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Proteomic changes and molecular effects associated with Cr(III) and Cr(VI) treatments on germinating kiwifruit pollen

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ABSTRACT

The present study is aimed at identifying molecular changes elicited by Cr(III) and Cr(VI) on germinating kiwifruit pollen. To address this question, comparative proteomic and DNA laddering analyses were performed. While no genotoxic effect was detected, a number of proteins whose accumulation levels were altered by treatments were identified. In particular, the upregulation of some proteins involved in the scavenging response, cell redox homeostasis and lipid synthesis could be interpreted as an oxidative stress response induced by Cr treatment. The strong reduction of two proteins involved in mitochondrial oxidative phosphorylation and a decline in ATP levels were also observed. The decrease of pollen energy availability could be one of the causes of the severe inhibition of the pollen germination observed upon exposure to both Cr(III) and Cr(VI). Finally, proteomic and biochemical data indicate proteasome impairment: the consequential accumulation of misfolded/damaged proteins could be an important molecular mechanism of Cr(III) toxicity in pollen.

1. Introduction

Heavy metals are major industrial pollutants leading to phytotoxicity. Chromium is a heavy metal whose valency state ranges from −2 to +6. Trivalent [Cr(III)] and hexavalent [Cr(VI)] forms are of major environmental significance because of their stability in the natural environment where they are released in the form of liquid, solid and gaseous wastes (Kotas and Stasicka, 2000). Cr(III) prevails in effluents from tanneries and paint factories, whereas the sources of Cr(VI) include metallurgy, mining, wood preservation, cooling installation effluents and fossil fuel combustion. The metal is also present in the atmosphere in the form of particles and droplet aerosols which represent the major mechanism for long-range transfer of chromium (Nriagu et al., 1988). Chromium is taken up and accumulated by plant roots and aerial surfaces. As a consequence, it interferes with several metabolic and physiological processes. This causes alterations in seed germination as well as root, stem and leaf growth (Shanker et al., 2005; Scoccianti et al., 2006). Chromium was demonstrated to interfere with essential structures and functions of the male gametophyte (Speranza et al., 2007, 2009). In fact, both Cr(III) and Cr(VI) exerted a strong dose-dependent inhibitory effect on kiwifruit pollen germination and tube growth and produced profound alterations in pollen tube shape (Speranza et al., 2007). In particular, Cr(III) had dramatic effects on tube wall morphology and major polysaccharide assembly as indicated by the significant alteration of callose deposition and arabinogalactan protein distribution (Speranza et al., 2009). Contrary to many other systems (Cervantes et al., 2001; Han et al., 2004), Cr(III) was more toxic to kiwifruit pollen than Cr(VI), and complete germination inhibition was attained at much lower Cr(III) doses. Both the chromium species induced increases in lipid peroxide production, with a more pronounced effect upon Cr(VI) treatment (Scoccianti et al., 2008a). This result is consistent with the well-known Cr(VI) role as an oxidizing agent. Previously, it was reported that Cr(VI) reduction to Cr(III) generates the whole spectrum of ROS (Kotas and Stasicka, 2000). Both GSH and GSSG content increased in the presence of increasing chromium concentrations, but glutathione pool dynamics appeared to vary depending on chromium species (Scoccianti et al., 2008a). Finally, chromium exposure of kiwifruit pollen resulted in a marked increase of ubiquitin adducts and a

Abbreviations: 2-DE, two-dimensional electrophoresis; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; Hepes, [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]; IEF, isoelectrofocusing; ACN, acetonitril; DMF, dimethylformamide; CBB, Coomassie brilliant blue; LC–ESI-MS/MS, liquid chromatography–electro spray tandem mass spectrometry.

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parallel reduction of free ubiquitin content. This suggests a role for the ubiquitin–proteasome 26S proteolytic system in the chromium stress response (Scoccianti et al., 2008b). This proteolytic pathway plays an important role in eukaryotic cell growth and development, as well as plant stress response and environmental adaptation through the degradation of short-lived and abnormal proteins (Belknap and Garbarino, 1996; Callis and Vierstra, 2000; Vierstra, 2003).

Heavy metals such as Pb^{2+}, Ga^{3+}, and Cu^{2+} enhanced proteasomal activity in human SH-SY5Y cells (Grunberg-Etkovitz et al., 2009). Furthermore, a proteasome alpha (α)-subunit was found to be over-expressed in the alga *Scytosiphon gracilis* (Phaeophyceae). This may help to remove damaged proteins resulting from copper-mediated oxidative stress (Contreras et al., 2010). Cadmium (Cd) increased the 20S proteasome activity in maize (Pena et al., 2007). By contrast, Cd impact caused an upregulation in the gene expression and proteolytic activity of the proteasome and various proteases in *Arabidopsis* (Polge et al., 2009).

In addition to metabolic changes, ultrastructural alterations including swelling and loss of mitochondrial cristae, cytoplasmic vacuolization, perturbed endoplasmic reticulum cisternae arrangement and chromatin condensation were also observed as early as 30 min after incubation in the presence of 50 μM Cr(III) and 150 μM Cr(VI) (Speranza et al., 2007). These features have been observed in plant cells, including pollen, undergoing programmed cell death (PCD) (Wuang and Cui, 1998; Coimbra et al., 2004; Geitmann et al., 2004; Varnier et al., 2005).

The heavy metal genotoxicity has been reported for various crops including blackgram, soybean, paddy, tobacco, potato and kiwifruit (Gichner et al., 2006; Sankar Ganesh et al., 2006; Chidambaram et al., 2009; Sundaramoorthy et al., 2010). At higher concentrations, both Cr(VI) and Cr(III) lead to DNA damage by inducing DNA fragmentation, single- and double-strand breaks, DNA–protein crosslinks and oxidative DNA base modifications (Burkhardt et al., 2001; Rudolf and Červinka, 2003; Xie et al., 2005; Chidambaram et al., 2006; Sundaramoorthy and Sankar Ganesh, 2007). These previous studies led us to investigate the pollen response to metal by verifying DNA fragmentation (laddering). This is one of the characteristic features of PCD that occurs in cells during normal development and in response to biotic and abiotic stresses such as pathogen attacks or toxic compounds (Varnier et al., 2005; Chidambaram et al., 2009; Serrano et al., 2010).

Abiotic stresses, including heavy metals, induce changes in plant protein expression (Cuypers et al., 2005; Amme et al., 2006; Ndimba et al., 2005). Proteomic changes in response to Cr(VI) toxicity have been recently reported in bacteria, in the freshwater green alga *Pseudokirchneriella subcapitata* and in the leaves of *Typha angustifolia* (Chourey et al., 2006; Kılıç et al., 2010; Vannini et al., 2009; Bah et al., 2010). However, up to now there is no comparative proteomic report on protein pattern changes during Cr(III) and Cr(VI) stress in plants cells. Proteomics of pollen development and germination have been extensively investigated (see for example Fernando, 2005; Sheoran et al., 2006; Dai et al., 2006, 2007; Zou et al., 2009), whereas information on heavy metal (in particular chromium)-responsive proteins in the male gametophyte is still missing. Therefore, in the present study we use a proteomic approach to identify specific changes in the protein pattern of kiwifruit pollen during Cr(III) and Cr(VI) exposure. The purpose was to obtain insights into the molecular mechanism of chromium toxicity to pollen.

2. Results and discussion

2.1. 2-DE analysis of chromium treated pollen

High resolution 2-DE was used to separate soluble proteins from kiwifruit pollen treated for 1 h with 30 μM Cr(III) or 90 μM Cr(VI). These experimental conditions were based on our previous data in this area (Speranza et al., 2007, 2009; Scoccianti et al., 2008a).

About 600 spots were highly resolved and detected by colloidal CBB staining, over a pH range of 4–7 and a size range of 10–100 kDa. All spots were matched by gel to gel comparisons and differences in the relative abundance (vol%) of each spot were analyzed. We focused our attention on spots whose abundance varied at least ±1.5-fold between the Cr-treated and non-treated pollen. Using this criterion, we selected 28 statistically confirmed protein variations between control and treated samples.

The differentially expressed proteins are marked on the representative 2-DE gel shown in Fig. 1. Excised spots were in-gel digested and analyzed by LC–ESI-MS/MS. In total, 18 proteins were successfully identified: in 10 cases it was not possible to determine the protein spot identity. Some representative images of spot changes as induced by the chromium-treatments are shown in Fig. 1. The predicted molecular masses and pIs for the majority of the identified proteins were generally consistent with the experimental data, as judged from the location of spots on 2-D gels; however, there were some exceptions. For example, spot 883 had an apparent molecular mass lower than the corresponding identified protein, whereas spots 1756, 883, 1430, 2556 and 980 had a molecular pI very different from the predicted value. These deviations in molecular mass and pI, as well as multiple spots for the same protein (551/554 and 1658/749) could be due to various factors, including alternative RNA splicing, post-translational modifications, and protein degradation. Ten proteins were up-regulated and eight down-regulated in chromium treated versus non-treated pollen. Seven protein spots exhibited significant changes under both Cr-treatments, while five and six proteins changed specifically only in the presence of Cr(III) or Cr(VI), respectively. The identified proteins were categorized into three functional groups based on predicted protein function.

2.2. Differentially expressed proteins were involved in stress response

Cr(VI) exposure led to the accumulation of three spots corresponding to a dehydroascorbate reductase (DHAR, spot 475) and a thioredoxin-dependent peroxidase (PRX, spots 551 and 554) (Table 1). DHAR catalyzes the reduction of DHA into ascorbic acid (AsA) using glutathione as the reductant. DHAR allows plants to recycle oxidized AsA. Increased DHAR activity was earlier reported in pea plants and green gram leaves exposed to Cr(VI) stress (Pandey et al., 2009; Shanker et al., 2004). The authors attributed the increased DHAR activity to a possible signal transduction mechanism operational due to increased ROS generation by Cr(VI). PRXs are known to exert a protective antioxidant role through their peroxidase activity. Proteomic analysis of maize seeds and roots and germinating rice seeds showed that PRX-type proteins were up-regulated by potassium dichromate, arsenic and copper treatment respectively (Labra et al., 2006; Requejo and Tena, 2005; Ahsan et al., 2007).

In Cr(III)-treated pollen, we also found the up-regulation of an electron transporter protein (spot 599) containing a classic thioredoxin domain with a redox active CXXC motif. Its function could be to alter the redox state of target proteins via the reversible oxidation of its dithiol active site.

The overexpression of all these proteins following Cr exposure is in agreement with the ROS production generated by Cr, causing oxidative damage to plants (Panda and Patra, 2000; Panda et al., 2003; Choudhury and Panda, 2005). The differential defensive response caused by Cr(VI) and Cr(III) could putatively be due to a higher ROS production by Cr(VI) (Stohs and Bagchi, 1995).

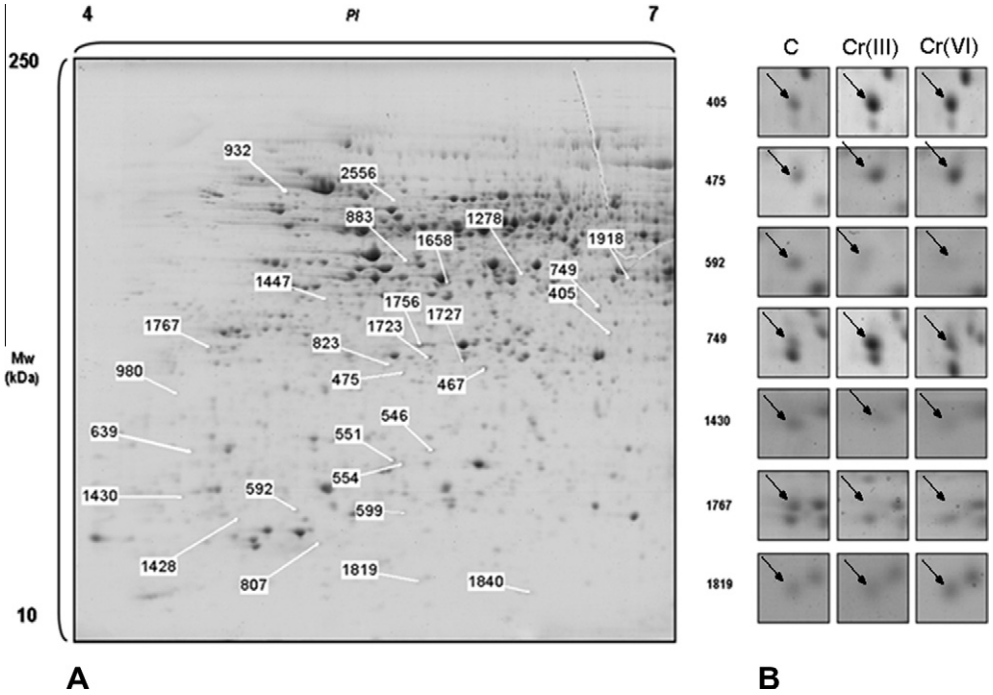

Fig. 1. Colloidal Coomassie brilliant blue-stained 2-DE map. (A) Image of a representative gel: Proteins showing significant ($p < 0.05$) changes in abundance are marked by their relative numbers. (B) Selected differentially accumulated protein spots in kiwifruit pollen cells exposed to Cr(III) and Cr(VI).

2.3. Differentially expressed proteins were involved in protein synthesis and fate

In eukaryotes, the ubiquitin/proteasome proteolytic system is responsible for the degradation of the bulk of the cellular proteins, including misfolded, oxidatively damaged and regulatory proteins (Smalle and Vierstra, 2004). In pollen the proteasome plays a pivotal role in regulating tube development and morphology (Speranza et al., 2001; Sheng et al., 2006). Indeed, proteasome inhibitors significantly decrease tube growth and alter tube structure. Interestingly, similar functional and structural alterations of pollen tubes have been described upon Cr treatment (Speranza et al., 2007), suggesting that Cr pollen toxicity may occur through an impairment of the proteasome function.

Proteomic data, obtained in this study, indicate that Cr causes alterations in the expression of two proteasome subunits: the alpha type subunit (spot 1723), belonging to the catalytic 20S core and the 26S non-ATPase regulatory subunit Rpn11 (spots 1658 and 749), belonging to 19S regulatory complex. Together, the 20S and 19S particles form the ubiquitin-dependent 26S proteasome (Kurepa and Smalle, 2008). We found that the peptidase activity of the 20S proteasome was significantly reduced in pollen exposed to Cr(III) but not to Cr(VI) (Fig. 2). It has been suggested that the accumulation of ubiquitin–protein conjugates under metal stress likely reflects a decrease in the proteasome activity rather than an increase in the ubiquitination process (Vierstra, 2003). If this is correct, and considering that 20S proteasome activity in kiwifruit pollen treated with Cr(VI) is unaffected (Fig. 2), the accumulation of ubiquitinated proteins in Cr(VI)-treated pollen, as previously reported (Scoccianti et al., 2008b), is quite unexpected. However, when looking at the proteomic data, a noticeable decrease in the 26S regulatory subunit Rpn11 (spot 1658) level occurs under Cr(VI) stress. The Rpn11 protein is a metalloprotease that deubiquitinates substrates bound to the proteasome, an essential step for their channeling into the catalytic particle (Yao and Cohen, 2002). Indeed, it has been demonstrated that mutation of Rpn11 active site is lethal and stabilizes ubiquitin pathway substrates in yeast, suggesting that failure to remove ubiquitin can be rate-limiting for degradation (Yao and Cohen, 2002; Verma et al., 2002). In addition, knockdown of Rpn11 in mammalian cells has been shown to determine a significant accumulation of polyubiquitinated proteins, to impair the degradation of synthetic ubiquitin adducts and to impede 26S proteasome assembly, but has little effect on 20S peptidase activity (Koulich et al., 2008).

Thus, in light of these findings, the downregulation of this subunit could explain the accumulation of ubiquitinated adducts in Cr(VI)-treated pollen. Interestingly, in addition to its function as deubiquitinating enzyme, Rpn11 plays a role in maintaining mitochondrial integrity and proper function (Rinaldi et al., 2004). The decrease of this subunit in Cr(VI)-treated pollen may contribute to more severe mitochondria alterations induced in the same experimental system by Cr(VI) with respect to Cr(III) (Speranza et al., 2007).

With respect to Cr(VI), the effects of Cr(III) on the ubiquitin pathway appear different. Previous evidence, obtained in sunflower leaves, has demonstrated that Cr(III) and other metals strongly inhibit 20S proteasome activity without a decrease in the proteasome protein abundance (Pena et al., 2008). Although, a direct effect of heavy metals, including Cr(III), on proteasome function cannot be excluded (Pena et al., 2008), our data demonstrate that the proteasome activity reduction (40% decrease with respect to control) induced by Cr(III) pollen treatment is paralleled by a 40% decrease of spot 1723, which corresponds to 20S proteasome α-subunit. Thus, the observed Cr(III)-induced loss of proteasome function would explain the accumulation of ubiquitinated proteins previously reported (Scoccianti et al., 2008b). On the other hand, although Cr(III) does not seem to produce a relevant oxidative damage to proteins (Pena et al., 2008), the induction of the molecular chaperone Hsp20 (spot 546) correlates with the observation that proteasome inhibition leads to heat shock genes activation, including those encoding hsp, in the absence of a heat shock stress response (Kim et al., 1999). There is greater consensus for the idea that upon inhibition of the proteasome, newly synthesized misfolded or aberrant proteins accumulate in the cells mimicking a

Table 1
List of proteins identified by MS/MS in Cr-treated Kiwifruit pollen (NCBI Nos. 04/29/2011: 13841106 sequences, 4750259772 residues. Viridiplantae: 881708 sequences. Plants_EST: 144355992 sequences, 25218209504 residues).

Spot	NCBI Accession No.	Protein name	Organism	Loc. [b]	MS/MS (MASCOT)		MW (Da)/pI		Fold of variation (treated versus control)[c]		EST Accession No.
					Score	Pep.	Theor.	Exp.	Cr(III)	Cr(VI)	
Stress/detoxification											
475	gi|284437870	Dehydroascorbate reductase	*Actinidia deliciosa*	cyt	201	7	24.0/5.2	24.0/5.6	=	1.95(0.7)	
551	gi|52851172[a]	Thioredoxin-dependent peroxidise	*Plantago major*	cyt	494	6	17.5/5.3	17.5/5.5	=	1.6(0.1)	gi|195318812
554	gi|52851172[a]	Thioredoxin-dependent peroxidise	*Plantago major*	cyt	310	5	17.5/5.3	17.5/5.5	=	2.5(0.3)	gi|195318812
599	gi|255544266	Electron transporter, putative	*Ricinus communis*	cyt	160	2	14.9/6.3	15.0/5.6	2.2(0.5)	=	
546	gi|87240494[a]	Heat shock protein Hsp20	*Medicago truncatula*	cyt	124	2	18.2/5.4	18.0/5.8	2(0.4)	=	gi|31394109
Protein synthesis/fate											
592	gi|1346180	Glycine-rich RNA-binding protein GRP1A	*Sinapis alba*	nuc	115	2	16.1/5.2	15.0/5	<0.01	<0.01	
1723	gi|255544626[a]	Proteasome subunit alpha type, putative	*Ricinus communis*	cyt	613	10	25.6/5.5	25.0/5.8	0.6(0.1)	=	gi|195220099
1658	gi|255538376	26S proteasome non-ATPase regulatory subunit Rpn11, putative	*Ricinus communis*	cyt	170	8	34.8/6.3	35.0/5.9	=	0.6(0.1)	
749	gi|255538376[a]	26S proteasome non-ATPase regulatory subunit Rpn11, putative	*Ricinus communis*	cyt	175	4	34.8/6.3	33.0/6.6	5.6(0.3)	=	gi|195211369
1767	gi|255564428[a]	Elongation factor 1-beta, putative	*Ricinus communis*	Nuc	282	4	24.4/4.6	26.0/4.6	<0.01	<0.01	gi|195293057
1756	gi|21593028[a]	Putative elongation factor beta-1	*Arabidopsis thaliana*	cyt	282	5	25.3/4.5	27.5/5.7	0.6(0.1)	=	gi|195237682
Metabolism											
883	gi|255571784[a]	Glutamate-1-semialdehyde 2,1-aminomutase, putative	*Ricinus communis*	chl/cyt	232	4	50.5/5.9	45.0/5.6	=	2.4(0.6)	gi|195294653
1278	gi|10798652	Malate dehydrogenase	*Nicotiana tabacum*	cyt	269	4	35.4/5.9	40.0/6.1	<0.01	<0.01	
1819	gi|118484826	Acyl CoA binding protein	*Populus trichocarpa*	cyt	120	3	35.6/6.4	10/5.6	=	6.5(2.3)	
1430	gi|82623399[a]	Cytochrome *c* oxidase family protein-like	*Solanum tuberosum*	mit	187	3	18.4/5.4	17/4.5	<0.01	<0.01	gi|195217379
2556	gi|255560715[a]	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase, putative	*Ricinus communis*	chl/mit	340	6	59/8.3	60/5.5	=	>100	gi|195275007
980	gi|255577993[a]	Mitochondrial glycoprotein family protein	*Ricinus communis*	mit	147	3	20.0/4.1	23/4.5	<0.01	<0.01	gi|195236024
Unknown function											
405	gi|255552037[a]	Unknown protein aluminum-induced	*Ricinus communis*	cyt/nuc	368	7	27.0/6.0	30/6.6	3.9(0.8)	3.2(1.5)	gi|195260956

[a] Proteins identified in EST database. The name and the accession number are annotated by BLAST search and the theoretical Mw/pI was calculated with Expasy Protein Tools (http://www.expasy.ch/tools/pi_tool.html).
[b] Cyt, cytoplasm; Chl, chloroplast; Mit, mitochondrion, nuc, nucleus.
[c] = Means that the spot variation was not significantly >1.5 or <1.5 with respect to the control. The value '>100' ('<0.01') means that the protein spot is detected (is not present) exclusively under the condition reporting this value.
Standard Deviations are reported in brackets.

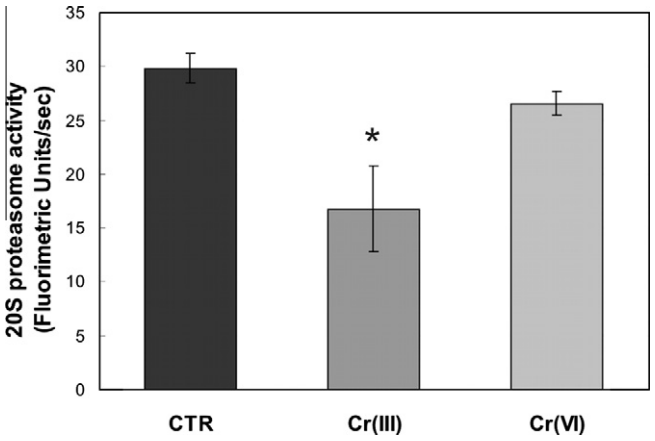

Fig. 2. 20S proteasome activity in untreated (CTR) and Cr-treated kiwifruit pollen [Cr(III) and Cr(VI)]. Equal amounts of cell extracts were incubated in the presence of 200 μM sLLVY-NH-Mec at 30 °C. The breakdown of the fluorigenic peptide was monitored using a fluorescence microplate reader. Proteasome activity, expressed as Fluorimetric Units/s, has been calculated by submitting data to regression analysis. Data are presented as means (±SD) of three independent experiments. Asterisk indicates statistical significance ($p < 0.05$) versus CTR.

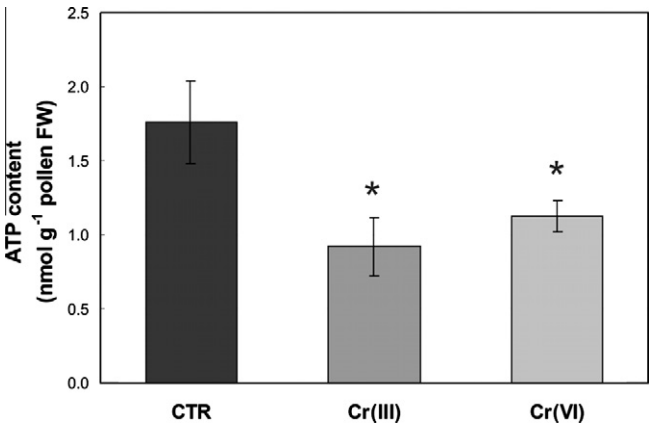

Fig. 3. ATP content in untreated (CTR) and in 30 μM Cr(III) and 90 μM Cr(VI) treated kiwifruit pollen. The values reported are means ± SD of three independent experiments and were analyzed by one-way ANOVA and Tukey post test using GraphPad Prism software. *Indicate statistically significant differences ($p < 0.05$) between treatments and the control.

condition of proteotoxic stress (McConkey and Zhu, 2008). Unfolded and misfolded proteins are inherently toxic to cells and must be quickly and efficiently eliminated before the intracellular environment is intoxicated. An important defense mechanism is the specific destruction of these proteins by the ubiquitin–proteasome system (Dantuma and Lindsten, 2010). In light of these observations it could be hypothesized that proteasome dysfunction and the consequent accumulation of misfolded proteins could be one of the possible molecular mechanisms of Cr(III) toxicity in pollen.

To maintain cellular integrity against stress causing direct or indirect protein misfolding, cells control not only the quality but also the quantity of proteins synthesis. Interestingly, two elongation factors (EFs, spots 1767 and 1756) were markedly decreased in Cr-treated samples. Protein synthesis in the cell involves multiple steps and numerous components, some of which, such as EFs, are important targets for regulating protein translation (Sonenberg and Dever, 2003).

In parallel, chromium drastically reduces the levels of a nuclear glycine-rich RNA-binding protein (GRP1A, spot 592). RNA-binding proteins are ubiquitous cellular proteins that regulate gene expression primarily at the post-transcriptional level, involving pre-mRNA splicing, nucleocytoplasmic mRNA transport, mRNA stability and decay, and translation (Dreyfuss et al., 1993; Simpson and Filipowicz, 1996). The under-expression of GRP1A and EFs in the presence of Cr illustrates that protein synthesis may be down-regulated at both post-transcriptional and translational levels.

2.4. Differentially expressed proteins were involved in the metabolism

Proteomic analysis showed a drastic decrease of two proteins involved in energy production: a cytochrome *c* oxidase family protein-like (spot 1430) and a mitochondrial glycoprotein family protein/MAM33 family protein (spot 980). The later protein is believed to be involved in mitochondrial oxidative phosphorylation and in nucleus–mitochondrion interactions in mammals (Jiang et al., 1999). There is very little information on metal action sites in the linear electron transport chain between NADH/succinate and oxygen in plant mitochondria. However, there are reports of Pb-, Cd- and Hg-induced inhibition of cytochrome oxidase (Vallee and Ulmerd, 1972). Monitoring partial electron transport reactions, Dixit et al. (2001) showed that cytochrome oxidase was the most susceptible complex of the root mitochondrial membranes to Cr(VI).

In light of these findings, the effect of Cr on kiwifruit pollen ATP content was evaluated. Both Cr(III) and Cr(VI) caused a reduction in ATP content with respect to the control, corresponding to 48% and 36%, respectively (Fig. 3). Mitochondria are the major source of damaging reactive oxygen species (ROS) in heterotrophic cells. The increase in ROS induced by Cr can be correlated with respiratory chain damage, resulting in an impairment of respiration and diverting more electrons to oxygen. The drastic decrease of two proteins involved in mitochondrial oxidative phosphorylation, as well as the decrease in ATP content, could be related to the swelling of mitochondria and loss of cristae previously observed in kiwifruit pollen treated with Cr(III) and Cr(VI) (Speranza et al., 2007).

Pollen tube tip growth places distinct demands on energy production and biosynthetic capacity, due to a respiration level greater than 10 times that of green leaf tissue (Tadege et al., 1999). Our data indicate that, in kiwifruit pollen, the chromium treatment severely affected respiratory chain function. A decrease in the pollen energy supply could be one of the causes of the severe inhibition of the pollen germination observed under Cr(III) and Cr(VI) treatments.

In Cr(VI)-treated pollen we found a strong accumulation of two proteins involved in fatty acid biosynthesis: a dihydrolipoamide acetyltransferase (E2 component of the plastidial pyruvate dehydrogenase complex (plPDC), spot 2556) and an acyl CoA binding protein (ACBP, spot 1819). plPDC supplies NADH and acetyl-CoA for *de novo* fatty acid biosynthesis. Elevated levels of plPDC expression were observed in mature pollen grain (Tovar-Mendez et al., 2003). The identified ACBP is very similar to ACBP6 of Arabidopsis. ACB6-overexpressing plants showed increased phospholipase D (PLD) expression and elevations in phosphatidic acid (PA) in comparison to wild type (Chen et al., 2008). PLD-derived PA plays a role in stimulating pollen germination and tube elongation (Potocky et al., 2003). The expansion of the pollen tube apical region is associated with a several fold increase in PA, generated by PLD (Zonia and Munnik, 2004). The accumulation of two proteins involved in fatty acid biosynthesis following Cr(VI) treatment could be interpreted as a response to oxidative damage. In fact, in kiwifruit pollen, Cr(III) and Cr(VI) induces an increase in lipid peroxide production and this increase is significantly greater upon exposure to Cr(VI) (Scoccianti et al., 2008a).

2.5. Protein with unknown function

Both Cr-treatments induced the accumulation of a spot corresponding to an unknown protein (spot 405). Interestingly, this

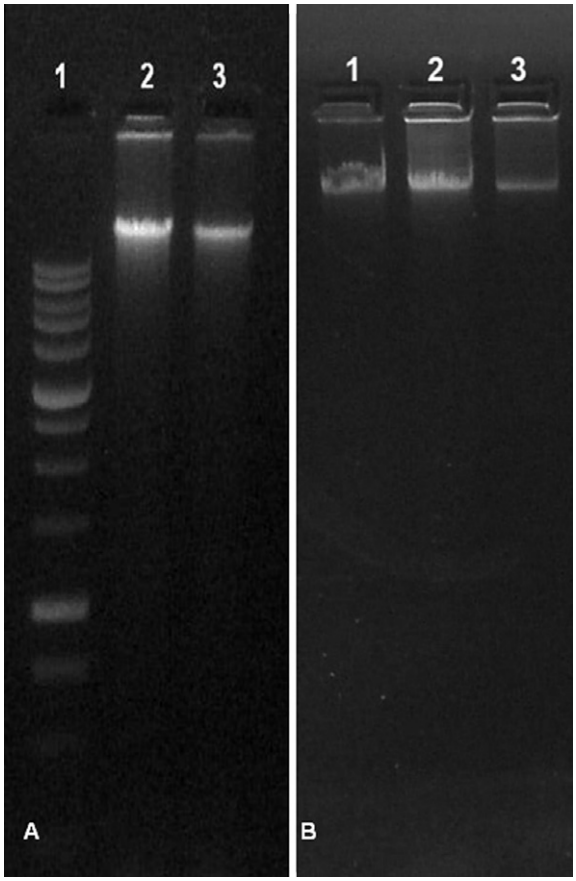

Fig. 4. 1% Agarose gel of DNA extracted from pollen of control and treated samples. (A) Lane 1, 1 kb DNA ladder; lane 2, DNA from non treated pollen; lane 3, DNA from pollen treated with Cr(III) 50 μM. (B) Lane 1, 100 ng λDNA uncut; lane 2, DNA from non treated pollen; lane 3, DNA from pollen treated with Cr(VI) 150 μm.

protein contains a domain present in Wali7, a protein of unknown function that is induced in wheat by aluminum (Al) treatment (Richards et al., 1994). The overexpression of the foxtail millet *Si69* gene, coding for a protein containing a conserved Wali7 domain, increases Al tolerance in transgenic Arabidopsis plants (Zhao et al., 2009). Our results indicate that Wali7-containing proteins may also play a role in the tolerance/detoxification of other metals.

2.6. Effect on DNA

Although Cr(III) and Cr(VI) were previously demonstrated to induce ultrastructural alterations and chromatin condensation of kiwifruit pollen DNA at the same concentration used in this work (Speranza et al., 2007), they were not able to induce DNA fragmentation (Fig. 4). A recent study reported that the aberration rate induced by chromium is strongly dose-dependent and gradually increases with increases in chromium concentrations (Chidambaram et al., 2009). This implies that the Cr(III) and Cr(VI) concentrations used in this study may not be sufficient to induce DNA fragmentation in kiwifruit pollen or that they may act through the formation of complexes that do not possess DNA fragmentation ability (Chatterjee and Luo, 2010). In tobacco and potato heavy metal treatments caused small but significant increases in DNA fragmentation associated with morphological and cellular damage (Gichner et al., 2006). However, no DNA damage was observed in the root or shoot cells of *Phaseolus vulgaris* treated with uranium (Vandenhove et al., 2006). The chemically killed cells do not necessarily exhibit DNA laddering as reported in cowpea leaves treated with chemicals (Ryerson and Heath, 1996).

3. Conclusion

Overall, our results demonstrated that both Cr(III) and Cr(VI) likely affect cellular pathways essential in cell metabolism leading to alteration of microgametophyte performance (Fig. 5). In particular, Cr exposure induces a drastic decrease of two proteins involved in mitochondrial oxidative phosphorylation, as well as a decline in ATP content. Pollen tube emergence and growth have a high energy requirement. Thus, a decrease in ATP intracellular levels could be one of the causes of the severe inhibition of kiwifruit pollen germination observed under Cr(III) and Cr(VI) treatments. Another major finding is that the ubiquitin proteolytic pathway is affected by both Cr species, although through different molecular mechanisms. In Cr(VI)-treated kiwifruit pollen the activity of the 20S proteasome is unaffected but the decrease of the Rpn11 proteasome subunit could impair protein turnover by affecting 26S-mediated proteolysis. Conversely, the 20S proteasome dysfunction in terms of activity and protein abundance could be one of the possible molecular mechanisms of Cr(III) toxicity in pollen. According to Kristen (1996), the energy compartment (mitochondria, respiration, ATP) and protein synthesis can be considered as primary targets of toxic impact to pollen tubes; in this view, at least the protein changes relevant to these cellular activities described by us in kiwifruit pollen could be considered as being direct consequences of chromium toxicity. On the other hand, the other protein changes could be also interpreted as indirect effects due to a deeply altered cell environment, where the main task, that is, germination, was severely impaired by the chromium treatment.

The lack of DNA laddering observed in Cr-treated pollen supports that the chromium damage is not due to a genotoxic effect.

4. Experimental

4.1. Plant material

Pollen was obtained from male kiwifruit plants [*Actinidia deliciosa* var. deliciosa (A. Chev.), C.F. Liang et. A.R. Ferguson] and maintained at −20 °C until use. Pollen was rehydrated for 30 min at 30 °C under 100% humidity. Germination was performed by suspending rehydrated pollen (1 mg ml^{-1}) in liquid medium containing 0.29 M sucrose and 0.4 mM boric acid (Speranza et al., 2007). Different concentrations of Cr(III) and Cr(VI) were separately administered at the beginning of incubation as aliquots from freshly made 5 mM $CrCl_3$ or 50 mM CrO_3 stock solutions, respectively. Controls without chromium were run in parallel. Cultures were incubated for 60 min or up to 90 min, as specified, at 30 °C in the dark. The duration of chromium treatment was definitely appropriate to obtain a substantial inhibition of pollen performance, being the *in vitro* growth conditions of kiwifruit pollen well established, and the time-course of chromium inhibition already assessed (Speranza et al., 2007). After incubation, pollen cultures were centrifuged at 1400*g* for 2 min, the supernatants were discarded and the cells were washed with fresh medium containing 0.29 M sucrose. Cells were then collected by Millipore vacuum filtration (8.0 μm pore size). Pollen samples were detached from the filter membrane and immediately used for analyses or frozen in liquid nitrogen and stored at −80 °C until use.

4.2. Protein sample preparation

Controls and chromium-treated samples (30 μM $CrCl_3$ and 90 μM CrO_3, respectively), were collected after 60 min of

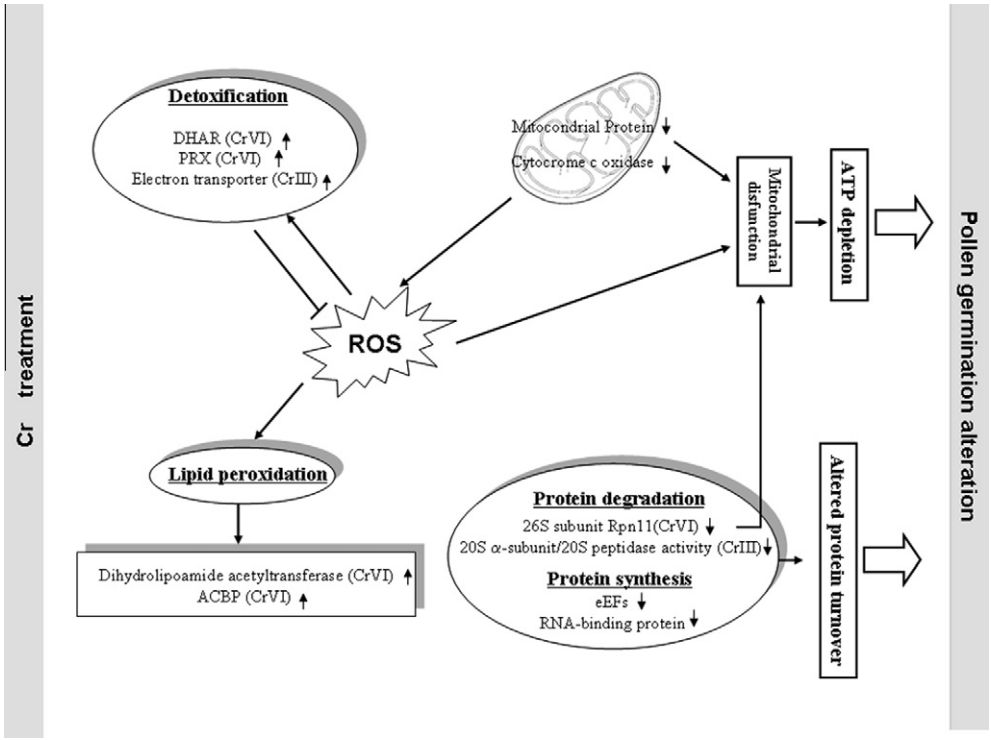

Fig. 5. A putative model of the response of kiwifruit pollen to Cr(III) and Cr (VI) treatments. Only some of the responsive proteins are indicated. Those up-accumulated, with respect to untreated cells, are marked by ↑ and those down-accumulated by ↓. DHAR, dehydroascorbate reductase; eEFs, eukaryotic elongation factors; PRX, tioredoxin-dependent peroxidase; ACBP, acyl CoA binding protein.

incubation as reported above. Such concentrations were previously shown to be seriously inhibitory on pollen tube emergence and elongation. They caused in fact nearly 65–70% inhibition on pollen tube emergence and elongation; being the hexavalent form less effective, as we assessed previously, the CrO_3 concentration was necessarily higher than that of the trivalent metal form (Speranza et al., 2007). Frozen samples were then homogenized by using mortar and pestle in liquid nitrogen with an addition of sand quartz. Total proteins were extracted as described by Marsoni et al. (2008). After the sample clarification at 13,000*g* for 10 min, the protein concentration was measured by Bio-Rad protein assay (Hercules, CA, USA), using bovine serum albumin as a standard. The samples were directly loaded for isoelectrofocusing (IEF) or stored in aliquots at −80 °C until use. Three independent experiments and extraction for each stress condition were performed.

4.3. Two-dimensional IEF/SDS–PAGE

IEF was carried out with 700 μg of total protein extract by using an immobilized 4–7 pH gradient (Immobiline DryStrip, 18 cm; Amersham Biosciences, Uppsala, Sweden). The strips were rehydrated in the IPGphor system (Amersham Biosciences, Bucks, UK) for 1 h at 0 V, 20 °C and 10 h at 30 V, 16 °C with the solubilization buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 50 mg/ml DTT, 0.5% of carrier ampholyte (3–10 NL IPG buffer; Amersham Biosciences), bromophenol blue 0.005% and the protein extracts. IEF was performed at 16 °C in the IPGphor system (Amersham Biosciences) for 4 h at 200 V, from 200 to 3500 V in gradient during 30 min, 3 h at 3500 V, from 3500 to 8000 V in gradient during 30 min, after which the run was continued at 8000 V to give a total of 70 kVh.

Each focused strip was equilibrated for 30 min against 6 M urea, 30% glycerol, 2% SDS, 50 mM Tris–HCl pH 8.8, 2% DTT and then a further 30 min with the substitution of the DTT with 2.5% iodoacetamide in the equilibration buffer. The equilibrated strips were placed on top of vertical 12.5% polyacrylamide gels (acrylamide/PDA 12.5% T, 2.6% C, 0.375 M Tris–HCl pH 8.8, TEMED 0.05% v/v, APS 0.1% w/v). The molecular weight markers, covering a 10–250 kDa range, were run on the acidic side of each gel. Electrophoresis was performed at 4 °C in a Laemmli running buffer (25 mM Tris–HCl pH 8.3, 192 mM glycine, 0.1% SDS) for 30 min at 15 mA/gel then at 45 mA/gel until the dye front reached the bottom of the gel. Each extraction was analyzed by three gel replicates.

4.4. Staining and analysis of 2-D gels

Proteins were detected with colloidal Coomassie brilliant blue (CBB) modified as described in Marsoni et al. (2010). Thereafter gels were digitalized by ImageScanner (Amersham Bioscience). Image and data analysis of the scanned (300 dpi, 16-bit greyscale pixel depth) gels as TIFF files were performed by using the Image Master 2D Platinum imaging software version 5.0, which allows spot detection, spot matching among multiple gels, background subtraction and quantization. For this purpose, data were normalized by expressing protein abundance as percent spot volume relative to volume of total protein in the gel (vol%). Gel replicas for each condition tested were averaged and the resulting gels contain only spot presents in all the replicates. The average-gels were compared and only proteins with a fold change of ±1.5, significant in Student's *t*-test at a level of 95%, were accepted as differentially expressed. These spots were selected for MS/MS analysis.

4.5. Mass spectrometry analysis and protein identification

Spots digestion was performed as described in Marsoni et al. (2010). The extracted tryptic fragments were analysed by MS/MS after reverse phase separation of peptides (liquid chromatography–electro spray tandem mass spectrometry, LC–ESI-MS/MS).

For all experiments, a Finnigan LXQ mass spectrometer, equipped with a Finnigan Surveyor MS Pump Plus HPLC system (Thermo Electron Corporation, CA, USA) was used. Chromatography separations were conducted on a BioBasic C18 column (150 μm I.D. × 150 mm length and 5 μm particle size; Thermo Electron Corporation, USA), using a linear gradient from 5% to 75% ACN, containing 0.1% formic acid with a flow of 2 μl/min. Including the regeneration step, one run lasted 80 min. Acquisitions were performed in the data-dependent MS/MS scanning mode (full MS scan range of 400–1400 *m*/*z* followed by Zoom scan for the most intense ion from the MS scan and full MS/MS for the most intense ion from the zoom scan), thus enabling a dynamic exclusion window of 3 min.

Protein identifications were conducted by correlation of uninterpreted tandem mass spectra to the entries of a non-redundant protein and/or EST-viridiplantae database downloaded from the National Center for Biotechnology Information (NCBI Nos. 04/29/2011: 13841106 sequences, 4750259772 residues. Viridiplantae: 881708 sequences. Plants_EST: 144355992 sequences, 25218209504 residues) using MASCOT open source (http://www.matrixscience.com). The parameters were set to allow one missed cleavages and considering as variable modification cysteine carbamido-methylation and methionine oxidation. The precursor ion tolerance was set at 1.2 Da and fragment ion tolerance 0.6 Da. Only proteins with a minimum of two matching peptides were considered and Mowse scoring system was used to assign correct identification.

4.6. Proteasome activity

The activity of the 20S proteasome was assayed using the fluorogenic substrate *N*-Succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methylcoumarin (Sigma–Aldrich, Italy), as previously reported (Speranza et al., 2001). In brief, untreated or Cr-treated pollen samples were collected after 60 min of germination and immediately extracted in cold 50 mM Hepes/KOH buffer pH 7.8, containing 2 mM DTT and 0.25 M sucrose using a Potter–Elvehjem apparatus, and were then centrifuged for 10 min at 12,000 rpm in a refrigerated Eppendorf centrifuge. Aliquots of the supernatants corresponding to 100 μg of proteins were pre-incubated at 30 °C for 5 min in 100 mM Hepes–KOH buffer, pH 7.8, containing 5 mM $MgCl_2$ and 10 mM KCl (final volume 200 μl) The reaction was initiated by adding the fluorigenic substrate to a final concentration of 0.2 mM. The breakdown of the peptide was monitored for 30 min using a fluorescence microplate reader (FLUOstar OPTIMA, BMG Labtech GmbH, Offenburg, Germany) with an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Proteasome activity in each sample, expressed as fluorimetric units/s, has been calculated by submitting data to regression analysis. Statistical analysis was performed by ANOVA for repeated measurement, followed by the Tukey–Kramer multiple comparison test using GraphPad InStat version 3.0.6 for Windows (GraphPad Software, San Diego, CA). Differences between means were set as statistically significant for $p < 0.05$.

4.7. ATP content determination

Pollen incubated for 60 min without treatment (control) and incubated for 60 min with 30 μM $CrCl_3$ or 90 μM CrO_3 was collected as reported above, and immediately extracted in cold 7% perchloric acid using a Potter–Elvehjem apparatus. The extracts were centrifuged at 14,000*g* for 10 min at 4 °C, and the supernatants were removed for assay. Aliquots of the supernatants (500 μl) were neutralized with 3 M K_2CO_3 solution and centrifuged at 5000*g* for 10 min at 4 °C. The ATP content in the supernatants was determined spectrophotometrically following the reduction of $NADP^+$ to NADPH at 340 nm ($\varepsilon = 6.22$ for NADPH), as reported by Beutler (1984).

4.8. DNA extraction and analysis

Control and chromium-treated sample (50 μM $CrCl_3$ and 150 μM CrO_3, respectively), were collected after 90 min of incubation as reported above. Total genomic DNA was isolated from untreated or treated kiwifruit pollen grains by grinding 150 mg of the material with a plastic pestel in an pre-chilled Eppendorf tube, at room temperature without buffer for two to three times. The powder was suspended by adding 750 μl of CTAB extraction buffer (2% CTAB, 100 mM Tris pH 8, 1.4 M NaCl, 20 mM EDTA pH 8.0, 0.001 mg/mL protease K) and 5 μl/ml of β-mercaptoethanol. Samples were vigorously shaked and incubated at 60 °C for 60 min. An equal volume of chloroform/isoamyl alcohol (24:1, v/v) was added and then the samples were centrifuged at 16,000*g* per 10 min. The upper phase was transferred in a new tube and 0.10 volume of 3 M ammonium acetate (NH_4OAc) and 0.7 volume of ice-cold isopropanol were added. After gently mix, samples were centrifuged at 18,000*g* for 10 min. The pellet was washed with 70% ethanol, resuspended in TE (Tris·Cl 10 mM, pH 8; EDTA 10 mM, pH 8.0) and added by RNAse (1 mg/ml) to remove contaminating RNAs. After incubation at 37 °C for 60 min, an equal volume of chloroform/isoamyl alcohol (24:1, v/v) was added and then the samples were centrifuged at 16,000*g* per 5 min. The upper phase was added by 3 M ammonium acetate and equal volume of ice-cold isopropanol, incubated at −20 °C for 20 min and then centrifuged at 16,000*g* for 30 min. The pellet was washed with 80% ethanol and resuspended in TE. The DNA concentration was measured in a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA). Samples were run on a 1% (w/v) agarose gel containing 0.5 g/ml ethidium bromide in 1× TBE buffer for 45 min at 80 V. The gels were documented using the PC based gel documentation system Kodak 1D 3.6. A 1 kbp ladder was used as standard marker.

Conflict of interest

The authors declare that there are no conflicts of interest.

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