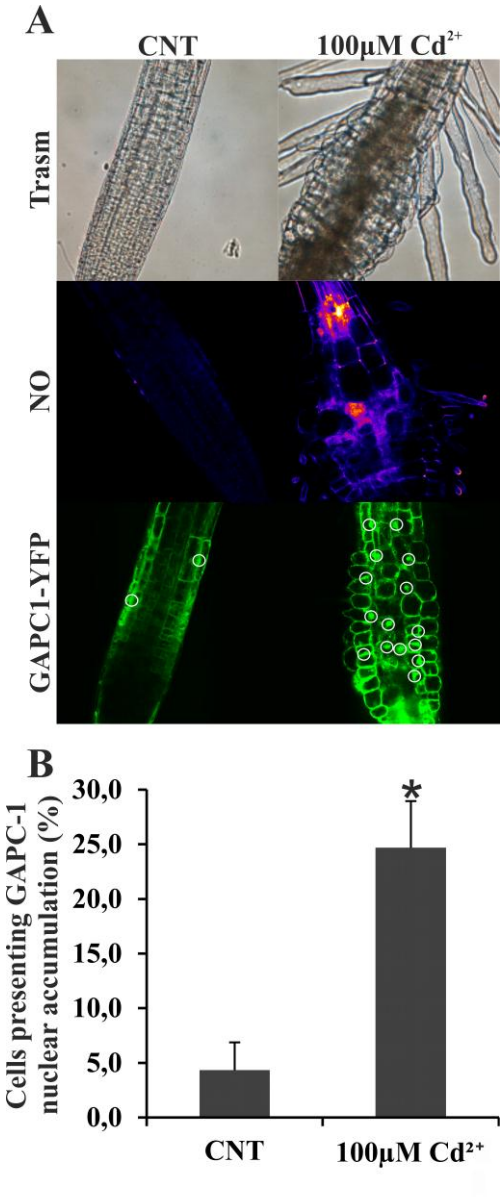

Figure 17: Subcellular localization of GAPC1-YFP chimeric protein in cadmium treated roots. (A) 7-day-old *Arabidopsis* wild-type and *pGAPC1::GAPC1-YFP* transgenic seedlings were treated with 100 µM $CdCl_2$ for 72 hours. In the first row, pictures of wild-type root tips observed by an optical microscope, in the second row, pictures of wild-type root tips stained with DAF-FM DA and observed by a confocal microscope, and in the third row, pictures of *pGAPC1::GAPC1-YFP* transgenic root tips observed by a confocal microscope. White circles indicate cells which present GAPC1-YFP nuclear accumulation. Pictures are representative of at least 18 roots from 3 independent experiments. (B) Percentage of cells presenting GAPC1-YFP nuclear accumulation, values reported in the graph represent mean ± SD of the percentage of cells presenting GAPC1-YFP nuclear accumulation. Data come from the analysis of 18 roots from 3 independent experiments asterisks indicate values that are significantly different from those found in untreated roots as calculated by Student's *t* test (* $p < 0.001$).

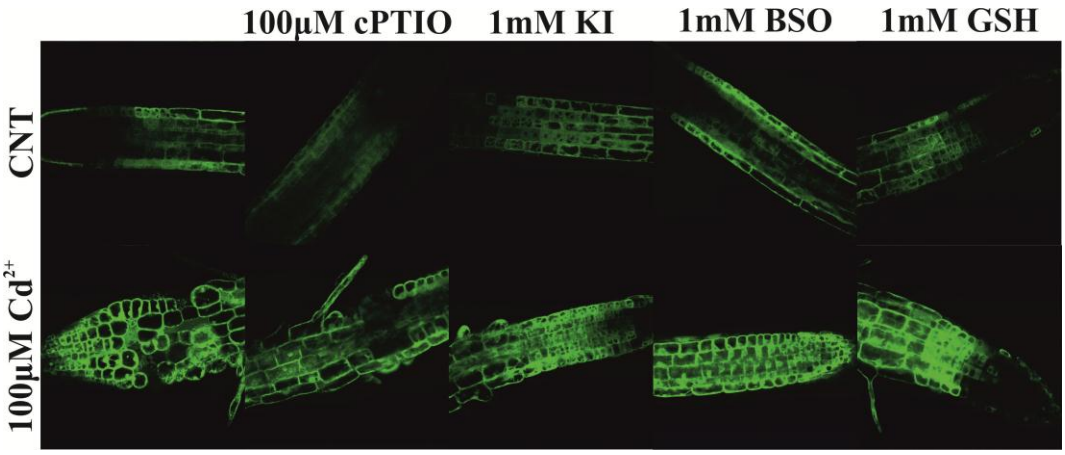

Figure 18: Effects of scavenging NO, H_2O_2 and altering glutathione pool on GAPC1-YFP subcellular localization. 7-day-old *Arabidopsis pGAPC1::GAPC1-YFP* transgenic seedlings were treated with 100 µM $CdCl_2$, 100 µM CPTIO, 1 mM KI, 1 mM BSO, 1 mM GSH, 100 µM $CdCl_2$ + 100 µM CPTIO, 100 µM $CdCl_2$ + 1 mM KI, 100 µM $CdCl_2$ + 1 mM BSO and 100 µM $CdCl_2$ + 1 mM GSH for 48 hours. Then, root tips were observed by a confocal microscope. Pictures are representative of at least 18 roots from 3 independent experiments.

Effects of scavenging NO and H_2O_2, and of altering the glutathione pool on root morphology and viability

In order to understand whether morphological changes induced by cadmium could be induced by the accumulation of NO and H_2O_2 and/or alteration of GSH pool, we treated 7-day-olds wild-type *Arabidopsis* seedlings as described in the previous paragraphs (Fig. 19). The viability of control and 3-day-treated roots was analyzed by Evan's blue staining, a dead cell specific dye. cPTIO, KI, and BSO treated roots did not reveal any morphological changes when compared with untreated roots. Instead the GSH treatment induced the formation of several root hairs along the elongation zone of roots. Moreover, the Evan's blue staining of cPTIO, KI, BSO, and GSH treated roots revealed that these molecules did not affect cell viability. As previously described, cadmium treatment induced strong morphological changes inducing the enlargement of root tip cells and the

formations of root hairs along the differentiation and elongation zone of root tips. Moreover, cadmium treatment caused the death of some cells localized in the differentiation zone. The addition of cPTIO to cadmium treatment did not prevent the effect of cadmium since both morphological changes and death cells were still present. Instead, the addition of KI to cadmium treatment avoided the elongation of root hairs, and partially inhibited the enlargement of root tip cells. By analyzing the cell viability, it was observed that KI prevented the death of cells localized in the differentiation zone, but, death cells were still present in the elongation zone.

Altering the GSH pool of cadmium treated roots caused more severe effects. In fact, the addition of BSO dramatically affected the viability of the entire root tip, and probably due to this high mortality, no morphological changes occurred. While, the addition of GSH completely prevented the enlargement of root tip cells and cell death.

These results suggest that the main actor in triggering the development of root hairs and the enlargement of root tip cells could be H_2O_2, because the KI-mediated scavenging of H_2O_2 reduced both processes. However, the scavenging of H_2O_2 did not prevent cell death, while, increasing the GSH pool completely prevented cell death.

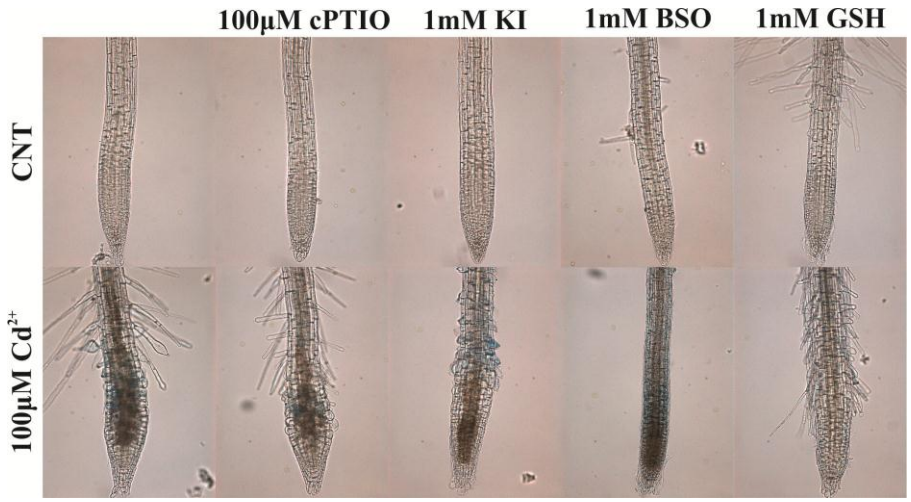

Figure 19: Effects of scavenging NO, H_2O_2 and altering glutathione pool on root tip morphology and viability. 7-day-old *Arabidopsis* wild-type seedlings were treated with 100 µM $CdCl_2$, 100 µM CPTIO, 1 mM KI, 1 mM BSO, 1 mM GSH, 100 µM $CdCl_2$ + 100 µM CPTIO, 100 µM $CdCl_2$ + 1 mM KI, 100 µM $CdCl_2$ + 1 mM BSO and 100 µM $CdCl_2$ + 1 mM GSH for 72 hours, and then stained with Evan's blue. Roots were observed by an optical microscope. Pictures are representative of at least 18 roots from 3 independent experiments.

Involvement of specific GAPC-1 residues in oxidative stress perception and signalling

Hara and co-workers (2005) described specific residues in the rat GAPDH were responsible to its nuclear relocalization occurring upon oxidative stress. In particular, they showed that the nitrosylation of the catalytic Cys 150 was required for the GAPDH interaction with the nuclear carrier ubiquitin ligase Siah 1. Moreover, the Lys 225 was also required for the proper GAPDH-Siah-1 binding. Wawer and co-workers (2010) observed that introducing tobacco NtGAPCa or NtGAPCb mutated in the cysteines of the catalytic domain in *Arabidopsis* protoplasts prevented the nuclear relocalization of the enzymes after stress induced by 250 mM NaCl treatment.

In order to study the role of the homologous aminoacid residues in the *Arabidopsis* GAPC-1, four different point mutations of the *pGAPC1::GAPC1-YFP* expression cassette were generated. Specifically: i) in the first cassette the catalytic Cys 155 was mutated into a serine (C155S); ii) in the second one the Cys 159 was replaced with a serine (C159S); iii) in the third construct both C155S and C159S mutations were introduced and finally iv) in the last construct the Lys 230, homologous to the animal GAPDH-Siah-1 binding domain, was mutated into an alanine (K230A). In order to test the different generated constructs, we transiently transformed *Arabidopsis* protoplasts and performed subcellular localization analyses by means of CLSM. The results presented in Figure 20 show that 16 hours after transformation, in all cases the GAPC-1s were localized only in the cytoplasm without showing any nuclear accumulation. In order to test the effect of the oxidative cadmium induced oxidative stress on the relocalization of the different mutated GAPC-1 isoforms, we then introduced these constructs in *Arabidopsis* wild-type plants by the generation of stable transgenic lines. The analyses of the mutated GAPC-1 subcellular localization in control conditions did not reveal any differences (data not shown). When we treated all the lines with cadmium, we were still able to see the GAPC-1 induction, in terms of expression level, and the typical nuclear relocalization observed with the GAPC-1-YFP (data not shown). These evidences do not confirm previous results, in which mutations in the two cysteines prevented the nuclear accumulation of the NtGAPCa and NtGAPCb (Wawer et al., 2010). The explanation for these different results could simply depend on the fact that the GAPCs studied by Wawer and co-workers where from tobacco and not from *Arabidopsis*. In our case indeed we introduced in *Arabidopsis* wild-type plants the different mutated versions of the *Arabidopsis*

GAPC-1. This strategy could be potentially problematic due to the presence of the endogenous *Arabidopsis* GAPCs. In fact, cytosolic GAPDHs are usually present as homotetramers (Fermani et al., 2007), but, in transgenic plants expressing mutated GAPC-1, the formation of heterotetramers hosting both endogenous and artificially introduced GAPCs could occur. In order to overcome this problem, we crossed plants expressing the chimeric proteins (GAPC1-YFP and GAPC1-C155S-YFP) with the *gapc-1* mutant and we selected F2 lines where no wild type GAPC-1 was present *(GAPC1::GAPC1-YFP-gapc-1-/-; GAPC1::GAPC1-C155S-YFP-gapc-1-/-*). With the obtained transgenic-mutant lines we studied the effect of cadmium treatment, and we evaluated the number of cells showing the chimeric GAPC-1 nuclear accumulation (Fig. 21). The most evident results are that 3-day cadmium treatment induced both the GAPC-1 expression and the GAPC-1 nuclear accumulation among all the transgenic line here analyzed. In particular, we observed that the chimeric proteins were accumulated into the nucleus at the highest extent in mutant genetic background. Moreover, it is worth to underline that the biggest increase in GAPC-1 nuclear accumulation, in terms of number of stained nuclei, was detected in the *GAPC1::GAPC1-C155S-YFP-gapc-1-/-* line where the percentage of cells showing GAPC-1 nuclear accumulation reached up the 49%. In the same transgenic-mutant line, even under control conditions, a bigger percentage of cells showing GAPC-1 nuclear accumulation was observed. This suggests that the catalytic cysteine does not seem to be essential for *Arabidopsis* GAPC-1 nuclear relocalization, on the contrary, the lack of this residue seems even to enhance its nuclear accumulation. All together these results demonstrate that the study of the role of specific GAPC-1 residues is quite difficult in a wild-type background leading to erroneous

conclusions. In fact, chimeric mutated proteins introduced in wild-type background did not show different localization pattern when compared to normal chimeric protein. Indeed, the formation of heterotetramers with the endogenous GAPC-1 could drive the complex into the right subcellular domain. This hypothesis is confirmed by the analyses of percentage of cells showing GAPC-1 nuclear accumulation, by which we observed that treating *GAPC1::GAPC1-YFP-gapc-1-/-* and *GAPC1::GAPC1-C155S-YFP-gapc-1-/-* plants led to a major increase of nucleated cells than in *GAPC1::GAPC1-YFP* and *GAPC1::GAPC1-C155S-YFP* transgenic plants.

Further analyses will be necessary to confirm the results obtained. Moreover, it will be also interesting to better investigate the role of Cys 159 and Lys 230.

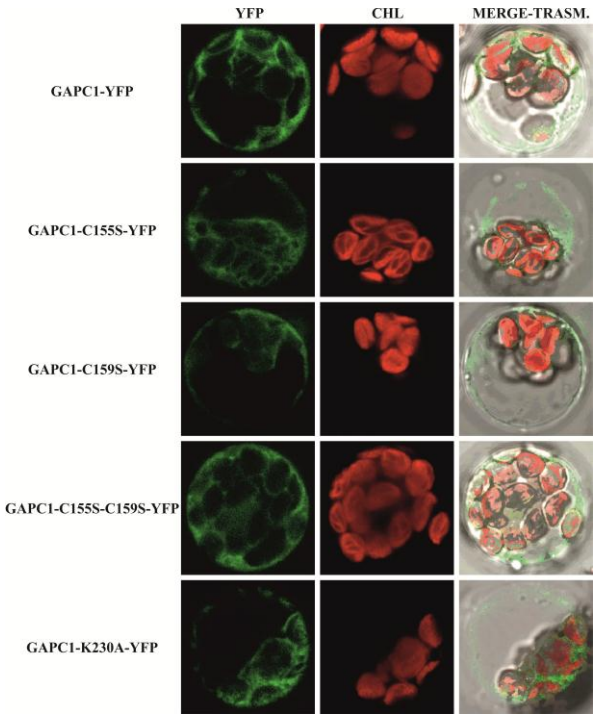

Figure 20: Transient expression of mutated GAPC1-YFP chimeric proteins in *Arabidopsis thaliana* protoplasts. Isolated protoplasts were transformed with 1 µg of plasmids containing the following expression cassettes: *GAPC1::GAPC1-YFP*, *GAPC1::GAPC1-C155S-YFP*, *GAPC1::GAPC1-C159S-YFP*, *GAPC1::GAPC1-C155S-C159S-YFP* and *GAPC1::GAPC1-K230A-YFP*. Then, the protoplast were incubated in the dark for at least 16 hours, and observed at the confocal microscope.

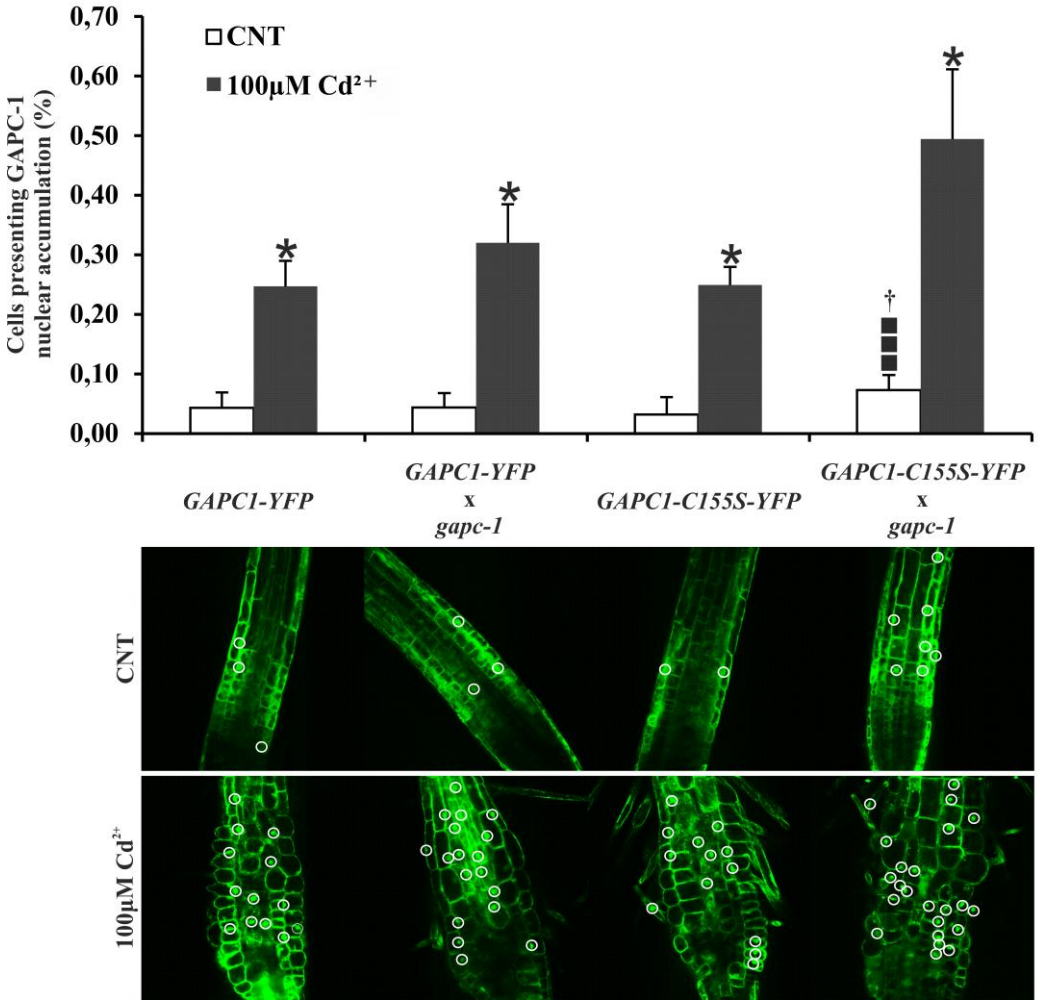

Figure 21: Mutation of the catalytic Cys 155 affects the subcellular localization of GAPC1-YFP chimeric protein. 7-day-old *Arabidopsis pGAPC1::GAPC1-YFP, pGAPC1::GAPC1-YFP* X *gapc-1, pGAPC1::GAPC1C-155-YFP* and *pGAPC1::GAPC1-C155S-YFP* X *gapc-1* transgenic seedlings were treated with 100 µM $CdCl_2$ for 72 hours, then, root tips were observed by a confocal microscope. White circles indicate cell which present GAPC1-YFP nuclear accumulation. Pictures are representative of at least 18 roots from 3 independent experiments. In the graph are reported the percentages of cells presenting GAPC1-YFP nuclear accumulation, values represent mean ± SD of the percentages of cells presenting GAPC1-YFP nuclear accumulation. Data come from the analysis of 18 roots from 3 independent experiments, asterisks indicate values that are significantly different from those found in untreated roots as calculated by Student's *t* test (* $p < 0.001$). Squares indicate values that are significantly different from those found in the *pGAPC1::GAPC1-YFP* X *gapc-1* transgenic line as calculated by Student's *t* test (■■■ $p < 0.05$). Cross indicates values that are significantly different from those found in *pGAPC1::GAPC1C-155-YFP* transgenic line as calculated by Student's *t* test († < 0.001).

Conclusions and future perspectives

In this report we have investigated the role of the *Arabidopsis* cytosolic enzyme GAPCs in oxidative stress signalling. GAPC-1 and GAPC-2 are enzymes involved in glycolytic pathway, however, in previous studies the involvement of these enzymes in other pathways has already been demonstrated (Yang et al., 1993; Hancock et al., 2005; Baek et al., 2008; Holtgrefe et al., 2008; Rius et al., 2008; Wawer et al., 2010). In order to study the roles of these two enzymes in *Arabidopsis* plants, the knock out homozygous mutants for both *GAPC-1* and *GAPC-2* genes were isolated and characterized. Our analyses revealed that GAPC enzymes had a redundant catalytic function, and the presence of only one GAPC enzyme was necessary and sufficient to develop vital plants. The analysis of wild-type and mutant seedling phenotypes showed that the disruption of *GAPC* genes affected the root length, in particular *gapc-1* and *gapc-2* lines had longer and shorter roots, respectively, when compared with wild-type. We also demonstrated that the increase and decrease of root length correlated with the increase and decrease, respectively, of catalase activity, suggesting an involvement of GAPCs in the control of H_2O_2 levels as already demonstrated by Hancock et al. (2005) and Baek et al. (2008). Then, in order to study the effects of cadmium-induced oxidative stress on GAPC enzyme, we exposed wild-type *Arabidopsis* seedlings to 100 µM $CdCl_2$, and the NO and H_2O_2 levels were monitored in root tips. The analyses revealed 24 hours after treatment an accumulation of both NO and H_2O_2, and this increase probably contributed to modulate the root tip redox potential turning it into a more oxidized condition, as assessed by analyses performed with roGFP2 *Arabidopsis* transgenic line. At the same time, we observed the up-regulation of both *GAPC-1* and *GAPC-2* transcripts in wild-type *Arabidopsis*

seedlings exposed to these oxidative stress conditions, showing *GAPC-1* affected at higher extent. The last result suggested that *GAPC-1* gene was more sensitive to oxidative stress, thus, the studies were focused on this isoform, and to do so we generated *Arabidopsis* transgenic lines expressing the β-glucuronidase transcriptional reporter and the GAPC1-YFP translational reporter. These transgenic lines were used to study the effects of oxidative stress on *GAPC-1* expression and post-transcriptional regulation. The analyses revealed that oxidative stress affected *GAPC-1* expression and GAPC-1 level, turnover or activity. In particular, the treatment caused an increase of the promoter activity and a dramatic accumulation of the GAPC-1 enzyme without affecting the GAPDH activity. Since the protein accumulation occurred in the same root cells where both NO and H_2O_2 levels increased, we investigated the effect of these two molecules on the GAPC-1 activity. Indeed, we demonstrated that, the treatment of a recombinant GAPC-1 produced in *E.coli* with a NO-donor and H_2O_2 led to its inactivation. These evidences suggested that the oxidative stress condition induced by the accumulation of NO and H_2O_2 could affect the catalytic activity of the GAPC-1 enzyme, activating a signal cascade that enhances its expression and then its accumulation. This hypothesis was confirmed by the fact that the natural reducing agent GSH restored the catalytic activity of NO-inactivated recombinant GAPC-1. Furthermore, GSH treatment also reduced GAPC-1 expression and accumulation *in vivo*, while decreasing glutathione biosynthesis, by BSO treatment, led to the increase of GAPC-1 expression and accumulation.

Besides the accumulation of GAPC-1 enzyme after oxidative stress exposure, we also observed that in the cells of the root differentiation zone, the enzyme strongly accumulated into the nucleus in the same place where NO level

increased. This result was in agreement with the results already described by Hara et al. (2005), suggesting that NO could also play a crucial role in GAPC-1 nuclear relocalization in plant cells. The previous identification by Hara and co-workers (2005) of key residues of the GAPDH involved in its nuclear relocalization such as Cys 150 and Lys 225 led us to test the role of the homologous aminoacid residues in the *Arabidopsis* GAPC-1. Transgenic lines, expressing GAPC1-YFP proteins mutated in that homologous aminoacid residues were therefore produced, and the subcellular localization of mutated chimeras were analyzed. The introduction of mutated chimeras in wild-type background did not affect their nuclear relocalization neither in control nor under stress conditions. Instead, the introduction of the mutated chimeric protein GAPC1-C155S-YFP in the *gapc-1* mutant background led to a different nuclear accumulation pattern both in control and treated conditions. In fact, in control conditions, and after cadmium treatment we observed a strong increase of cells with the protein accumulated into the nucleus. These results suggest that the lack of this residue seems to enhance its nuclear localization, meaning that in *Arabidopsis* plants the inactivation of the enzyme is a sufficient condition to drive it to the nucleus.

In this work, we have demonstrated that GAPC-1 enzyme, besides its glycolytic function is involved in oxidative stress sensing, and under this stress condition it is relocalized into the nucleus where its role is still unknown. The understanding of the mechanisms involved in GAPC-1 nuclear relocalization, the role played by the enzyme in the nucleus, along with the study of the role played by the Cys 155, Cys 159 and Lys 230 in GAPC-1 post-translational modification, nuclear carrier interaction and nuclear relocalization will be investigated in the future in more detail.

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CONCLUSIONS

In this PhD thesis, the study of two stress signalling mechanisms has been approached. In particular, I have investigated the role of *Arabidopsis* CK receptors (AHK2, AHK3, and AHK4/CRE1), and *Arabidopsis* cytosolic glyceraldehyde-3-phosphate dehydrogenases (GAPC-1) in stress signalling pathways activated by high concentrations of CKs and by oxidative stress conditions, respectively.

Considering the research activities focused on CK receptors, the reported results clearly demonstrate their involvement in triggering the PCD signalling in *Arabidopsis thaliana* cell cultures treated with high concentrations of CKs. In particular, it has been demonstrated that CRE1/AHK4 is specifically involved in mediating this PCD signal, since it is sufficient to abolish its expression to make *Arabidopsis* cell cultures insensitive to high concentrations of CKs. Besides this important result, by analysing cell growth parameters of different CK receptor mutants, it has also been demonstrated that CK receptors are not involved in the control of cell cycle progression in *Arabidopsis* cell cultures.

Considering the study carried out on GAPC-1 enzyme, the reported results shed more light on the *in vivo* functions of GAPC-1 in *Arabidopsis* plants. It has been demonstrated that, besides its role in glycolysis, it is also involved in oxidative stress sensing. In particular, it has been observed that under oxidative stress conditions, its transcription is up-regulated, and its steady state level increases without affecting the total GAPDH catalytic activity of the cell. Moreover, it has been demonstrated that under continuative oxidative stress

conditions GAPC-1 is translocated into the nucleus, where its role is still unclear. Altogether these results reported suggest for GAPC-1 a role in sensing and triggering the oxidative stress signal in *Arabidopsis* plants.

UNIVERSITÀ DEGLI STUDI DI PADOVA

SCUOLA DI DOTTORATO IN BIOSCIENZE E BIOTECNOLOGIE INDIRIZZO DI BIOTECNOLOGIE

Estratto del verbale del Collegio

Scuola di Dottorato in Bioscienze e Biotecnologie

Indirizzo di BIOTECNOLOGIE

Seduta del 7 Dicembre 2011

Alle ore 14.30 del giorno 7 Dicembre 2011 nell'aula seminari del 5° piano sud del Dipartimento di Biologia, si è riunito il Collegio dei Docenti dell'indirizzo di Biotecnologie della Scuola di Dottorato in Bioscienze e Biotecnologie.

Risultano presenti:

Nome	*Presenze*	*Assenze giustificate*	*Assenze*
Mariano Beltramini	X		
Pietro Benedetti	X		
Elisabetta Bergantino	X		
Luigi Bubacco	X		
Francesco Filippini	X		
Angelo Fontana	X		
Fiorella Lo Schiavo	X		
Stefano Mammi		X	
Emanuele Papini	X		
Patrizia Polverino De Laureto	X		
Andrea Squartini		X	
Giorgio Valle	X		
Paola Venier	X		
Giuseppe Zanotti	X		
Chiara Fecchio (*Rappresentante dei dottorandi*)	X		

Il Coordinatore dell'Indirizzo, Prof. Giorgio Valle, riconosce valida la seduta e la dichiara aperta per trattare, come dall'avviso di convocazione, il seguente ordine del giorno:

1. Comunicazioni.
2. Approvazione del verbale precedente.
3. Riorganizzazione della scuola di dottorato.
4. Ammissione dei dottorandi del 1° e 2° anno all'anno successivo.
5. Ammissione dei dottorandi del 3° anno all'esame finale.
6. Pratiche studenti.

...omissis...

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SCUOLA DI DOTTORATO
IN BIOSCIENZE E BIOTECNOLOGIE
INDIRIZZO DI BIOTECNOLOGIE

Oggetto 5: Ammissione dei dottorandi del 3° anno all'esame finale

Due dottorandi del terzo anno hanno chiesto di posticipare l'esame finale: Albiero per motivi di ricerca ha chiesto il rinvio di un anno, già approvato dal Collegio nella riunione del 25 Novembre scorso; Moret ha chiesto il rinvio per maternità.

I restanti dottorandi sono Albania, Damiano, Pivato, Quarantini, Reghelin, Rodio, Rosani, Rosselli, Shaik, Telatin, Vescovi, Zamperin, Zatti. Tutti hanno recentemente presentato i loro risultati al Collegio Docenti, sia mediante esposizione, sia con una relazione scritta. Il giudizio del Collegio Docenti è unanime nell'ammettere tutti all'esame finale.

Il Collegio constata tuttavia che le presentazioni di alcuni dottorandi potrebbero essere meglio valorizzate; pertanto sarà chiesto ad alcuni dottorandi di migliorare il modo con cui esporre i risultati ottenuti.

...omissis...

Non essendoci altri punti da discutere, il verbale viene approvato seduta stante e la seduta del Collegio si conclude alle ore 13,45.

Prof. Giorgio Valle
Coordinatore dell'Indirizzo

Giudizio sull'attività del dottorando Marco Vescovi

Marco Vescovi ha svolto il suo dottorato di ricerca nel mio laboratorio conducendo degli studi sulla caratterizzazione delle vie di signalling indotte nelle piante da stress. In particolare, ha indagato il ruolo di recettori noti delle citochinine nel processo di morte programmata cellulare indotta in colture cellulari da alte concentrazioni di questo ormone e il ruolo della GAPC in piante esposte a stress ossidativo.

Nel corso del suo dottorato, Marco ha avuto l'opportunità di divenire competente in aree della biochimica e di biologia cellulare e molecolare delle piante. Marco ha mostrato nel corso dei tre anni una grande serietà nel lavoro e capacità di apprendere e divenire esperto di innumerevoli tecniche. Per quanto riguarda il suo progetto sulle citochinine, si è recato per alcuni mesi a Berlino nel laboratorio di Thomas Schmulling, esperto internazionale negli studi su questi ormoni vegetali. Il giudizio sul lavoro di Marco da parte del prof. Schmulling è stato eccellente. Inoltre tale periodo svolto da Marco a Berlino ha offerto la possibilità al nostro laboratorio di stabilire relazioni di collaborazioni stabili con questo laboratorio. Per quando riguarda il lavoro sul ruolo della GAPC nello stress ossidativo, Marco ha lavorato in stretta collaborazione con il laboratorio del prof. Paolo Trost a Bologna, un esperto internazionale nel campo dell'enzimologia e biochimica vegetale. Anche in questo caso Marco è riuscito ad ampliare le sue competenze e far fiorire nuove collaborazioni tra i nostri lavoratori.

Alla fine di questo dottorato, Marco mostra il profilo di un giovane ricercatore che ha maturità scientifica nel condurre le sue ricerche in maniera autonoma e competente e capacità a condurre programmi scientifici in diverse aree della moderna biologia delle piante.

Pertanto il mio giudizio sul candidato è ottimo.

In fede,

Fiorella Lo Schiavo

Prof. Fiorella Lo schiavo

Padova, 13 Dicembre 2011

SCIENTIFIC PUBLICATIONS

Vescovi M, Riefler M, Gessuti M, Novák O, Schmülling T, Lo Schiavo F. (2012). Programmed cell death induced by high levels of cytokinin in *Arabidopsis* cultured cells is mediated by the cytokinin receptor CRE1/AHK4. *Journal of Experimental Botany.*
DOI: 10.1093/jxb/ers008

Bastianelli F, Costa A, **Vescovi M**, D'Apuzzo E, Zottini M, Chiurazzi M, Lo Schiavo F. (2009). Salicylic acid differentially affects suspension cell cultures of Lotus japonicus and one of its non-symbiotic mutants. *Plant Molecular Biology*
DOI: 10.1007/s11103-009-9585-8

POSTER

Marco Vescovi, Alex Costa, Mirko Zaffagnini, Paolo Trost, Fiorella Lo Schiavo (2010). *Arabidopsis thaliana* glyceraldehyde-3-phosphate dehydrogenase as an oxidative stress sensor.
14th International Biotechnology Symposium and Exhibition.
Rimini, Italy, 14th – 18th September 2010.

MEETINGS

2011 *Joint Meeting AGI – SIBV – SIGA 2011*
Assisi, Italy, 19th – 22th september 2011. **Oral presentation.**

2010 *14th International Biotechnology Symposium and Exhibition.*
Rimini, Italy, 14th – 18th September 2010.

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Verona, Italy, 30th June – 2nd July 2009.

Plant Mol Biol
DOI 10.1007/s11103-009-9585-8

Salicylic acid differentially affects suspension cell cultures of *Lotus japonicus* and one of its non-symbiotic mutants

Fiorenza Bastianelli · Alex Costa · Marco Vescovi · Enrica D'Apuzzo · Michela Zottini · Maurizio Chiurazzi · Fiorella Lo Schiavo

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Abstract Salicylic acid (SA) is known to play an important role in the interaction between plant and micro-organisms, both symbiotic and pathogen. In particular, high levels of SA block nodule formation and mycorrhizal colonization in plants. A mutant of *Lotus japonicus*, named *Ljsym4-2*, was characterized as unable to establish positive interactions with *Rhizobium* and fungi (NOD^-, MYC^-); in particular, it does not recognize signal molecules released by symbiotic micro-organisms so that eventually, epidermal cells undergo PCD at the contact area. We performed a detailed characterization of wild-type and *Ljsym4-2* cultured cells by taking into account several parameters characterizing cell responses to SA, a molecule strongly involved in defense signaling pathways. In the presence of 0.5 mM SA, *Ljsym4-2* suspension-cultured cells reduce their growth and eventually die, whereas in order to induce the same effects in wt suspension cells, SA concentration must be raised to 1.5 mM. An early and short production of nitric oxide (NO) and reactive oxygen species (ROS) was detected in wt-treated cells. In contrast, a continuous production of NO and a double-peak ROS response, similar to that reported after a pathogenic attack, was observed in the mutant *Ljsym4-2* cells. At the molecular level, a constitutive higher level of a SA-inducible pathogenesis related gene was observed. The analysis in planta revealed a strong induction of the *LjPR1* gene in the *Ljsym4-2* mutant inoculated with *Mesorhizobium loti*.

Keywords *Lotus japonicus* cell cultures · Salicylic acid · Cell death · H_2O_2 · NO

Fiorenza Bastianelli and Alex Costa are contributed equally to this work.

This report is dedicated to the memory of Prof. M. Terzi.

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Introduction

Plant-interacting microbes differ with respect to the nature of the response that they elicit in their respective hosts. In an incompatible plant-pathogen interaction, the host plant induces a defense response—be it the hypersensitive response (HR), systemic acquired resistance or both—that limits pathogen invasion and spreading. However, in the case of symbiotic bacteria, such as those of the genus *Rhizobium*, an obvious defense response is usually not elicited. Instead, a beneficial relationship is established that results in nodule formation and atmospheric nitrogen fixation.

An emerging picture from recent studies indicates that legumes utilize similar mechanisms to recognize pathogens and symbiotic microbes (Miya et al. 2007; Wan et al. 2008). Both *rhizobia* and successful pathogens suppress plant defenses when establishing an infection. Plant defense-like phenotypes are induced by legumes or by bacterial mutants unable to carry out an efficient nodulation program (Carlson et al. 1987; Campbell et al. 2002; Veershlingam et al. 2004). In the same way, normally, after initial nodule formation, the host inhibits the progress of

[P-P&F.43]

Arabidopsis thaliana Glyceraldehyde-3-Phosphate Dehydrogenase As An Oxidative Stress Sensor

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[1] *Padova University, Italy*
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Keywords: *Arabidopsis thaliana*; GAPDH; Cadmium; Oxidative stress

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a well known enzyme mainly involved in the glycolytic process. In mammalian cells, the GAPDH has been demonstrated also to play a role in the induction of apoptotic events. In particular, stimuli inducing oxidative stress have shown to induce the nitrosylation of the GAPDH catalytic cysteine, leading to the enzyme inactivation and its relocalization into the nucleus, where it participates in the induction of apoptotic processes. In plants, both cytoplasmic and chloroplast GAPDHs isoforms have been described, but up to now there are no evidence of their involvement in the induction of plant cell death events, even if also in this case the nitrosylation of cysteine has been reported.

Cd^{2+} is a common environmental pollutant able to induce oxidative stress in plant cells with production of both reactive oxygen species and nitric oxide, leading to the induction of a senescence-like program in leaves and in cell cultures. In order to investigate the possible involvement of plant GAPDHs in the Cd^{2+}-induced plant cell death, we focused on the *Arabidopsis* GAPC-1, one the cytosolic GAPDH isoforms. *Arabidopsis* transgenic plants, transformed with the *GUS* reporter gene and the chimeric *GAPC1:YFP* construct, both under the control of the *GAPC-1* promoter, have been generated and exposed to Cd^{2+} stress. Preliminary analyses showed that seedlings treated with 100 μM Cd^{2+}, enhanced the *GAPC-1* transcription and GAPC-1:YFP chimeric protein accumulation when compared with control seedlings. Moreover, a nuclear GAPc1 relocalization was observed in root cells. We have also isolated homozygous *gapc-1* and *gapc-2* null mutants in order to study the effect of Cd^{2+} treatment in these genetic backgrounds.

The understanding of the pathway/s leading to plant cell death induced by Cd^{2+} could have important biotechnology applications because the increasing tolerance of plants to abiotic stress could help in developing new phytoremediation strategies.

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Oral Communication Abstract – 2B.06

ARABIDOPSIS THALIANA GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AS AN OXIDATIVE STRESS SENSOR

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GAPC, cadmium, oxidative stress, Arabidopsis thaliana

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a well known enzyme mainly involved in the glycolytic process. In mammalian cells, the GAPDH has been demonstrated also to play a role in the induction of apoptotic events. In particular, stimuli inducing oxidative stress have shown to induce nitrosylation of the GAPDH catalytic cysteine, leading to the enzyme inactivation and its relocalization into the nucleus, where it participates in the induction of apoptotic processes. In plants, both cytoplasmic and chloroplast GAPDH isoforms have been described, but up to now there are no evidences of their involvement in the induction of plant cell death events, even if nitrosylation of the same cysteine has been reported.

Cd^{2+} is a common environmental pollutant able to induce oxidative stress in plant cells with production of both reactive oxygen species and nitric oxide, leading to the induction of a senescence-like programme in cell cultures.

In order to investigate the possible involvement of plant GAPDHs in the Cd^{2+}-induced oxidative stress sensing, we focused on the *Arabidopsis* GAPC-1, one the two cytosolic GAPDH isoforms.

By performing *in vitro* analyses, using recombinant GAPC-1, we observed a reversible enzyme inactivation mediated by H_2O_2 and NO administration. The exposure of *Arabidopsis* seedlings to Cd^{2+} led to an accumulation of H_2O_2 and NO in roots where also an enhanced *GAPC-1* transcription and GAPC-1-YFP chimeric protein was detected, followed by its nuclear relocalization. In the *gapc-2* null mutant, where only the GAPC-1 enzyme is present, the Cd^{2+} stress determined an increase of GAPDH activity. Scavenging of H_2O_2 and NO in Cd^{2+} treated seedlings prevented the GAPC-1 accumulation.

Together these results support the hypothesis that the regulation of expression and activity of GAPC-1, in response to Cd^{2+}-induced oxidative stress, is mediated by the levels of H_2O_2 and NO in the cell that are directly sensed by the GAPC-1 enzyme.

We therefore propose that the GAPC-1 can be considered as an oxidative stress sensor.