Biochemical and Biophysical Research Communications 445 (2014) 457–462

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The *Arabidopsis thaliana* cysteine-rich receptor-like kinases CRK6 and CRK7 protect against apoplastic oxidative stress

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ARTICLE INFO

Article history:
Received 3 February 2014
Available online 12 February 2014

Keywords:
CRK
RLK
ROS signaling
Oxidative stress
Redundancy
Arabidopsis thaliana

ABSTRACT

Receptor-like kinases are important regulators of many different processes in plants. Despite their large number only a few have been functionally characterized. One of the largest subgroups of receptor-like kinases in *Arabidopsis* is the cysteine-rich receptor like kinases (CRKs). High sequence similarity among the CRKs has been suggested as major cause for functional redundancy. The genomic localization of *CRK* genes in back-to-back repeats has made their characterization through mutant analysis unpractical. Expression profiling has linked the CRKs with reactive oxygen species, important signaling molecules in plants. Here we have investigated the role of two CRKs, CRK6 and CRK7, and analyzed their role in extracellular ROS signaling. CRK6 and CRK7 are active protein kinases with differential preference for divalent cations. Our results suggest that CRK7 is involved in mediating the responses to extracellular but not chloroplastic ROS production.

1. Introduction

Plants have developed sophisticated signaling systems for environment-to-cell and cell-to-cell communication to cope with changing environmental conditions. Biotic and abiotic environmental cues can induce specific signaling pathways and trigger downstream signal transmission. In addition to external stimuli, plants use intra- and intercellular messenger molecules including peptides, hormones but also reactive oxygen species (ROS) for the control of growth, development and survival [1,2].

ROS are highly reactive oxygen-based molecules that include hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$), singlet oxygen ($^1O_2$) and superoxide ($O_2^{\cdot-}$). ROS are constantly produced as by-products of cellular metabolism. Due to their ability to oxidize a wide range of biomolecules, ROS levels are tightly controlled to avoid cellular damage [2]. The role of ROS in signal transduction is currently not well understood but recent research has identified redox modification of target proteins and lipids as one major mechanism [2]. However, despite the recent advances in ROS research the perception of extracellular ROS is still unclear.

Several distinct systems have been proposed to be involved in sensing ROS in the plant extracellular space [2–6]. Changes in extracellular ROS levels affect the ascorbate gradient and lead to changes in cellular redox homeostasis [7,8]. Superoxide, produced by NADPH oxidases, is dismutated to H_2O_2 which can cross plasma membranes through aquaporins [9]. ROS can cause direct redox modifications to secreted and membrane-localized proteins and lipids. These changes could be sensed through conformational changes or breakdown products, which might act as ligands for receptors. Receptor-like protein kinases (RLKs), one group of potential receptors, are membrane proteins that participate in many important signaling processes including plant growth and development, hormone signaling, and stress responses. A growing body of evidence suggests that a significant number of RLKs is involved in the response to environmental cues [10,11]. The more than 600 RLKs encoded in the *Arabidopsis* genome are divided into subfamilies according to their extracellular domain [12]. The variable extracellular domain is typically responsible for signal perception but also protein–protein interaction; the variety ensures perception of a wide range of signals.

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http://dx.doi.org/10.1016/j.bbrc.2014.02.013
0006-291X/

One of the biggest RLK subfamilies is the cysteine-rich receptor-like protein kinase (CRK, also known as domain of unknown function 26 [DUF26] RLK) subfamily, which are defined by two copies of the DUF26 (recently renamed to stress-antifung PF01657) domain in the extracellular region. Several CRKs are suggested to be involved in ROS signaling on the basis of the possibility of redox regulation of their extracellular thiol groups and their transcriptional regulation patterns [2,5,6,13]; several CRKs show elevated transcript levels in response to oxidative stress, pathogens and salicylic acid (SA) [14]. While the characterization of *crk* mutants has been difficult due to genetic redundancy [15,16], overexpression studies have suggested that CRKs are involved in disease resistance [17,18] and cell death [15,17]. Comprehensive phenotypic characterization of a T-DNA insertion collection for the *crks* revealed several processes where CRKs are involved including biotic and abiotic stresses as well as development [19].

Arabidopsis contains 44 *CRKs* arranged in several clusters. Chromosome IV contains the majority of *CRKs* and the biggest *CRK* gene cluster with 19 genes arranged one after the other (Fig. 1A). Smaller clusters and isolated *CRKs* are located on chromosomes I, III–V. Most CRKs share the general features typical for RLKs consisting of a signal peptide, an extracellular region, a transmembrane domain and a conserved intracellular serine/threonine kinase domain (Fig. 1B). The DUF26 domains in the extracellular region contain three conserved cysteine residues arranged in a C-X_8-C-X_2-C motif [20] (Fig. S1). The biochemical function of the domain is unknown but the structure of the DUF26 domain-containing antifungal protein ginkbilobin-2 (Gnk2) from *Ginkgo biloba* has provided first evidence for intramolecular disulfide bridges between cysteine residues [21].

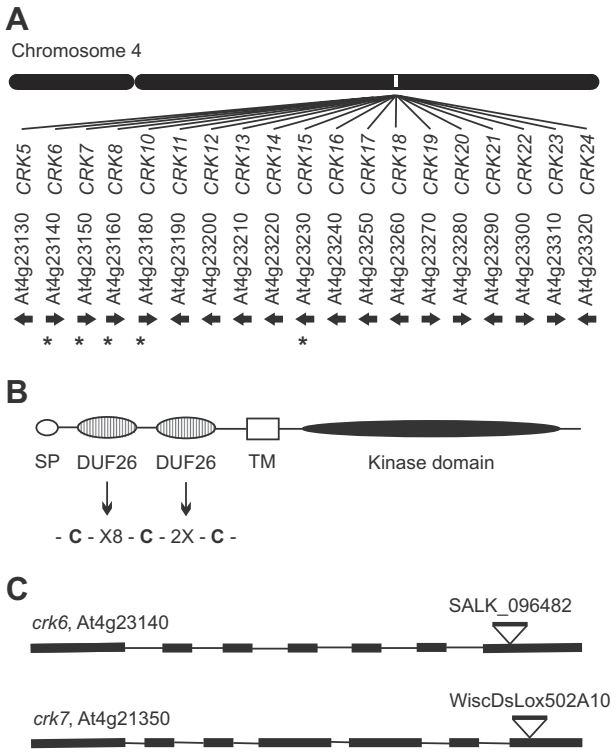

Fig. 1. Location of *CRK6* and *CRK7* genes, domain structure of CRK6 and CRK7 proteins and insertion sites in *crk6* and *crk7* mutants. (A) *CRK6* and *CRK7* are located in the largest tandem array on chromosome IV (*CRK5–CRK24*), separated only by 1500 bp promoter area of *CRK7*. Genes marked with asterisks (*) are silenced in the ami-RNA lines *crk6/7/8/10/15-1/2*. (B) CRKs share a conserved protein structure: a signal peptide (SP), extracellular domain containing two cysteine-rich DUF26 domains, transmembrane domain (TM), intracellular kinase domain. (C) T-DNA insertion sites in *crk6* and *crk7* are located in the intracellular kinase domain.

In this study we have characterized the role of two closely related CRKs, CRK6 and CRK7, in ROS signaling using T-DNA insertion lines (Fig. 1C). We provide evidence that CRK6 and CRK7 are active kinases that are involved in signaling in response to extracellular ROS. CRK6 and CRK7 share strong sequence similarity and can at least partially act in a redundant fashion.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds were vernalized for 2 days at 4 °C and grown on 1:1 mixture of peat and vermiculite at 23 °C/19 °C (day/night) under 70% relative humidity (±20%) with a 12 h photoperiod (170 μmol m^{-2} s^{-1}) in controlled growth chambers (Weiss Bio 1300, Weiss Umwelttechnik, Reiskirchen-Lindenstruth, Germany) or on *in vitro* plates in controlled growth chambers (Sanyo, Sakata, Japan) under a 12 h photoperiod (130 μmol m^{-2} s^{-1}) at 23 °C/19 °C (day/night).

2.2. Transgenic plant lines

For ectopic overexpression coding regions of *CRK6* and *CRK7* were cloned and placed under the control of the CaMV 35S-promoter and tagged with c-myc and StrepII (35S::CRK6-6x-cMyc/StrepII). Artificial micro-RNA (amiRNA) lines under the control of the CaMV 35S were constructed as described [22]. For genomic complementation complete genomic regions of CRK6 and CRK7 including promoter (3600 and 4000 bp, respectively) were cloned into the pGreenII0029 [23] binary vector. Promoter regions of *CRK6* and *CRK7* (1200 and 1400 bp, respectively) were used to drive expression of the *uidA* gene (in pGreenII0029). Constructs were transformed into wild type Col-0 (for ectopic overexpression) or T-DNA insertion (for complementation) plants using *Agrobacterium*-mediated floral dipping [24].

2.3. In vitro kinase assay

Cytoplasmic domains of CRK6 and CRK7 were expressed as a glutathione *S*-transferase (GST)-tagged recombinant proteins in *Escherichia coli* BL21. One μg of purified protein was incubated with [γ-^{32}P]-ATP for 1 h at 22 °C in the presence of phosphorylation buffer (50 mM HEPES, pH 7.4, 1 mM DTT) supplemented as indicated with 10 mM Mg^{2+}, 10 mM Mn^{2+} and 50 mM NaCl. Myelin basic protein (MBP) was used as artificial substrate. The mixture was subsequently separated by SDS–PAGE and analyzed by autoradiography.

2.4. Stainings

2.4.1. GUS staining

Samples were immersed in ice-cold fixing solution (90% acetone) for 1 h, washed three times for 1 h with ice-cold sodium phosphate buffer (0.05 M, pH 7.2) and then vacuum-infiltrated with GUS staining solution (0.05 M sodium phosphate buffer, pH 7.2; 0.5 mM ferrocyanide, 0.5 mM ferricyanide, 1 mM X-glucuronic acid, 0.1% Triton X-100). Samples were incubated overnight at 37 °C in darkness, washed with water and cleared in ethanol.

After staining, samples were fixed in 1% glutaraldehyde, 4% formaldehyde, 0.05 M sodium phosphate pH 7.2 at 4 °C overnight. Fixed samples were dehydrated in an ethanol series (10%, 30%, 50%, 70%, 90%, twice 100%; 30 min each), and incubated overnight in a solution 1:1 100% ethanol:Solution A (Leica Historesin Embedding Kit). Samples were embedded for 3 h in Solution A and incubated overnight in plastic chambers [25] filled with 14:1 Solution A:Hardener (Leica Historesin Embedding Kit). Ten-μm-sections

were prepared on a Leica JUNG RM2055 microtome using a microtome knife (Leica Disposable blades TC-65), rinsed for 5 s in 0.05% Ruthenium Red (Sigma–Aldrich) and then washed with water. Sections were mounted in water and visualized with a Leica 2500 Microscope.

2.4.2. Trypan blue (TB) staining

Samples were boiled with TB-lactophenol solution for 5 min and cleared by chloral hydrate (2.5 mg/mL) solution. Samples were stored in 60% glycerol and examined by stereomicroscopy.

2.5. Phenotyping

To assay germination, seeds were sown onto 0.5 MS + 2% sucrose + 0.8% agar plates with 0, 0.1, 0.5 and 1.0 μM methyl viologen (MV), respectively, vernalized 2 days at +4 °C and grown in Sanyo chamber for 14 days. The biomass of 10 plants per line was measured. For ozone (O_3) analysis twenty-one-day old plants were exposed to O_3 (350 ppb) for 6 h. Whole rosettes were collected 2 h after the end of fumigation for electrolyte leakage and analyzed as previously described [19,26]. For assaying ROS-induced electrolyte leakage, leaf discs from four-week-old plants were infiltrated with xanthine (X, 1 mM) and xanthine oxidase (XO, 0.1 U/mL) as previously described [19]. Leaf discs were washed three times with 15 mL water prior to electrolyte leakage measurement with a conductivity meter (Mettler Toledo).

3. Results

3.1. CRK6 and CRK7 are active kinases

The intracellular kinase domain is highly conserved between the RLK subfamilies and the general receptor activation mechanism is phosphorylation dependent. CRK6 and CRK7 possess the conserved consensus motifs of active serine/threonine protein kinases [27,28]. To determine whether CRK6 and CRK7 are enzymatically active protein kinases, full cytoplasmic domains were produced as GST-tagged recombinant proteins in *Escherichia coli*. Both GST-CRK6 and GST-CRK7 phosphorylated the artificial substrate MBP (Fig. 2) but only GST-CRK7 displayed detectable autophosphorylation activity. In spite of their high sequence identity (75.68%) and similarity (82.51%; http://imed.med.ucm.es/Tools/sias.html; Fig. S2) GST-CRK6 exhibited a preference for manganese (Mn^{2+}) while GST-CRK7 displayed stronger autophosphorylation and MBP phosphorylation with magnesium (Mg^{2+}) compared to Mn^{2+}. Together, these results demonstrate that GST-CRK6 and GST-CRK7 were active protein kinases with a preference for Mn^{2+} and Mg^{2+}, respectively, as divalent cations.

3.2. CRK7 gene expression is induced by O_3

O_3 induces extracellular ROS production and has been successfully used to study ROS signaling [6,13,26,29] and to identify novel ROS signaling components [6,30]. *CRK6* and *CRK7* have previously been reported to show elevated transcript levels in response to O_3 [6]. Tissue-specific expression patterns of *CRK6* and *CRK7* in response to O_3, were studied with promoters of *Arabidopsis CRK6* and *CRK7* fused to the β-glucuronidase (*uidA*) reporter gene. *CRK6::uidA* and *CRK7::uidA* lines and Col-0 plants were exposed to 350 ppb O_3 for 6 h and samples were harvested after a two-hour recovery period (6 + 2 h) for GUS staining.

Under control conditions, only *CRK7::uidA* lines presented staining located in the apical meristem area while *CRK6::uidA* was not detectable (Fig. 3A, pictures 1–4). O_3 led to a slight induction of *CRK6::uidA* expression visible in microsections but not whole-plant images (Fig. 3A pictures 5–6; Fig. S3A pictures 1–2). *CRK7::uidA* expression was strongly induced after 6 h ozone treatment and 2 h recovery (Fig 3A pictures 7–8, Fig. S3B pictures 3–5). Expression was localized to leaf areas typical for O_3-induced damage and intriguingly to tissue surrounding leaf vasculature (primary midvein, secondary and higher order veins; Fig. 3A pictures 6–8 and Fig. S3A and B pictures 1–5). Notably, O_3-induced GUS expression in *CRK7::uidA* lines was stronger in young leaves, which rarely display O_3 damage, compared to older leaves.

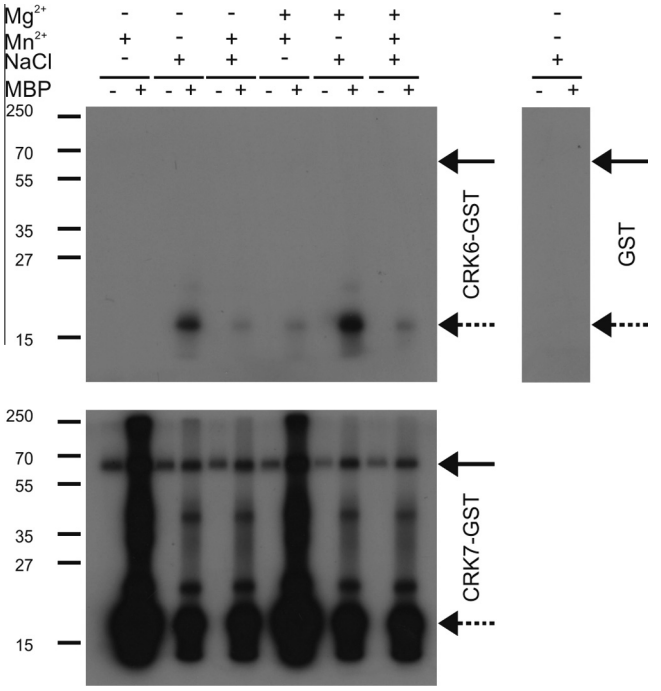

Fig. 2. CRK6 and CRK7 kinase domains displayed kinase activity *in vitro*. Autophosphorylation and substrate phosphorylation activities of the recombinant GST-tagged CRK6 and CRK7 cytoplasmic domains were tested with [γ-^{32}P]-ATP in the absence (−) or presence (+) of the artificial kinase substrate myelin basic protein (MBP) followed by SDS–PAGE and autoradiography. Phosphorylation buffer was supplemented with 10 mM Mn^{2+}, 10 mM Mg^{2+}, and 50 mM NaCl as indicated. Solid arrows show autophosphorylation and dashed arrows show phosphorylated MBP. Numbers on the left side indicate molecular weight in kDa. The experiment was repeated three times with similar results.

3.3. O_3 sensitivity of crk6 and crk7 is disguised by redundancy effect

High sequence similarity between CRKs has been suggested as the primary reason for the difficulties in identifying loss-of-function phenotypes for *crk* mutants. Furthermore, the tight genetic linkage affecting the majority of the CRK genes creates difficulties with double mutant approaches [2,12,15]. To overcome these problems, artificial microRNA (ami-RNA) lines were created where the closely related *CRK6*, *CRK7*, *CRK8*, and *CRK15* (and to a small extent also *CRK10*) showed reduced transcript abundance (Figs. 1A and S4).

To gain further insight into the function of CRK6 and CRK7 in ROS signaling, the response of *crk6* and *crk7* T-DNA insertion and *crk6/7/8/10/15-1/2* ami-RNA lines to O_3 was analyzed by measuring electrolyte leakage and by trypan blue (TB) staining; both methods assess cell death. Although *CRK6* and *CRK7* showed elevated transcript levels in response to O_3 [6], corresponding mutants displayed only subtle phenotypes after O_3 treatment. After 6 h O_3 and 2 h recovery, the *crk6* and *crk6/7/8/10/15-1/2* plants displayed more TB-stained dead cells in response to O_3 compared to Col-0 (Fig. 3B). O_3 sensitivity of *crk7* was not drastically different from wild type

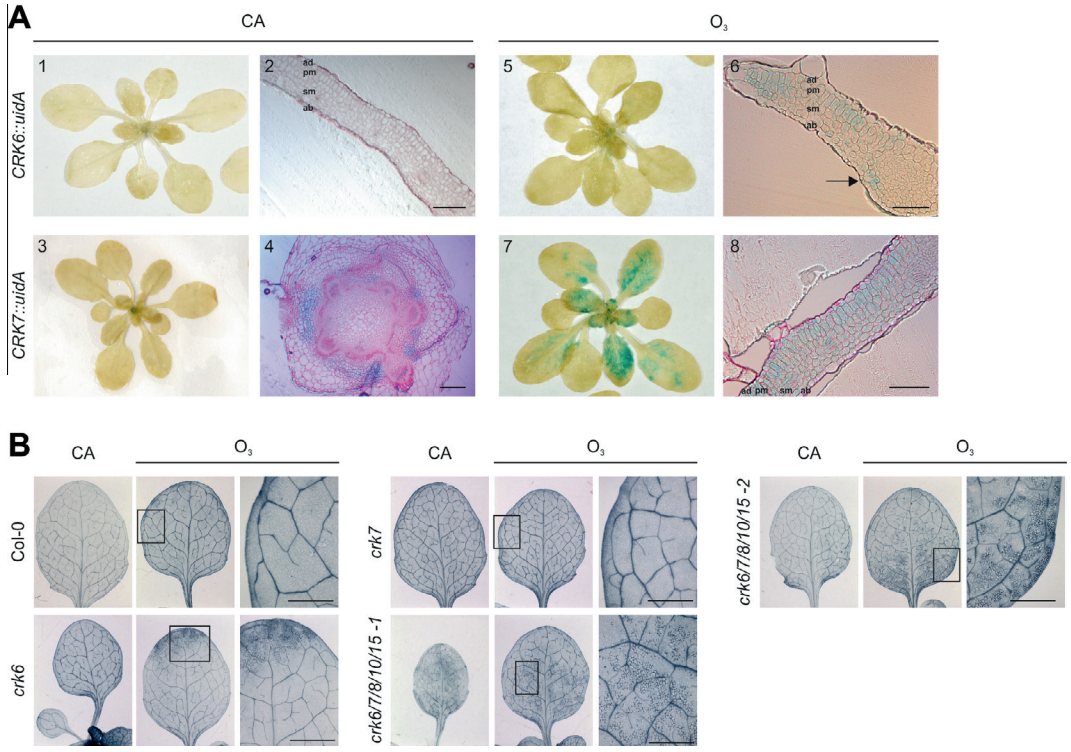

Fig. 3. GUS expression and localization of cell death after O_3 exposure. (A) O_3-induced expression of *CRK6::uidA* and *CRK7::uidA* in transgenic plants at 8 hpt. GUS staining localizes close to stomata and mostly in young leaves. ab: abaxial epidermis, ad: adaxial epidermis, CA: clean air, pm: palisade mesophyll, sm: spongy mesophyll. (B) O_3-induced cell death visualized by TB staining in *crk6*, *crk7*, *crk6/7/8/10/15-1/2* and Col-0 plants. *crk6*, *crk7* and especially *crk6/7/8/10/15-1/2* lines display more TB stained dead cells than wild-type Col-0. Bar = 1 mm. Experiments were repeated three times with similar results.

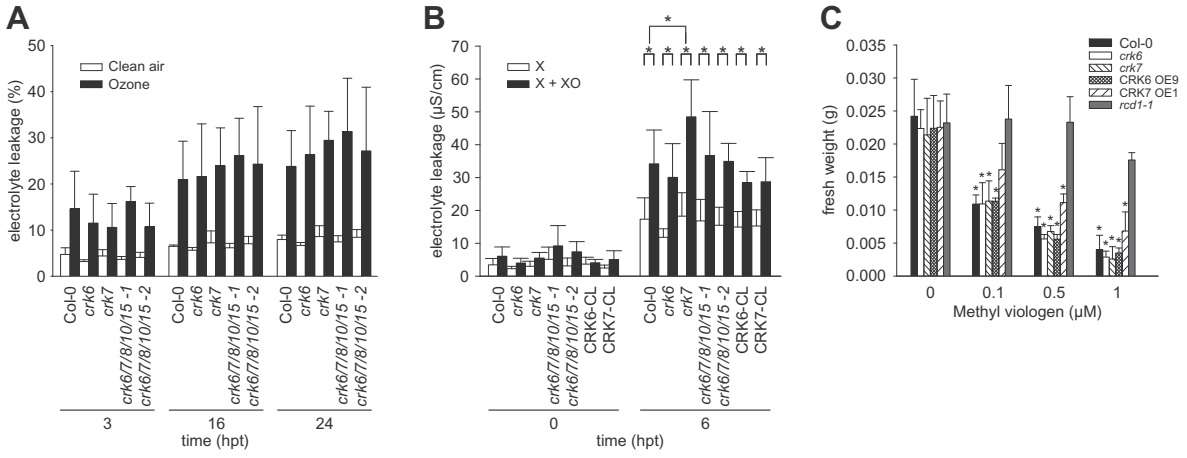

Fig. 4. *crk7* is sensitive to apoplastic but not chloroplastic ROS. (A) Electrolyte leakage increased in Col-0, *crk6*, *crk7* and *crk6/7/8/10/15-1/2* plants after exposure to O_3. Electrolyte leakage is plotted as % of total ion content. Differences are not significant ($P > 0.05$; ANOVA with Tukey's honestly significant differences (HSD) post hoc analysis). (B) Xanthine oxidase (X + XO) treatment slightly increased electrolyte leakage at 6 hpt in *crk7* but not in *crk6*, *crk6/7/8/10/15-1/2* and Col-0. X: xanthine, XO: xanthine oxidase. Data from four independent experiments has been combined. CL: complementation line. (C) Effect of methyl viologen (MV) on fresh weight. Fresh biomass of plants grown for 14 days in the presence of increasing amounts of MV was measured. *rcd1-1* was used as a MV tolerant line. OE: overexpression lines. In all data points are mean ± SD (in A $n = 4$; B $n = 12$, C $n = 10$). Differences labeled with asterisks are significant ($P \leqslant 0.05$; ANOVA with Tukey's honestly significant differences (HSD) post hoc analysis).

(Fig. 3B). However, the increased cell death visible in trypan blue staining of *crk6* and *crk6/7/8/10/15-1/2* was not reflected in the electrolyte leakage measurements for cell death quantification where *crk6*, *crk7* and *crk6/7/8/10/15*-1/2 plants showed similar O_3 sensitivity as Col-0 wild-type (Fig. 4A).

O_3 induces increased ROS production in plants [31]. We investigated whether *crk6*, *crk7* and *crk6/7/8/10/15-1* lines showed differences in O_3-induced H_2O_2 production compared to Col- using DAB staining of clean air and O_3-treated plants. After exposure to O_3, *crk6, crk7* and *crk6/7/8/10/15-1* showed stronger accumulation of H_2O_2 than Col-0 and corresponding clean air controls (Fig. S5A and B). These results suggest an involvement of CRK6 and CRK7 in O_3-induced cell death signaling.

3.4. crk7 is sensitive to ROS produced by xanthine oxidase

To analyze sensitivity of the mutant lines to extracellular ROS, an enzymatic system, xanthine–xanthine oxidase (X + XO), was used to generate extracellular O_2^- and H_2O_2, similar to O_3. X + XO treatment has a more dramatic effect on ROS production compared

to O_3 since access to the cells is not controlled by the stomatal aperture. Interestingly, *crk7* but not *crk6* or *crk6/7/8/10/15-1/2* showed increased X + XO sensitivity compared to Col-0 wild type (Figs. 4B and S6). The increased X + XO sensitivity of *crk7* was rescued by genomic complementation (Figs. 4B and S6). Increased ROS production, frequently referred to as oxidative burst, is a component of pathogen defense in plants [2]. However, *crk6*, *crk7* and *crk6/7/8/10/15-1* showed no differences in pathogen responses compared to wild type (Fig. S6). Since extracellular and chloroplastic ROS production is connected [5] we tested the response of *crk6* and *crk7* to elevated ROS accumulation in the chloroplast. Methyl viologen (MV) accents electrons from photosystem I and transfers them to molecular oxygen thereby forming superoxide. *crk6* and *crk7* did not display different tolerance to MV compared to Col-0 (Fig. 4C) suggesting that they are not involved in the response to chloroplastic ROS. However, plants overexpressing CRK7 showed slightly, reproducible but not statistically significant, increased tolerance to MV (Fig. 4C). This suggested that chloroplastic ROS production could affect extracellular ROS signaling. Taken together our results demonstrate that CRK6 and CRK7 are required for a precise and fine-tuned response to extracellular ROS.

4. Discussion

ROS signaling has been under extensive research for the last two decades. New signaling components and pathways have been identified and our understanding of the function of ROS has shifted from harmful side-products to important signaling intermediates with diverse roles [2,3]. However, fundamental questions still remain unanswered: how are ROS signals sensed [2] and how is ROS signaling specificity achieved [30]?

RLKs are involved in many important processes and members of the CRK subfamily have been suggested as candidates for ROS perception [2]. We have analyzed the roles of CRK6 and CRK7 which were found to be active protein kinases (Fig. 2). Intriguingly, despite their high sequence identity CRK6 and CRK7 had different preferences for divalent cations *in vitro*. So far no substrates for CRKs or interacting proteins have been identified. Based on their kinase activities however, it could be expected that CRK6 and CRK7 phosphorylate a different set of protein targets. Biochemical analysis of CRKs based on activation patterns, protein–protein interactions and phosphorylation targets might be a strategy to overcome the genetic redundancy which hampers the investigation of this protein family.

We analyzed the response of *crk6* and *crk7* to different ROS-inducing conditions to study the role of CRK6 and CRK7 in ROS signaling. *CRK6* and *CRK7* transcript accumulation was induced by O_3 and the corresponding single knockout mutants showed more cell death in cell death-specific staining indicating elevated sensitivity to O_3. The sequence similarity and subtle O_3 sensitivity of *crk6* and *crk7* suggest strong redundancy and overlapping functions. The loss of a single gene could be compensated by a sister gene(s) leading to an almost wild type O_3 phenotype. According to our previous study closely related CRK genes tend to share similar O_3 expression patterns [6]. *CRK6*, *CRK7*, *CRK8*, *CRK10* and *CRK15* show increased transcript levels after exposure to O_3 [6] which suggests also functional redundancy between homologous proteins. This redundancy has been suggested as the cause for the unaltered sensitivity of *crk* mutants towards O_3 and pathogens [15,17,19]. Silencing of five most homologous genes (*CRK6/7/8/10/15*) led to elevated sensitivity to O_3. However, *crk7* showed enhanced X + XO sensitivity suggesting also specific functions for individual CRKs. The chloroplast plays an essential role in ROS signaling [5,11]. However, our analysis of MV sensitivity together with recent investigation of *crk* sensitivity to light stress [19] suggested that CRK6 and CRK7 are not directly involved in chloroplastic ROS signaling. This is supported by previous transcriptional analysis where most *CRKs*, including *CRK6* and *CRK7*, displayed lower transcript levels in response to light stress [6].

Our analyzes demonstrate that CRKs have important and overlapping roles in oxidative signaling induced by apoplastic ROS. CRK6 and CRK7 help the plants to cope with increased ROS levels in the apoplast caused by O_3 and X + XO suggesting protective roles for CRK6 and CRK7. This is supported by the rapid increase in expression after O_3 treatment in *CRK7::uidA* line, where *CRK7* expression was co-localized with areas in which O_3 damage is typically observed. The results suggest that in the presence of CRK6 and CRK7 O_3 damage can be avoided. Surprisingly, we did not detect enhanced O_3-tolerance in overexpression lines (Fig. S7). Protein kinase activity is frequently regulated post-translationally, which could obscure the effect of CRK6 and CRK7 overexpression. Alternatively, the amount of potential ligands for CRKs could be rate-limiting.

Taken together, our results show that CRK6 and CRK7 are active protein kinases. CRK6 and CRK7 are, together with their closest homologs (CRK8, CRK10, CRK15), involved in the coordination of a proper response to extracellular ROS caused by O_3 and X + XO in *Arabidopsis thaliana*. We show that *crk6* and *crk7* phenotypes are partly disguised by redundancy, a prominent feature of the CRK family. This redundancy and overlapping functions are likely a result of evolutionary pressure to guarantee survival and the ability to adapt to changing environmental conditions. Nevertheless, some CRKs have specific and even antagonistic functions [19]. Further experiments will be required for determining the steps leading to receptor activation, possible complex formation and the role of DUF26 domain before the role of CRKs in ROS sensing is revealed. The large number of CRKs, their redundant yet specific function and the fine tuning opportunities for signal transduction provide many interesting research questions for the future years. Understanding the complex signaling network regulated *via* CRKs will provide new clues for improving plants' tolerance mechanisms against future environmental challenges.

Acknowledgments

Tuomas Puukko, Leena Grönholm and Airi Lamminmäki are acknowledged for technical assistance. Dr. Maija Sierla is acknowledged for critically commenting on the manuscript. N.I. was supported by the Finnish Graduate School in Plant Biology; A.G. is supported the Academy of Finland (decision #140187). R.S. is funded by the Integrative Life Science Graduate School and A.P.M. is supported by the Academy of Finland. M.W. acknowledges University of Helsinki for funding. J.K. acknowledges funding from the Academy of Finland (ERA-PG research grant 129940, Centre of Excellence program 2006–2010), Biocentrum Helsinki and the University of Helsinki.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.013.

References

[1] M.J. Considine, C.H. Foyer, Redox regulation of plant development, Antioxid. Redox Signal. (2013).
[2] M. Wrzaczek, M. Brosché, J. Kangasjärvi, ROS signaling loops-production, perception, regulation, Curr. Opin. Plant Biol. 16 (2013) 575–582.
[3] R. Mittler, S. Vanderauwera, N. Suzuki, et al., ROS signaling: the new wave?, Trends Plant Sci 16 (2011) 300–309.
[4] I.M. Møller, L.J. Sweetlove, ROS signalling–specificity is required, Trends Plant Sci. 15 (2010) 370–374.
[5] A. Shapiguzov, J.P. Vainonen, M. Wrzaczek, J. Kangasjärvi, ROS-talk-how the apoplast, the chloroplast, and the nucleus get the message through, Front. Plant Sci. 3 (2012) 292.

[6] M. Wrzaczek, M. Brosché, J. Salojärvi, et al., Transcriptional regulation of the CRK/DUF26 group of receptor-like protein kinases by ozone and plant hormones in Arabidopsis, BMC Plant Biol. 10 (2010) 95.
[7] C.H. Foyer, A.J. Bloom, G. Queval, et al., Photorespiratory metabolism: genes, mutants, energetics, and redox signaling, Annu. Rev. Plant Biol. 60 (2009) 455–484.
[8] S. Munné-Bosch, G. Queval, C.H. Foyer, The impact of global change factors on redox signaling underpinning stress tolerance, Plant Physiol. 161 (2013) 5–19.
[9] G.P. Bienert, A.L. Møller, K.A. Kristiansen, et al., Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes, J. Biol. Chem. 282 (2007) 1183–1192.
[10] Y. Osakabe, K. Yamaguchi-Shinozaki, K. Shinozaki, et al., Sensing the environment: key roles of membrane-localized kinases in plant perception and response to abiotic stress, J. Exp. Bot. 64 (2013) 445–458.
[11] M. Sierla, M. Rahikainen, J. Salojärvi, et al., Apoplastic and chloroplastic redox signaling networks in plant stress responses, Antioxid. Redox Signal. 18 (2013) 2220–2239.
[12] S.H. Shiu, W.M. Karlowski, R. Pan, et al., Comparative analysis of the receptor-like kinase family in Arabidopsis and rice, Plant Cell 16 (2004) 1220–1234.
[13] J.P. Vainonen, J. Kangasjärvi, Plant signaling in acute ozone exposure, Plant Cell Environ. (2014), http://dx.doi.org/10.1111/pce.12273.
[14] P. Czernic, B. Visser, W. Sun, et al., Characterization of an *Arabidopsis thaliana* receptor-like protein kinase gene activated by oxidative stress and pathogen attack, Plant J. 18 (1999) 321–327.
[15] K. Chen, B. Fan, L. Du, et al., Activation of hypersensitive cell death by pathogen-induced receptor-like protein kinases from Arabidopsis, Plant Mol. Biol. 56 (2004) 271–283.
[16] L. Ederli, L. Madeo, O. Calderini, et al., The *Arabidopsis thaliana* cysteine-rich receptor-like kinase CRK20 modulates host responses to *Pseudomonas syringae* pv. tomato DC3000 infection, J. Plant Physiol. 168 (2011) 1784–1794.
[17] B.R. Acharya, S. Raina, S.B. Maqbool, et al., Overexpression of CRK13, an Arabidopsis cysteine-rich receptor-like kinase, results in enhanced resistance to *Pseudomonas syringae*, Plant J. 50 (2007) 488–499.
[18] K. Chen, L. Du, Z. Chen, Sensitization of defense responses and activation of programmed cell death by a pathogen-induced receptor-like protein kinase in Arabidopsis, Plant Mol. Biol. 53 (2003) 61–74.
[19] G. Bourdais, P. Burdiak, A. Gauthier, et al., Large-scale phenotyping of the CRK receptor-like protein kinase family in Arabidopsis reveals roles in plant development and stress response. Submitted manuscript (2014).
[20] Z. Chen, A superfamily of proteins with novel cysteine-rich repeats, Plant Physiol. 126 (2001) 473–476.
[21] T. Miyakawa, K. Miyazono, Y. Sawano, et al., Crystal structure of ginkbilobin-2 with homology to the extracellular domain of plant cysteine-rich receptor-like kinases, Proteins 77 (2009) 247–251.
[22] R. Schwab, S. Ossowski, N. Warthmann, et al., Directed gene silencing with artificial microRNAs, Methods Mol. Biol. 592 (2010) 71–88.
[23] R.P. Hellens, E.A. Edwards, N.R. Leyland, et al., PGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation, Plant Mol. Biol. 42 (2000) 819–832.
[24] S.J. Clough, A.F. Bent, Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*, Plant J. 16 (1998) 735–743.
[25] B. Scheres, H. Wolkenfelt, V. Willemsen, et al., Embryonic origin of the Arabidopsis primary root and root meristem initials, Development 120 (1994) 2475–2487.
[26] K. Overmyer, H. Tuominen, R. Kettunen, et al., Ozone-sensitive arabidopsis *rcd1* mutant reveals opposite roles for ethylene and jasmonate signaling pathways in regulating superoxide-dependent cell death, Plant Cell 12 (2000) 1849–1862.
[27] S.K. Hanks, A.M. Quinn, T. Hunter, The protein kinase family: conserved features and deduced phylogeny of the catalytic domains, Science 241 (1988) 42–52.
[28] J.M. Stone, J.C. Walker, Plant protein kinase families and signal transduction, Plant Physiol. 108 (1995) 451–457.
[29] M. Wrzaczek, M. Brosché, H. Kollist, et al., *Arabidopsis* GRI is involved in the regulation of cell death induced by extracellular ROS, Proc. Natl. Acad. Sci. USA 106 (2009) 5412–5417.
[30] L. Vaahtera, M. Brosché, M. Wrzaczek, J. Kangasjärvi, Specificity in ROS signaling and transcript signatures, Antioxid. Redox Signal. (2013), http://dx.doi.org/10.1089/ars.2013.5662.
[31] K. Overmyer, H. Kollist, H. Tuominen, et al., Complex phenotypic profiles leading to ozone sensitivity in *Arabidopsis thaliana* mutants, Plant Cell Environ. 31 (2008) 1237–1249.