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# Transcriptional regulation of the CRK/DUF26 group of Receptor-like protein kinases by ozone and plant hormones in *Arabidopsis*

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## Abstract

**Background:** Plant Receptor-like/Pelle kinases (RLK) are a group of conserved signalling components that regulate developmental programs and responses to biotic and abiotic stresses. One of the largest RLK groups is formed by the Domain of Unknown Function 26 (DUF26) RLKs, also called Cysteine-rich Receptor-like Kinases (CRKs), which have been suggested to play important roles in the regulation of pathogen defence and programmed cell death. Despite the vast number of RLKs present in plants, however, only a few of them have been functionally characterized.

**Results:** We examined the transcriptional regulation of all *Arabidopsis* CRKs by ozone (O<sub>3</sub>), high light and pathogen/elicitor treatment - conditions known to induce the production of reactive oxygen species (ROS) in various subcellular compartments. Several CRKs were transcriptionally induced by exposure to O<sub>3</sub> but not by light stress. O<sub>3</sub> induces an extracellular oxidative burst, whilst light stress leads to ROS production in chloroplasts. Analysis of publicly available microarray data revealed that the transcriptional responses of the CRKs to O<sub>3</sub> were very similar to responses to microbes or pathogen-associated molecular patterns (PAMPs). Several mutants altered in hormone biosynthesis or signalling showed changes in basal and O<sub>3</sub>-induced transcriptional responses.

**Conclusions:** Combining expression analysis from multiple treatments with mutants altered in hormone biosynthesis or signalling suggest a model in which O<sub>3</sub> and salicylic acid (SA) activate separate signaling pathways that exhibit negative crosstalk. Although O<sub>3</sub> is classified as an abiotic stress to plants, transcriptional profiling of CRKs showed strong similarities between the O<sub>3</sub> and biotic stress responses.

## Background

Receptor-like/Pelle kinases (RLKs) are important components in the regulation of plant development, hormone signalling, abiotic, and biotic stress responses in plants. RLKs are serine-threonine protein kinases that typically contain a signal peptide, a variable extracellular domain, a transmembrane region, and a conserved intracellular protein kinase domain. The extracellular ligand-binding domain perceives signals and is commonly used to classify RLKs into distinct subgroups [1]. The RLKs are one of the largest gene families in *Arabidopsis* with more than

600 members, [1-4], but only relatively few of them, mostly leucine-rich repeat RLKs (LRR-RLK), have been functionally characterized. CLAVATA1, a LRR-RLK, binds the small extracellular protein CLAVATA3 to regulate meristem proliferation [5]. FERONIA (a member of a previously uncharacterized group of RLKs) is central to the regulation of male-female interactions during pollen tube reception in *Arabidopsis* [6] and in *Brassica* the S-locus Receptor Kinase and its ligand are critical determinants of self-incompatibility [7,8]. In *Arabidopsis*, ERECTA (a LRR-RLK) is a multifaceted regulator of development and physiological processes as well as environmental responses [9]. BRASSINOSTEROID INSENSITIVE 1 (BRI1, a LRR-RLK) binds the plant hormone brassinosteroid and dimerizes with BRI1-ASSOCIATED RECEPTOR KINASE 1/SOMATIC EMBRYOGENESIS

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RECEPTOR KINASE 3 (BAK1/SERK3) [10,11]. BAK1 also inducibly dimerizes with the RLK FLAGELLIN SENSITIVE 2 (FLS2, a LRR-RLK), which recognizes bacterial flagellin and is important in plant immunity [12,13]. Other RLKs contributing to pathogen recognition include EFR (the *Arabidopsis* receptor for EF-Tu) and rice Xa21 (a LRR-RLK), which recognizes a sulfonated peptide produced by the pathogen *Xanthomonas oryzae* pv. *oryzae* [14-18].

The DUF26 (Domain of Unknown Function 26; PFAM domain PF01657) RLKs, also known as Cysteine-rich RLKs (CRKs), form a large subgroup of the RLK family with more than 40 members [1,19]. The extracellular region of the protein contains two copies of the DUF26 domain which has four conserved cysteines (three of them form the motif C-8X-C-2X-C) that may form disulfide bridges as potential targets for thiol redox regulation. The CRKs are transcriptionally induced by oxidative stress, pathogen attack and application of salicylic acid (SA) [19-22]. Accordingly several members of the CRK subgroup of RLKs are involved in the regulation defence reactions and cell death in *Arabidopsis* leaves. Constitutive over-expression of CRK5 led to increased resistance to the virulent bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 but also to enhanced growth of the plant leaves [22]. Over-expression of CRK4, CRK5, CRK19 and CRK20 by a chemically inducible promoter, on the other hand, caused cell death [19,22]. Genetic analysis suggested that CRK5 regulated cell death independently of SA [22]. Conversely the enhanced resistance to *Pseudomonas* upon overexpression of CRK13 required increased SA levels [23].

Reactive oxygen species (ROS) have been established as important signalling molecules for inter- and intracellular communication in plants, animals and yeast [24-26]. ROS are produced in strictly defined locations in response to specific stimuli [25]. Pathogen infection rapidly induces an extracellular oxidative burst while light stress and specific chemicals, including paraquat and norflurazon, induce ROS production in the chloroplast [27-29]. Plant cells can differentiate between the type and localization of ROS resulting in very specific responses. Furthermore, ROS production in specific cellular compartments can have impact on ROS generation and signalling in other locations [30,31]. This crosstalk is likely accomplished through interplay between separate signalling pathways rather than direct interaction of the ROS molecules themselves [30,31]. However, the molecular components and mechanisms involved are still poorly defined [31,32]. In addition, it is unknown how ROS are sensed and how specificity in ROS signalling is achieved. The gaseous molecule ozone (O<sub>3</sub>) induces a burst of ROS in the apoplast similar to the oxidative burst in plant-pathogen interactions [24]. Other similarities between O<sub>3</sub> and

pathogen infection include the production of SA and ethylene (ET) [24]. O<sub>3</sub> is a convenient system to experimentally address the effects of apoplastic ROS since the plant is not exposed to other effector proteins or toxins which might induce defence responses. O<sub>3</sub> permits the study of the apoplastic oxidative burst undisturbed by manual manipulation of the plant material.

Plant hormones are a group of unrelated small compounds which are central to signalling during environmental adaptation and developmental regulation [33,34]. SA, jasmonic acid (JA) and ET are viewed as the main hormonal determinants of plant pathogen defence [35,36]. Abscisic acid (ABA) modulates plant defence and is a negative regulator of SA responses [37]. In addition, ABA is a key regulator of the high light response [38]. The interaction of hormone and ROS signalling is well documented. ROS can induce cell death in a SA-dependent and independent manner [24]. Cell death and ROS induce ET synthesis, which feeds into a positive forward amplification loop enhancing ROS production [39]. ROS-induced JA is critical in limiting cell death [24]. Thus, the successful outcome of a given response is not determined by one hormone, but is achieved through balance, interaction and constant recalibration of different plant hormones.

Despite extensive research on ROS signalling, the exact components mediating ROS signalling, ROS sensing, and perception in particular are still unknown. Here we have analysed transcriptional regulation and the involvement of hormonal signalling in regulating the expression of the whole *Arabidopsis* CRK gene subfamily by ROS. The effects of ROS production in different subcellular compartments was analysed by using O<sub>3</sub>- and light stress treated plant material and publicly available microarray data. We show that O<sub>3</sub>-induced transcriptional responses are blocked in the *defense, no death 1 (dnd1)* mutant, and they are altered in hormone biosynthesis or signalling mutants. Collectively this reveals alternate pathways in the regulation of ROS responses.

## Results

### CRK transcriptional response to O<sub>3</sub>

Several groups of RLKs are transcriptionally regulated in response to biotic stresses [40]. We identified several CRKs which were differentially regulated by O<sub>3</sub> (MB and JK unpublished microarray data). These results suggest a strong transcriptional regulation of the CRKs during stress responses. Therefore we chose to investigate further the transcriptional regulation of the whole CRK subfamily by ROS.

According to Shiu and Bleeker [1], Chen *et al.* [19], and our analysis (see table 1 for nomenclature and reference), the CRK subfamily consists of 44 members. Previ-

**Table 1: Nomenclature of the CRKs/DUF26 RLKs.**

| CRK Nomenclature | AGI Code  | DUF26 Nomenclature |
|------------------|-----------|--------------------|
| CRK1             | At1g19090 | DUF26 40           |
| CRK2             | At1g70520 | DUF26 41           |
| CRK3             | At1g70530 | DUF26 39           |
| CRK4             | At3g45860 | DUF26 14           |
| CRK5             | At4g23130 | DUF26 13           |
| CRK6             | At4g23140 | DUF26 6            |
| CRK7             | At4g23150 | DUF26 8            |
| CRK8             | At4g23160 | DUF26 7            |
| CRK10            | At4g23180 | DUF26 9            |
| CRK11            | At4g23190 | DUF26 4            |
| CRK12            | At4g23200 | DUF26 1            |
| CRK13            | At4g23210 | DUF26 25           |
| CRK14            | At4g23220 | DUF26 2            |
| CRK15            | At4g23230 | DUF26 36           |
| CRK16            | At4g23240 | DUF26 22           |
| CRK17            | At4g23250 | DUF26 21           |
| CRK18            | At4g23260 | DUF26 20           |
| CRK19            | At4g23270 | DUF26 15           |
| CRK20            | At4g23280 | DUF26 11           |
| CRK21            | At4g23290 | DUF26 23           |
| CRK22            | At4g23300 | DUF26 5            |
| CRK23            | At4g23310 | DUF26 12           |
| CRK24            | At4g23320 | DUF26 24           |

**Table 1: Nomenclature of the CRKs/DUF26 RLKs.**

|       |           |          |
|-------|-----------|----------|
| CRK25 | At4g05200 | DUF26 10 |
| CRK26 | At4g38830 | DUF26 30 |
| CRK27 | At4g21230 | DUF26 43 |
| CRK28 | At4g21400 | DUF26 28 |
| CRK29 | At4g21410 | DUF26 29 |
| CRK30 | At4g11460 | DUF26 19 |
| CRK31 | At4g11470 | DUF26 17 |
| CRK32 | At4g11480 | DUF26 18 |
| CRK33 | At4g11490 | DUF26 16 |
| CRK34 | At4g11530 | DUF26 3  |
| CRK36 | At4g04490 | DUF26 31 |
| CRK37 | At4g04500 | DUF26 32 |
| CRK38 | At4g04510 | DUF26 35 |
| CRK39 | At4g04540 | DUF26 34 |
| CRK40 | At4g04570 | DUF26 33 |
| CRK41 | At4g00970 | DUF26 26 |
| CRK42 | At5g40380 | DUF26 38 |
| CRK43 | At1g70740 | DUF26 37 |
| CRK44 | At4g00960 | DUF26 27 |
| CRK45 | At4g11890 | DUF26 45 |
| CRK46 | At4g28670 | DUF26 42 |

Nomenclature of the CRK/DUF26 group of RLKs according to Chen et al. [19] and Shiu and Bleecker [1]. CRK35 was not listed in Chen et al. [19].

ously two additional genes have been included, but *At4g11500* (*DUF26 44*) was classified as a pseudogene in the current version of the *Arabidopsis* genome (TAIR9; <http://www.arabidopsis.org>[41]) and *At4g23170* (*CRK9*)

contains no identifiable extracellular domain, signal peptide or complete kinase domain; thus both genes were excluded from the analysis.

We analysed the transcriptional responses of all the 44 *CRKs* to extracellular ROS produced by O<sub>3</sub> by quantitative real-time RT-PCR (qPCR). Out of the 44 *CRKs*, 25 (nine with statistical significance FDR [False Discovery Rate]-corrected p-value ≤ 0.1; additional file 1) showed more than two-fold higher mRNA abundance after 1-hour exposure to O<sub>3</sub> (Figure 1). After a 6-hour O<sub>3</sub> exposure followed by a 2-hour recovery period, 26 *CRKs* exhibited a more than two-fold increase in expression (eight with statistical significance FDR-corrected p-value ≤ 0.1; additional file 1). Only *CRK22*, *CRK30*, *CRK32*, *CRK33* and *CRK46* showed decreased expression in response to O<sub>3</sub>-treatment. In order to analyze if transcriptional regulation after exposure to O<sub>3</sub> was a feature of a single subset of the *CRKs*, the protein sequence of the kinase domain of all *CRKs* was aligned to construct a Neighbour-joining tree representing the relations between the members of the *CRK* group of RLKs (Figure 2). *CRKs* that were transcriptionally regulated in response to O<sub>3</sub> are high-lighted. O<sub>3</sub>-regulated genes were distributed across the tree instead of forming a unique branch. However, closely related genes showed a tendency to share similar O<sub>3</sub> expression patterns.

#### **CRK transcriptional response to light stress**

To determine the effects of light stress-induced ROS production, we monitored the expression of *ASCORBATE PEROXIDASE 2 (APX2)*, encoding a ROS scavenger and established marker for light-induced ROS production [42]. *APX2* was strongly induced after 1- and 2-hour exposure to light stress conditions (Figure 3). In contrast to O<sub>3</sub> (Figure 1), light stress led to rapid transcriptional repression of several *CRKs* (Figure 3). Twenty *CRKs* were transcriptionally repressed while only eight exhibited increased expression. However, the light-dependent regulation of the *CRKs* was not statistically significant. The lack of transcriptional induction in response to light stress corresponds to results from Lehti-Shiu *et al.* [40], who reported that the *CRKs* were transcriptionally strongly induced in response to biotic stimuli but the expression level decreased in response to abiotic stress (including heat, cold, drought and salt). Of the abiotic treatments, only UV-B, osmotic stress and wounding resulted in increased expression of *CRKs* [40].

#### **CRK transcriptional response to PAMPs is similar to the O<sub>3</sub> response**

To more broadly address transcriptional regulation of the *CRKs*, we analyzed and compared their expression profiles from publicly available Affymetrix chip data. Raw

data files were obtained from several databases (see material and methods) and RMA (Robust Multi-Array Average) normalized. To take the sample variation into account, parametric bootstrapping combined with Bayesian hierarchical clustering [43] was applied. This results in a numerical measure of similarity between treatments and genes, which can be clustered hierarchically (Figure 4; for a related application, see [44]). The meta-analysis of the publicly available O<sub>3</sub> microarray data revealed high overlap with our qPCR data; all eight genes with more than 3-fold increased expression in the publicly available array data exhibited increased expression in our qPCR analysis. Treatment with norflurazon (which increases singlet oxygen [<sup>1</sup>O<sub>2</sub>] in the chloroplast causing excess ROS production) led to decreased expression of four *CRKs*. Norflurazon blocks carotenoid biosynthesis and thus removes this quencher of the triplet chlorophyll and <sup>1</sup>O<sub>2</sub>. Paraquat leads to superoxide O<sub>2</sub><sup>-</sup> production in the chloroplast by transferring electrons from photosystem I to oxygen. The O<sub>2</sub><sup>-</sup> is subsequently dismutated to H<sub>2</sub>O<sub>2</sub>. Paraquat had no effect on *CRK* expression with the exception of the latest time point tested (24 hr), whereupon five *CRKs* exhibited increased expression; four of which were also regulated in response to O<sub>3</sub>. However, at this time point paraquat had most likely induced cell death. H<sub>2</sub>O<sub>2</sub> treatment selectively led to increased expression of a few *CRKs* which also displayed increased expression by O<sub>3</sub>. Rotenone (an inhibitor of mitochondrial electron transport causing elevated ROS production in mitochondria) had little impact on *CRK* expression; only *CRK3* showed increased expression levels. Thus, the *CRK* expression profile triggered by O<sub>3</sub> was not related to expression profiles established by other ROS treatments. Instead, the O<sub>3</sub>-triggered *CRK* expression profile clustered together with that provoked by several biotic and PAMP treatments, including *Blumeria graminis* var. *hordei* (*Bgh*), harpin Z (HrpZ), and the flagellin elicitor-active epitope flg22 (Figure 4).

Our qPCR analysis confirmed the changes caused by flg22 in the expression profile of the *CRKs* obtained from publicly available microarray data (Additional file 2 and Figure 4). Treatments with benzothiadiazole S-methyl-ester (BTH; an active SA analog) resulted in two-fold or higher up-regulation of 12 *CRKs*, some of which also exhibited elevated expression in response to O<sub>3</sub>. Interestingly, in the *non-expressor of pathogenesis-related genes 1 (npr1)* mutant these genes were not regulated by BTH treatment (Figure 4), indicating that SA regulation of these genes was dependent on NPR1-mediated signalling. Application of methyl-jasmonate (MeJA) did not cause any major changes in *CRK* expression (Figure 4), whilst ABA treatment resulted in decreased expression of

*CRK25*, *CRK30*, *CRK28*, *CRK29*, *CRK19*, *CRK21* and *CRK22* at late time points. Overall, the *CRK* expression profile in response to BTH clustered together with that triggered by O<sub>3</sub>, pathogen and PAMP treatments; whereas *CRK* transcriptional regulation upon ABA application clustered together with paraquat, norflurazon, rotenone and MeJA treatments (Figure 4).

Taken together, these results demonstrate that the *CRK* expression profile in response to O<sub>3</sub> is not related to treatments which mediate ROS production in the chloroplast or the mitochondria. However, there is a substantial overlap between the transcriptional responses to O<sub>3</sub> and pathogen infection/PAMP perception, which may be a result of apoplastic ROS commonly generated by all these stimuli.

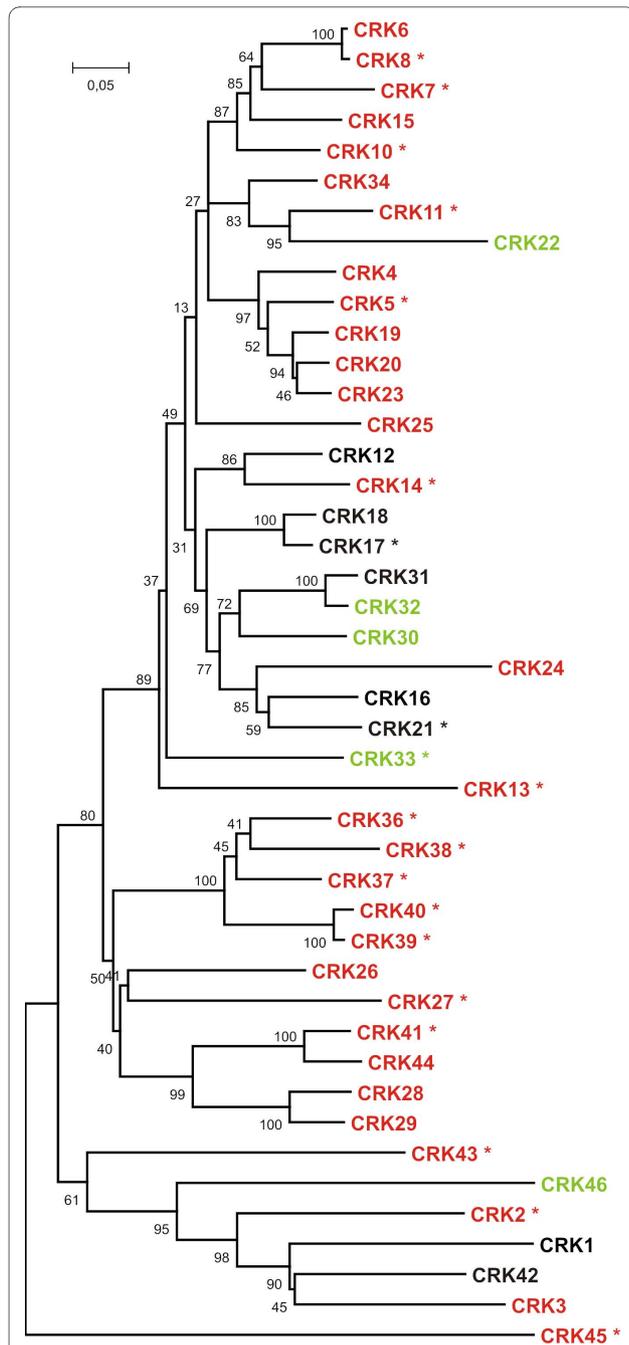
#### CRKs display different expression in hormone mutants

Altered transcriptional regulation of several *CRKs* has previously been shown following external application of the plant hormone SA or its active analog BTH (Figure 4 and [19]). In order to address the impact of hormone signalling on transcriptional regulation of *CRKs*, we used several mutants impaired in hormone biosynthesis and/or signalling. The *salicylic acid induction deficient 2* (*sid2*) mutant is deficient in SA biosynthesis (due to a mutation in the SA biosynthesis gene *ISOCHORISMATE SYNTHASE 1* [*ICS1*]), whilst *npr1* is impaired in SA signalling. The *dnd1* mutant fails to produce a hypersensitive response (HR), but has functional effector-triggered immunity, constitutive systemic resistance and accumulates elevated SA levels [45-47]. The *ethylene insensitive 2* (*ein2*) mutant is deficient in ET signalling, and the *fatty acid desaturase 3/7/8* (*fad3/7/8*) mutant is deficient in JA biosynthesis. We compared the transcript abundance of *CRKs* in these mutants to Col-0 wild type plants using qPCR. The obtained *Actin-2*-normalized threshold cycle values (Ct) were compared between Col-0 wild type and the mutants. Several *CRKs* showed lower expression in *sid2* and *npr1* (Figure 5A). *CRK29* displayed higher expression in *sid2* and ten *CRKs* (three with statistical significance FDR-corrected p-value ≤ 0.1) exhibited higher expression in *npr1*. In the *ein2* and *fad3/7/8* mutants, for nine and twelve *CRKs*, respectively, expression levels were elevated as compared to wild type plants. Only *CRK7* and *CRK8* showed lower expression in *ein2*. Along with several other defects, *dnd1* exhibits constitutive SA responses [48], which might be the cause for the increased transcript levels of 15 *CRKs* in *dnd1* signalling - however, other regulatory mechanisms cannot be ruled out due to the pleiotropic nature of the mutant [48]. Expression of some *CRKs* was unaltered or displayed only subtle changes in the *sid2* mutant, but was elevated in *npr1*, *ein2*, *fad3/7/8* and *dnd1* mutants (*CRK6*, *CRK23*, *CRK26*, *CRK36*, and *CRK45*). Interaction between hor-

|              | Ozone   |         |
|--------------|---------|---------|
|              | 1 h     | 6 + 2 h |
| <i>CRK1</i>  | NR      | NR      |
| <i>CRK2</i>  | 2.96 *  | 1.20    |
| <i>CRK3</i>  | 2.52    | 0.87    |
| <i>CRK4</i>  | 2.01    | 2.99    |
| <i>CRK5</i>  | 2.81 *  | 3.25    |
| <i>CRK6</i>  | 3.40    | 3.74    |
| <i>CRK7</i>  | 2.21    | 10.58 * |
| <i>CRK8</i>  | 7.72    | 4.40    |
| <i>CRK10</i> | 11.29 * | 1.92    |
| <i>CRK11</i> | 29.19 * | 3.27    |
| <i>CRK12</i> | NR      | NR      |
| <i>CRK13</i> | 2.34    | 9.68 *  |
| <i>CRK14</i> | 19.10 * | 3.39    |
| <i>CRK15</i> | 2.74    | 8.14    |
| <i>CRK16</i> | 1.55    | 1.36    |
| <i>CRK17</i> | 1.66 *  | 1.01    |
| <i>CRK18</i> | 1.27    | 1.68    |
| <i>CRK19</i> | 3.76    | 0.79    |
| <i>CRK20</i> | 4.39    | 0.90    |
| <i>CRK21</i> | 0.73 *  | 0.63    |
| <i>CRK22</i> | 0.57    | 1.2E-03 |
| <i>CRK23</i> | 2.25    | 5.84    |
| <i>CRK24</i> | 1.69 *  | 2.18    |
| <i>CRK25</i> | 0.96    | 2.39    |
| <i>CRK26</i> | 0.99    | 8.48    |
| <i>CRK27</i> | 4.96 *  | 4.27    |
| <i>CRK28</i> | 2.46    | 3.41    |
| <i>CRK29</i> | 5.98    | 2.24    |
| <i>CRK30</i> | 0.03    | 0.80    |
| <i>CRK31</i> | 0.69    | 1.64    |
| <i>CRK32</i> | 0.38    | 3.63    |
| <i>CRK33</i> | 0.83    | 0.12    |
| <i>CRK34</i> | 1.03    | 2.49    |
| <i>CRK36</i> | 4.90 *  | 10.64   |
| <i>CRK37</i> | 3.26    | 16.13 * |
| <i>CRK38</i> | 2.22 *  | 3.49    |
| <i>CRK39</i> | 5.95    | 30.45 * |
| <i>CRK40</i> | 1.49 *  | 3.59    |
| <i>CRK41</i> | 12.51 * | 1.96    |
| <i>CRK42</i> | 1.05    | 1.64    |
| <i>CRK43</i> | 2.72 *  | 2.01    |
| <i>CRK44</i> | 1.45    | 4.86    |
| <i>CRK45</i> | 3.09    | 23.31   |
| <i>CRK46</i> | 0.26    | 0.49    |

**Figure 1 Transcriptional regulation of the CRKs in response to O<sub>3</sub>.**

Expression of all members of the *CRK* group of plant RLKs was analyzed by quantitative real-time RT-PCR (qPCR) in Col-0 plants exposed to 250 ppb O<sub>3</sub> for 6 h. Samples were harvested at 1 or 8 h (6 h followed by 2 h recovery under clean air conditions) after the onset of the O<sub>3</sub> treatment. Transcript levels were calculated by comparison of O<sub>3</sub>-exposed plants with corresponding control plants grown under clean air conditions harvested in parallel with the O<sub>3</sub>-treated plants. An expression level of one indicates no change in expression, increased expression is indicated by values larger than one while decreased expression is shown by values smaller than one. Increase in expression by 2-fold or higher is high-lighted in red and decrease in expression by 2-fold or more in green. NR - no reproducible data could be obtained for this gene. The experiment was repeated four times; fold change was calculated from the average normalized cycle difference of all biological repeats. Statistical significance (Benjamini-Hochberg FDR corrected p-value ≤ 0.1) is indicated with asterisks (see additional file 1).



|              | 1 h            | 2 h           | 4 h + 4 h   |
|--------------|----------------|---------------|-------------|
| <b>APX2</b>  | <b>10.15 *</b> | <b>6.27 *</b> | 0.74 *      |
| <b>CRK1</b>  | NR             | NR            | NR          |
| <b>CRK2</b>  | 1.67           | 1.06          | 0.54        |
| <b>CRK3</b>  | 0.63           | 0.53          | 0.68        |
| <b>CRK4</b>  | 0.31           | 0.86          | 0.99        |
| <b>CRK5</b>  | 0.31           | 0.55          | 0.61        |
| <b>CRK6</b>  | 0.90           | 1.47          | 0.34        |
| <b>CRK7</b>  | 0.29           | 1.37          | 0.42        |
| <b>CRK8</b>  | 0.67           | <b>2.45</b>   | 0.59        |
| <b>CRK10</b> | 0.80           | 0.72          | 0.80        |
| <b>CRK11</b> | 0.79           | 0.49          | 0.67        |
| <b>CRK12</b> | NR             | NR            | NR          |
| <b>CRK13</b> | 0.24           | 1.73          | 1.64        |
| <b>CRK14</b> | 1.01           | 1.10          | 0.66        |
| <b>CRK15</b> | 0.56           | 0.90          | 0.57        |
| <b>CRK16</b> | 0.63           | 0.70          | 0.77        |
| <b>CRK17</b> | 0.40           | 0.78          | 0.85        |
| <b>CRK18</b> | 0.84           | 1.70          | 0.85        |
| <b>CRK19</b> | 0.80           | 1.03          | 0.41        |
| <b>CRK20</b> | 0.41           | 1.47          | 0.42        |
| <b>CRK21</b> | 0.54           | 0.36          | 0.38        |
| <b>CRK22</b> | 0.36           | 0.28          | 0.40        |
| <b>CRK23</b> | 0.18           | 1.27          | 0.24        |
| <b>CRK24</b> | 0.54           | 1.48          | 0.39        |
| <b>CRK25</b> | 0.92           | 1.38          | <b>2.74</b> |
| <b>CRK26</b> | 0.73           | 0.53          | 0.34        |
| <b>CRK27</b> | 1.19           | 1.18          | 1.54        |
| <b>CRK28</b> | 0.46           | 0.68          | 1.86        |
| <b>CRK29</b> | <b>3.00</b>    | 1.15          | 1.15        |
| <b>CRK30</b> | <b>2.03</b>    | 1.06          | 0.84        |
| <b>CRK31</b> | 1.57           | 1.27          | 0.82        |
| <b>CRK32</b> | <b>2.09</b>    | 1.26          | 1.22        |
| <b>CRK33</b> | <b>6.04</b>    | 0.47          | 0.26        |
| <b>CRK34</b> | 0.59           | 0.43          | 0.78        |
| <b>CRK36</b> | 0.70           | <b>2.08</b>   | 0.61        |
| <b>CRK37</b> | 0.53           | 1.27          | 0.32        |
| <b>CRK38</b> | 0.66           | 0.97          | 0.70        |
| <b>CRK39</b> | 0.33           | 1.03          | 1.32        |
| <b>CRK40</b> | 0.34           | 0.29          | 0.64        |
| <b>CRK41</b> | 1.78           | 1.33          | 0.95        |
| <b>CRK42</b> | 1.22           | 0.39          | 0.35        |
| <b>CRK43</b> | 0.64           | 0.74          | 1.56        |
| <b>CRK44</b> | <b>3.75</b>    | 1.47          | 0.40        |
| <b>CRK45</b> | 0.57           | 0.86          | 0.48        |
| <b>CRK46</b> | 1.42           | 1.09          | 0.89        |

**Figure 3 Transcriptional downregulation of CRKs in response to light stress.** Expression of *APX2* (a marker for light stress) and *CRKs* was analyzed by qPCR in plants after 1 h and 2 h exposure to light stress conditions and after 4 h light stress followed by 4 hours recovery at normal growth conditions. Transcript levels were calculated by comparison of light stress-treated plants with corresponding control plants grown under normal light conditions. An expression level of one indicates no change of expression, increased expression is indicated by values larger than one while decreased expression is shown by values smaller than one. Increase of expression by 2-fold or higher is high-lighted in red and decrease in expression by 2-fold or more in green. NR - no reproducible data could be obtained for this gene. The experiment was repeated twice; fold change was calculated from the average normalized cycle difference of all biological repeats. Statistical significance (Benjamini-Hochberg FDR corrected p-value ≤ 0.1) is indicated with asterisks (see additional file 1).

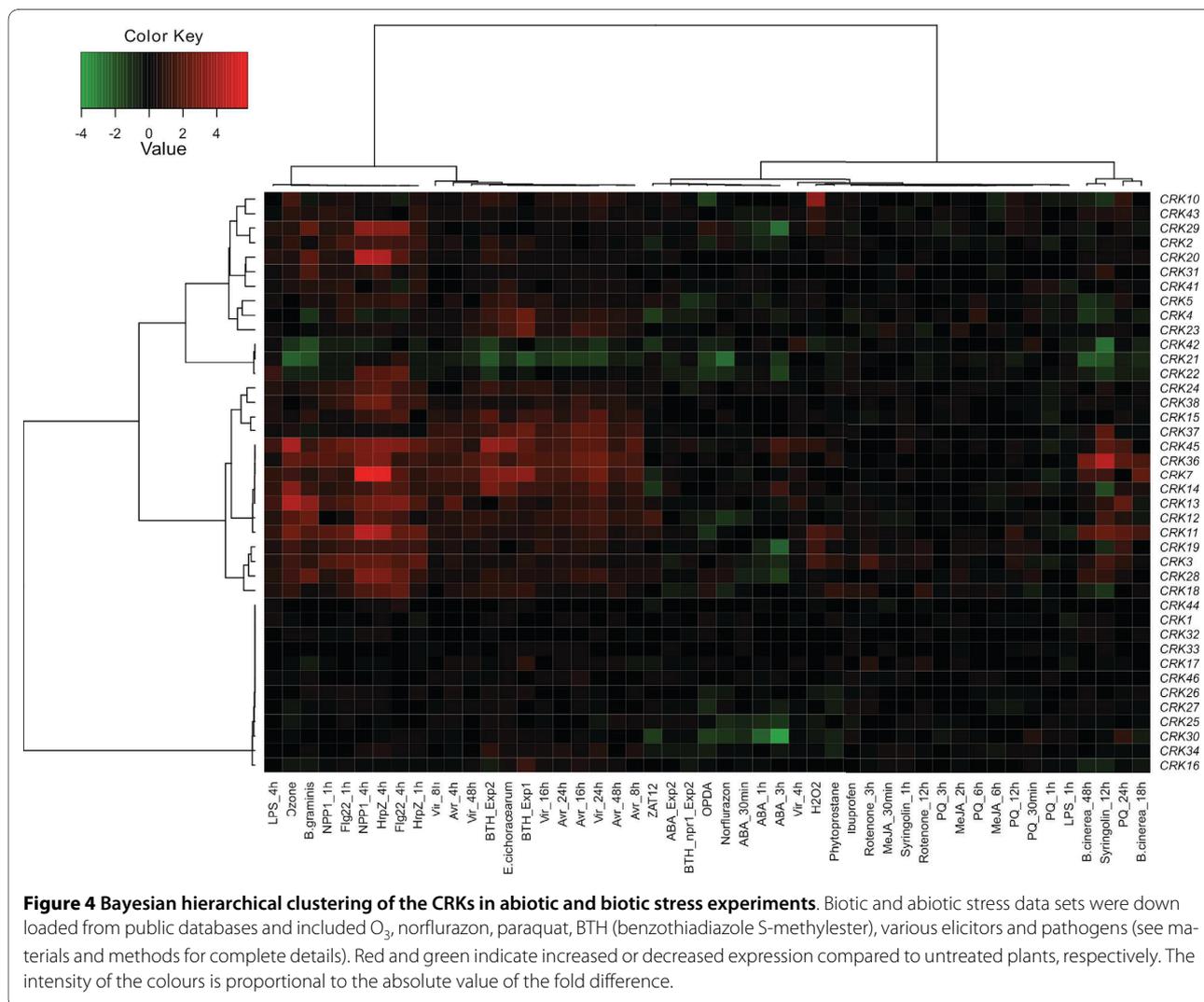
hormone signalling pathways is an established phenomenon [24,37], and the *CRKs* above exemplify that altering the balance of SA, JA or ET response leads to altered gene expression.

To test the robustness of gene expression in this set of hormone mutants, we compared two different growth conditions. These differed in photoperiod, light composition and intensity, soil composition and humidity (see Materials and Methods for a detailed description of the differences in the growth conditions), subsequently referred to as Weiss chamber (Figure 5A) and Phytotron (Figure 5B). Notably, the *dnd1* mutant did not grow under Phytotron conditions. The higher transcript abundance of *CRKs* in *ein2* and *fad3/7/8* observed in plants grown under Weiss chamber growth conditions was largely absent in plants grown under Phytotron growth conditions (Figure 5B). Moreover, the *CRKs* which showed higher gene expression in *npr1* under Weiss chamber growth conditions, were unaltered (or had even reduced transcript levels) in the Phytotron. Taken together, these results indicate that hormones play a

major role in the transcriptional regulation of many *CRKs*. However, environmental growth conditions also have a large impact on the extent of this regulation especially in soil grown plants [49,50].

#### *O<sub>3</sub>*-response of the *CRKs* in hormone mutants

To further study the role of SA, ET and JA in ROS signalling, wild type and the *sid2*, *npr1*, *dnd1*, *ein2* and *fad3/7/8* mutants were exposed to *O<sub>3</sub>*. A subset of 23 *O<sub>3</sub>*-induced and one *O<sub>3</sub>*-repressed *CRKs* were selected for expression analysis in the mutant backgrounds by qPCR (Figure 6). Most *O<sub>3</sub>*-induced *CRKs* exhibited even higher expression levels in *sid2* and *npr1* as compared to wild type, with the exception of *CRK10*, *CRK11*, *CRK20* and *CRK29*. In *ein2*, the magnitude of *CRK* induction was reduced. In the JA-deficient *fad3/7/8* mutant, the increased expression of *CRKs* in response to *O<sub>3</sub>* was in several cases reduced or even absent as compared to wild type plants. Remarkably, *O<sub>3</sub>*-triggered increase in expression of *CRKs* was absent in *dnd1* (Figure 6). In summary, these results suggest that the plant hormones SA, JA and ET play central roles in



the regulation of the expression of the *CRK* subfamily, both under control conditions (clean air), as well as in response to  $O_3$ .

To expand the model for  $O_3$  regulated gene expression, we tested several other  $O_3$  inducible marker genes. These genes were selected to represent "classical" marker genes for SA (including *PATHOGENESIS-RELATED GENE 1* [*PR-1*] and *PATHOGENESIS-RELATED GENE 2* [*PR-2*] and JA/ET (*PLANT DEFENSIN 1.2* [*PDF1.2*]). In addition we selected genes based on our previous  $O_3$  microarray data (*SENESCENCE-ASSOCIATED GENE 21* [*SAG21*] [51]), and genes which have previously been described as JA-regulated (*MONODEHYDROASCORBATE REDUCTASE* [*MDHAR*] [52]) or SA- and NPR1-regulated (*LECTIN-LIKE PROTEIN* [*LLP*] *At5g03350* [53]). The overall regulation of the marker genes was obtained by clustering them in response to biotic and abiotic stress and hormone treatments (Figure 7A). Most of the genes were regulated in response to BTH, biotic stress treatment and  $O_3$ , and the *MDHAR* gene was confirmed as a JA marker gene, as previously reported [52]. However, there was a lack of overall "specificity" in marker gene expression, i.e., several hormones or stresses were altering their expression. The marker genes were next tested with qPCR in the same  $O_3$  samples used for *CRK* expression. The genes were strongly induced in Col-0 wild type plants and in most mutants. However, in *dnd1* the  $O_3$ -induced signalling pathway(s) was evidently blocked since  $O_3$ -induced gene expression was not observed or it was severely reduced. Only *PATHOGENESIS-RELATED GENE 5* (*PR-5*) was weakly induced in *dnd1* at the later time point. The classical SA marker genes *PR-1* and *PR-2* had reduced  $O_3$ -induced increased expression in *sid2* and *npr1*, indicating a role for SA signalling in response to  $O_3$ . The loss of  $O_3$  induction of *MDHAR* in *fad3/7/8* confirmed the importance of JA in regulation of this gene.

#### Light stress response of the *CRKs* in hormone mutants

To elucidate the role of SA, JA and ET in the regulation of *CRK* expression in response to light stress, wild type and the *sid2*, *npr1*, *ein2* and *fad3/7/8* mutants were exposed to light stress and the subset of  $O_3$ -regulated *CRKs* was analyzed by qPCR. The transcriptional repression observed in response to light stress (Figure 3) for a majority of *CRK* family members was even more pronounced for some *CRKs* in *sid2* (Figure 8). Interestingly, several *CRKs* were specifically transcriptionally induced by light stress in the *ein2* mutant. In *fad3/7/8*, most *CRKs* exhibited a transient decrease in gene expression at early time points. However, statistical significance was overall low for the light-dependent regulation of the *CRKs* in the hormone signalling and biosynthesis mutants (Additional file 1).

#### *CRK* promoter analysis

Gene expression is regulated by transcription factors and the promoter elements they bind to. The 500 base pair (bp) and 1000 bp upstream promoter regions of the *CRKs* were inspected for significantly enriched promoter elements based on a list of verified *Arabidopsis* promoter elements (<http://arabidopsis.med.ohio-state.edu/AtcidsDB/bindingsites.html>[54]). The *CRKs* were divided into three groups ("*CRKs* all", "*CRKs*  $O_3$  up" -  $O_3$  increased expression and "*CRKs*  $O_3$  down" -  $O_3$  decreased expression) and searched for significant accumulation of single promoter elements or a combination of promoter elements. Statistical significance was measured with the Fisher exact test using false discovery rate correction [55]. The enrichment was calculated separately for the motifs in both forward and reverse orientations. No elements were enriched in the 1000 bp region for any of the groups or in the 500 bp region of  $O_3$  down genes. One element, the W-box, a target for WRKY transcription factors frequently found in the promoters of SA-regulated genes [56], was significantly overrepresented as a single motif in the group of "*CRKs* all" and "*CRKs*  $O_3$  up" in the 500 bp region (Table 2 and Additional file 3). Interestingly, several pairs of promoter elements were present with high statistical significance in the 500 bp region for the "*CRKs*  $O_3$  up" and "*CRKs* all" groups. Since these were mostly the same for both groups and had high statistical significance for the all group, this indicated that they were probably not responsible for the  $O_3$ -regulation of these genes. The W-box was the only element enriched as a single motif but also present in most pairs of promoter elements. This indicated that the W-box, alone or in combination with other elements, could be a target for the SA and/or pathogen regulation of *CRKs*.

#### Discussion

The RLK family is one of the largest gene families in the *Arabidopsis thaliana* genome. Several RLKs have previously been described to be involved in plant-microbe interactions [14,15,57-59] and abiotic stress [60,61]. Based on statistical analysis of gene expression data, RLKs in general, as well as the *CRK* subfamily, are more likely to have altered expression in response to abiotic and biotic stress than other *Arabidopsis* genes [40,62]. We analyzed the expression profile of the *CRKs* in detail using qPCR and array analysis under various stresses, growth conditions, and in different genetic backgrounds to obtain a better understanding of the signalling pathways leading to transcriptional regulation of the *CRKs* and to elucidate the role of apoplastic ROS in stress signalling.

The use of ROS as signalling molecules is a common feature of many stress responses [25]. Pathogen attack

| A            | Weiss Chamber |             |             |                 |             | B            | Phytotron   |             |             |                 |             |
|--------------|---------------|-------------|-------------|-----------------|-------------|--------------|-------------|-------------|-------------|-----------------|-------------|
|              | <i>sid2</i>   | <i>npr1</i> | <i>ein2</i> | <i>fad3/7/8</i> | <i>dnd1</i> |              | <i>sid2</i> | <i>npr1</i> | <i>ein2</i> | <i>fad3/7/8</i> | <i>dnd1</i> |
| <i>CRK1</i>  | NR            | NR          | NR          | NR              | NR          | <i>CRK1</i>  | NR          | NR          | NR          | NR              | ND          |
| <i>CRK2</i>  | 1.15          | 1.40        | 1.36 *      | 1.59            | 1.96 *      | <i>CRK2</i>  | 1.07        | 1.17        | 1.08        | 1.33            | ND          |
| <i>CRK3</i>  | 0.99          | 1.10        | 1.37 *      | 1.86            | 2.02 *      | <i>CRK3</i>  | 0.83        | 0.53        | 0.99        | 1.22            | ND          |
| <i>CRK4</i>  | 0.88          | 2.33        | 1.42 *      | 1.91            | 2.85 *      | <i>CRK4</i>  | 0.75        | 0.47 *      | 0.87        | 1.27            | ND          |
| <i>CRK5</i>  | 0.55          | 0.37        | 0.83 *      | 0.98 *          | 1.13 *      | <i>CRK5</i>  | 0.86        | 0.22 *      | 0.52        | 1.16            | ND          |
| <i>CRK6</i>  | 0.93 *        | 6.93 *      | 11.10       | 14.72           | 6.50        | <i>CRK6</i>  | 0.32 *      | 0.57        | 0.46        | 1.36            | ND          |
| <i>CRK7</i>  | 0.21          | 0.17        | 0.38 *      | 0.63 *          | 0.66 *      | <i>CRK7</i>  | 0.30 *      | 0.47 *      | 0.89        | 1.20            | ND          |
| <i>CRK8</i>  | 0.12          | 0.14        | 0.38 *      | 0.70 *          | 0.66 *      | <i>CRK8</i>  | 0.35        | 0.43 *      | 0.82        | 1.54            | ND          |
| <i>CRK10</i> | 0.80          | 0.80        | 0.86 *      | 1.08 *          | 1.30 *      | <i>CRK10</i> | 0.83        | 0.39 *      | 0.87        | 0.96            | ND          |
| <i>CRK11</i> | 0.70          | 0.82        | 1.13 *      | 1.60 *          | 1.82        | <i>CRK11</i> | 0.34 *      | 0.38 *      | 0.57        | 0.64            | ND          |
| <i>CRK12</i> | NR            | NR          | NR          | NR              | NR          | <i>CRK12</i> | NR          | NR          | NR          | NR              | ND          |
| <i>CRK13</i> | 0.56          | 0.51        | 1.30 *      | 2.62 *          | 1.43 *      | <i>CRK13</i> | 0.52        | 0.57 *      | 0.79        | 1.14            | ND          |
| <i>CRK14</i> | 0.46          | 0.43        | 0.63 *      | 0.84 *          | 1.16 *      | <i>CRK14</i> | 0.46 *      | 0.19 *      | 0.61        | 0.59            | ND          |
| <i>CRK15</i> | 0.34          | 0.29        | 0.96 *      | 1.35 *          | 1.09 *      | <i>CRK15</i> | 0.58 *      | 0.30 *      | 0.80        | 1.04            | ND          |
| <i>CRK16</i> | 0.85          | 0.68        | 1.50 *      | 1.95            | 2.03 *      | <i>CRK16</i> | 0.83        | 0.78 *      | 0.82        | 0.94            | ND          |
| <i>CRK17</i> | 0.81          | 0.64        | 1.40 *      | 1.78            | 1.87 *      | <i>CRK17</i> | 1.15        | 0.68        | 0.98        | 0.80            | ND          |
| <i>CRK18</i> | 0.69          | 0.71        | 1.25 *      | 1.52            | 1.46 *      | <i>CRK18</i> | 0.92        | 0.77        | 0.88        | 1.06            | ND          |
| <i>CRK19</i> | 1.26 *        | 1.18        | 1.03        | 1.21            | 1.38        | <i>CRK19</i> | 0.66        | 0.08 *      | 0.15        | 0.70            | ND          |
| <i>CRK20</i> | 1.35          | 1.17        | 1.25 *      | 1.80            | 1.74 *      | <i>CRK20</i> | 0.36 *      | 0.31 *      | 0.42        | 0.82            | ND          |
| <i>CRK21</i> | 0.69          | 1.27        | 1.16        | 1.40            | 1.14        | <i>CRK21</i> | 0.86        | 0.70 *      | 0.23        | 0.97            | ND          |
| <i>CRK22</i> | 0.67          | 0.87 *      | 1.42        | 1.75            | 1.58        | <i>CRK22</i> | 0.78        | 0.62 *      | 0.74        | 0.96            | ND          |
| <i>CRK23</i> | 0.65 *        | 6.02 *      | 3.77        | 8.03            | 3.86 *      | <i>CRK23</i> | 0.14 *      | 0.11        | 0.03 *      | 1.12            | ND          |
| <i>CRK24</i> | 0.25          | 0.27        | 0.87 *      | 1.22 *          | 0.95 *      | <i>CRK24</i> | 0.39 *      | 0.40 *      | 0.79        | 0.64            | ND          |
| <i>CRK25</i> | 0.59          | 0.60        | 1.27 *      | 1.56            | 1.82 *      | <i>CRK25</i> | 1.20        | 1.54        | 0.84        | 1.35            | ND          |
| <i>CRK26</i> | 1.68 *        | 2.80        | 2.58 *      | 3.37            | 5.13 *      | <i>CRK26</i> | 0.64        | 0.51        | 0.64        | 0.88            | ND          |
| <i>CRK27</i> | 1.34 *        | 1.58 *      | 1.65        | 2.29            | 2.36        | <i>CRK27</i> | 0.65        | 0.46 *      | 0.58        | 0.64            | ND          |
| <i>CRK28</i> | 1.57          | 1.82        | 1.85 *      | 2.73 *          | 3.13 *      | <i>CRK28</i> | 0.81        | 0.79        | 0.79        | 1.12            | ND          |
| <i>CRK29</i> | 28.94         | 6.33        | 1.82 *      | 3.60 *          | 6.78 *      | <i>CRK29</i> | 0.71 *      | 0.92        | 0.94        | 0.98            | ND          |
| <i>CRK30</i> | 1.01          | 1.29        | 4.21        | 5.14            | 1.21 *      | <i>CRK30</i> | 1.02        | 0.34 *      | 1.23        | 1.00            | ND          |
| <i>CRK31</i> | 0.86          | 0.95        | 1.04 *      | 1.23 *          | 1.36 *      | <i>CRK31</i> | 1.00        | 0.91        | 0.73        | 1.18            | ND          |
| <i>CRK32</i> | 0.96          | 1.15        | 2.76        | 3.88            | 1.78        | <i>CRK32</i> | 0.85        | 0.74        | 0.59        | 0.87            | ND          |
| <i>CRK33</i> | 0.65 *        | 0.47 *      | 1.22 *      | 0.90 *          | 0.44 *      | <i>CRK33</i> | 0.86        | 0.85        | 0.47        | 1.21            | ND          |
| <i>CRK34</i> | 0.68 *        | 0.89 *      | 1.66        | 1.97            | 1.70 *      | <i>CRK34</i> | 0.25        | 0.21 *      | 0.64        | 0.76            | ND          |
| <i>CRK36</i> | 1.51          | 4.78        | 2.76 *      | 4.76            | 4.99 *      | <i>CRK36</i> | 0.55        | 0.60        | 0.75        | 0.86            | ND          |
| <i>CRK37</i> | 1.77          | 5.93        | 1.72 *      | 2.71 *          | 6.59 *      | <i>CRK37</i> | 0.32        | 0.55        | 0.38        | 0.69            | ND          |
| <i>CRK38</i> | 1.18 *        | 2.21 *      | 1.82        | 2.14            | 2.19 *      | <i>CRK38</i> | 0.56 *      | 0.65        | 0.79        | 0.92            | ND          |
| <i>CRK39</i> | 1.11 *        | 4.54        | 5.18        | 11.16           | 3.36        | <i>CRK39</i> | 0.29 *      | 0.38 *      | 0.48        | 0.64            | ND          |
| <i>CRK40</i> | 1.47          | 1.82        | 1.92 *      | 2.48            | 2.31 *      | <i>CRK40</i> | 0.68 *      | 0.58        | 0.73        | 0.77            | ND          |
| <i>CRK41</i> | 0.71          | 0.86        | 1.32 *      | 1.58            | 1.14 *      | <i>CRK41</i> | 0.88        | 0.68        | 1.01        | 0.92            | ND          |
| <i>CRK42</i> | 1.74          | 1.67        | 1.16        | 1.22            | 1.36        | <i>CRK42</i> | 0.72        | 0.57        | 0.52 *      | 0.44            | ND          |
| <i>CRK43</i> | 1.24          | 1.10        | 0.51 *      | 0.67 *          | 1.58 *      | <i>CRK43</i> | 1.09        | 0.78        | 1.08        | 1.32            | ND          |
| <i>CRK44</i> | 0.38          | 0.44        | 1.17 *      | 1.28 *          | 1.37 *      | <i>CRK44</i> | 1.00        | 1.03        | 1.48        | 1.12            | ND          |
| <i>CRK45</i> | 1.33          | 6.03        | 2.01 *      | 3.03 *          | 5.51 *      | <i>CRK45</i> | 0.63        | 0.69        | 1.09        | 0.99            | ND          |
| <i>CRK46</i> | 1.39          | 0.76        | 16.73       | 10.29           | 1.91        | <i>CRK46</i> | 0.88        | 1.56        | 0.80        | 0.55            | ND          |

**Figure 5 Expression of CRKs is changed in hormone mutants.** The expression of all *CRKs* was analyzed by qPCR in the SA mutants *sid2* and *npr1*, the ET mutant *ein2*, the JA mutant *fad3/7/8* and the cell death mutant *dnd1* by qPCR and compared to Col-0 under two different growth conditions. (A) Weiss chamber conditions. (B) Phytotron. Transcript levels were calculated by comparison between mutants and Col-0 grown under control conditions. An expression level of one indicates no change of expression, increased expression is indicated by values larger than one while decreased expression is shown by values smaller than one. Increase of expression by 2-fold or higher is high-lighted in red and decrease in expression by 2-fold or more in green. NR - no reproducible data could be obtained for this gene. ND - The *dnd1* mutant did not grow in the phytotron. Fold-change is shown for the geometric mean of all biological repeats (n = 4). Statistical significance (Benjamini-Hochberg FDR corrected p-value ≤ 0.1) is indicated with asterisks (see additional file 1).

|              | Col-0   |         | <i>sid2</i> |         | <i>npr1</i> |         | <i>ein2</i> |         | <i>fad3/7/8</i> |         | <i>dnd1</i> |         |
|--------------|---------|---------|-------------|---------|-------------|---------|-------------|---------|-----------------|---------|-------------|---------|
|              | 1 h     | 6 + 2 h | 1 h         | 6 + 2 h | 1 h         | 6 + 2 h | 1 h         | 6 + 2 h | 1 h             | 6 + 2 h | 1 h         | 6 + 2 h |
| <i>CRK2</i>  | 2.96 *  | 1.20    | 4.33 *      | 1.61    | 4.39 *      | 2.59 *  | 2.78 *      | 1.49 *  | 1.18            | 0.56    | 1.23        | 1.39    |
| <i>CRK3</i>  | 2.52    | 0.87    | 4.55 *      | 1.82    | 3.24 *      | 2.44 *  | 3.19 *      | 1.61 *  | 0.72            | 0.91    | 0.99        | 1.29    |
| <i>CRK6</i>  | 3.40    | 3.74    | 15.31 *     | 9.16 *  | 13.91 *     | 21.49 * | 3.01 *      | 2.74 *  | 3.19            | 1.70    | 0.89        | 1.18    |
| <i>CRK7</i>  | 2.21    | 10.58 * | 7.87        | 10.44 * | 3.45 *      | 26.22 * | 1.29        | 1.43 *  | 1.86            | 3.02    | 1.14        | 1.18    |
| <i>CRK8</i>  | 7.72 *  | 4.40 *  | 14.52 *     | 8.82 *  | 10.99 *     | 17.59 * | 3.59 *      | 4.13 *  | 2.43            | 1.22    | 1.00        | 1.37    |
| <i>CRK10</i> | 11.29 * | 1.92    | 8.61 *      | 2.66    | 14.37 *     | 2.41 *  | 6.19 *      | 3.31    | 1.75            | 0.67    | 0.55        | 1.17    |
| <i>CRK11</i> | 29.19 * | 3.27 *  | 18.83 *     | 4.33    | 23.76 *     | 4.08 *  | 12.44 *     | 5.20 *  | 0.70            | 0.98    | 1.01        | 1.59    |
| <i>CRK13</i> | 2.34    | 9.68 *  | 13.06 *     | 12.17 * | 6.14 *      | 20.41 * | 1.26        | 1.77 *  | 0.94            | 4.16    | 0.33        | 2.36    |
| <i>CRK14</i> | 19.10 * | 3.39 *  | 24.13 *     | 10.11 * | 27.35 *     | 9.42 *  | 6.10 *      | 4.21 *  | 2.21            | 0.87    | 1.05        | 1.40    |
| <i>CRK15</i> | 2.74    | 8.14 *  | 14.13 *     | 10.84 * | 11.17 *     | 13.81 * | 2.58 *      | 2.80 *  | 3.20            | 2.68    | 1.25        | 1.36    |
| <i>CRK18</i> | 1.27    | 1.68    | 1.92 *      | 1.49 *  | 1.75 *      | 2.31 *  | 1.45 *      | 1.09 *  | 0.93            | 0.97    | 1.20        | 1.17    |
| <i>CRK19</i> | 3.76    | 0.79    | 1.34        | 2.19    | 1.05        | 2.11 *  | 1.45        | 1.03 *  | 0.67            | 1.21    | 1.10        | 1.29    |
| <i>CRK20</i> | 4.39    | 0.90    | 3.71        | 1.32    | 0.79        | 0.32    | 2.48        | 1.40    | 2.31            | 0.16    | 1.09        | 1.50    |
| <i>CRK22</i> | 0.57    | 1.2E-03 | 1.32        | 1.46    | 0.69 *      | 1.77 *  | 1.18        | 0.79 *  | 0.85            | 1.15    | 1.19        | 0.98    |
| <i>CRK27</i> | 2.46    | 3.41    | 1.26 *      | 2.31 *  | 1.38        | 3.34 *  | 1.51        | 1.12 *  | 1.88            | 1.27    | 1.00        | 1.44    |
| <i>CRK29</i> | 5.98    | 2.24    | 5.33        | 32.47   | 5.02        | 3.73    | 2.73 *      | 1.50    | 1.03            | 0.86    | 0.89        | 1.01    |
| <i>CRK31</i> | 0.69    | 1.64 *  | 3.62 *      | 2.40    | 4.78 *      | 3.56 *  | 3.46 *      | 2.38 *  | 0.85            | 0.71    | 0.96        | 1.66    |
| <i>CRK32</i> | 0.38    | 3.63    | 3.23 *      | 2.84 *  | 2.62        | 4.64    | 2.52 *      | 2.22 *  | 3.32            | 1.10    | 1.43        | 0.83    |
| <i>CRK36</i> | 4.90 *  | 10.64 * | 8.22 *      | 31.73 * | 11.89 *     | 29.70 * | 2.83 *      | 2.54 *  | 1.11            | 1.64    | 1.06        | 1.52    |
| <i>CRK37</i> | 3.26    | 16.13 * | 3.89 *      | 38.76 * | 5.32 *      | 47.12 * | 1.03        | 1.30 *  | 1.10            | 3.51    | 1.16        | 1.64    |
| <i>CRK39</i> | 5.95    | 30.45 * | 32.78 *     | 35.71 * | 44.74 *     | 76.96 * | 3.12        | 2.55 *  | 0.86            | 1.91    | 1.28        | 0.99    |
| <i>CRK41</i> | 12.51 * | 1.96    | 10.43 *     | 4.95 *  | 14.01 *     | 3.56 *  | 8.47 *      | 4.47 *  | 0.62            | 0.75    | 1.02        | 1.72    |
| <i>CRK45</i> | 3.09 *  | 23.31 * | 6.85 *      | 48.70 * | 6.49 *      | 56.52 * | 1.48        | 1.54 *  | 1.71            | 4.50    | 1.19        | 1.38    |
| <i>CRK46</i> | 0.26    | 0.49    | 0.84        | 1.48    | 1.55        | 1.19    | 0.42        | 1.37    | 1.52            | 0.83    | 0.68        | 1.40    |

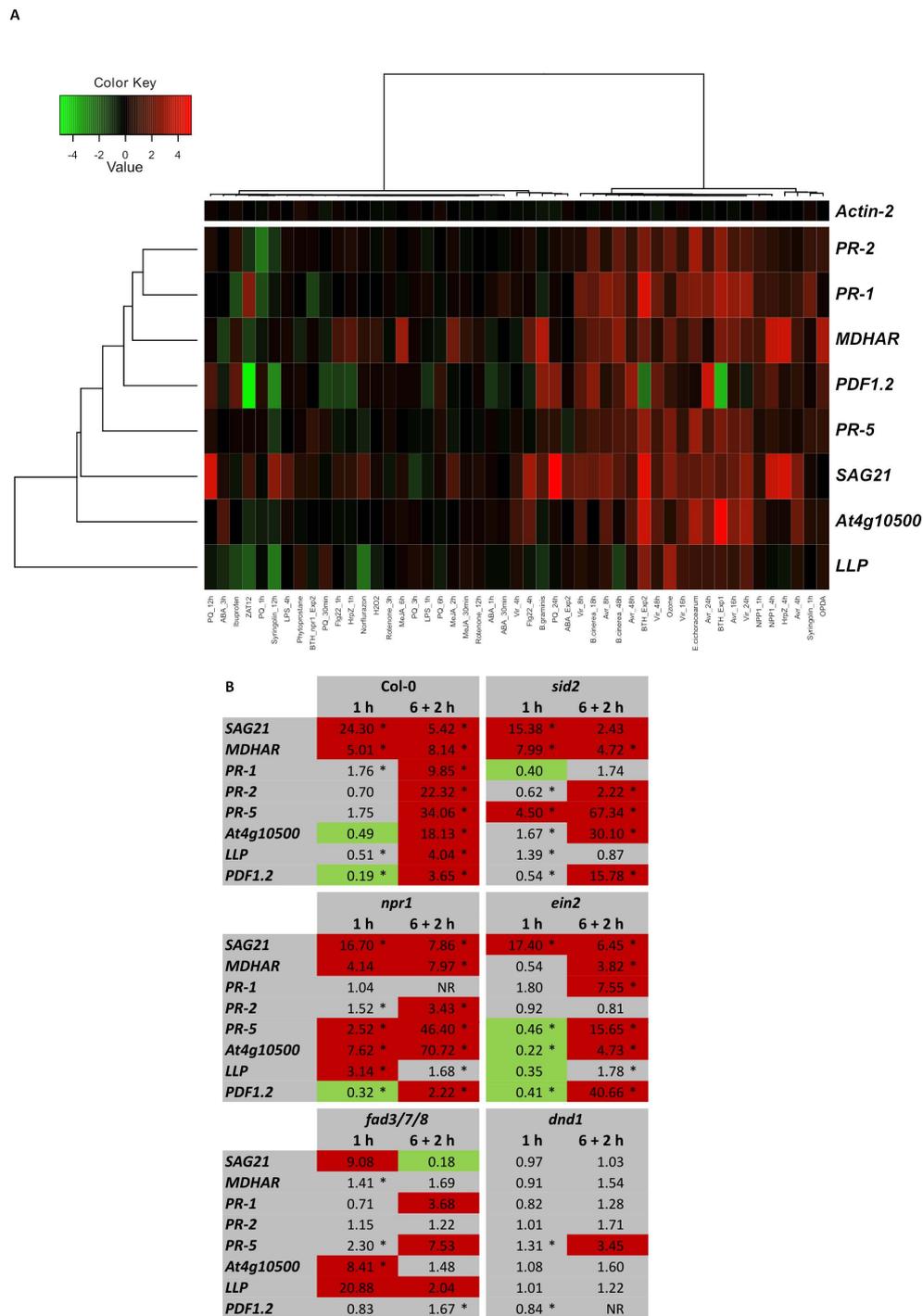
**Figure 6 O<sub>3</sub>-regulation of CRKs is different in hormone mutants.** The expression of 24 O<sub>3</sub>-regulated CRKs was analyzed by qPCR in Col-0 and *sid2*, *npr1*, *dnd1*, *ein2* and *fad3/7/8* exposed to 250 ppb O<sub>3</sub> for 6 h. Samples were harvested at 1 or 8 h (6 h plus 2 h recovery under clean air conditions) after the onset of the O<sub>3</sub> treatment. Transcript levels for Col-0 or each mutant line were calculated by comparison of O<sub>3</sub>-exposed plants with corresponding control plants of the same line grown under clean air conditions. An expression level of one indicates no change of expression, increased expression is indicated by values larger than one while decreased expression is shown by values smaller than one. Increase of expression by 2-fold or higher is high-lighted in red and decrease in expression by 2-fold or more in green. NR - no reproducible data could be obtained for this gene. The experiment was repeated four times; fold change was calculated from the average normalized cycle difference of all biological repeats. Statistical significance (Benjamini-Hochberg FDR corrected p-value ≤ 0.1) is indicated with asterisks (see additional file 1).

and perception of PAMPs are often associated with an oxidative burst in the apoplast [63]. Similarly, a hallmark of the early O<sub>3</sub> response is the generation of an oxidative burst in the apoplast [64]. ROS are also produced in other subcellular compartments, including the chloroplast, where light stress or treatments with the herbicides paraquat or norflurazon elicit elevated ROS production. In addition, crosstalk between pathways elicited by apoplastic ROS and chloroplast-derived ROS is important for the regulation of cell death [32]. The transcriptional response to apoplastic ROS, e.g. induced by O<sub>3</sub>, is strikingly different from chloroplast-derived ROS, e.g., induced by paraquat [30]. To further dissect the role of apoplastic ROS, we clustered several treatments triggering ROS production in distinct subcellular compartments together with various biotic stress experiments. Our results showed that the CRK expression profile upon O<sub>3</sub> exposure was most similar to those stimulated by PAMP perception (*flg22* and *HrpZ*) and pathogen infection (*Bgh*) (Figure 4). By contrast, treatments, which increased ROS levels in the chloroplast (norflurazon and paraquat) or mitochondria (rotenone; which might also lead to ROS production in the chloroplast [65]) either had no effect on CRK gene expression or resulted in down-regulation. These results

show that transcriptional induction of the CRKs can be triggered by apoplastic ROS, whereas chloroplastic ROS mainly lead to decreased expression. Furthermore, cluster analysis separated the effects of plant hormones: BTH (SA analog) caused a similar expression profile as O<sub>3</sub> and PAMP treatments, whereas CRK expression in response to ABA and MeJA was related to norflurazon and paraquat treatments.

To extend the microarray meta-analysis, transcript accumulation of the CRK subfamily was monitored in response to O<sub>3</sub> and light stress by qPCR. Out of 44 CRKs, 32 showed increased expression after exposure to O<sub>3</sub> at both time points while five members exhibited decreased expression. Light stress treatment led to a decrease in expression of the majority of the CRKs. Thus, in agreement with the results from array analysis, ROS production in different cellular compartments produces strikingly different transcriptional profiles on the CRK gene subfamily.

To further dissect the O<sub>3</sub> response, mutants deficient in biosynthesis, perception and signalling of SA (*sid2*, *npr1*), JA (*fad3/7/8*) and ET (*ein2*) were exposed to O<sub>3</sub> and the expression of a subset of CRKs was analyzed by qPCR. The O<sub>3</sub>-induced increase in transcript levels of the CRKs



**Figure 7 Clustering and qPCR analysis of the marker genes.** (A) The expression of eight O<sub>3</sub>-inducible genes and the qPCR normalization gene *Actin-2* were analyzed in public array data from biotic and abiotic stress and hormone treatments. Red and green indicate increased or decreased expression compared to untreated plants, respectively. The intensity of the colours is proportional to the absolute value of the fold difference. (B) Markers genes for O<sub>3</sub> responses were analyzed by qPCR in Col-0 and *sid2*, *npr1*, *dnd1*, *ein2* and *fad3/7/8* exposed to 250 ppb O<sub>3</sub> for 6 h. Samples were harvested at 1 or 8 h (6 h plus 2 h recovery under clean air conditions) after the onset of the O<sub>3</sub> treatment. Transcript levels were calculated by comparison of O<sub>3</sub>-exposed plants with corresponding control plants grown under clean air conditions. An expression level of one indicates no change of expression, increased expression is indicated by values larger than one while decreased expression is shown by values smaller than one. Increase of expression by 2-fold or higher is high-lighted in red and decrease in expression by 2-fold or more in green. NR - no reproducible data could be obtained for this gene. The experiment was repeated four times; fold change was calculated from the average normalized cycle difference of all biological repeats. Statistically significance (Benjamini-Hochberg FDR corrected p-value ≤ 0.1) is indicated with asterisks (see additional file 1).

|              | Col-0 |      |         | <i>sid2</i> |      |         | <i>npr1</i> |      |         | <i>ein2</i> |        |         | <i>fad3/7/8</i> |      |         |
|--------------|-------|------|---------|-------------|------|---------|-------------|------|---------|-------------|--------|---------|-----------------|------|---------|
|              | 1 h   | 2 h  | 4 + 4 h | 1 h         | 2 h  | 4 + 4 h | 1 h         | 2 h  | 4 + 4 h | 1 h         | 2 h    | 4 + 4 h | 1 h             | 2 h  | 4 + 4 h |
| <i>CRK2</i>  | 1.23  | 1.06 | 0.54    | 0.69        | 0.23 | 0.61    | 0.60        | 0.58 | 1.23    | 0.75        | 0.32   | 0.46    | 0.62            | 0.49 | 0.84    |
| <i>CRK3</i>  | 0.62  | 0.53 | 0.68    | 0.58        | 0.46 | 0.64    | 1.48        | 0.37 | 1.25    | 9.09        | 0.23   | 0.42    | 4.44            | 0.46 | 0.98    |
| <i>CRK6</i>  | 0.98  | 1.47 | 0.34    | 1.99        | 0.58 | 0.56 *  | 2.70        | 0.61 | 0.69    | 42.38       | 1.03   | 0.49    | 0.39            | 4.31 | 0.64    |
| <i>CRK7</i>  | 0.74  | 1.37 | 0.42    | 1.11        | 0.45 | 0.66    | 1.47        | 1.27 | 0.46    | 3.84        | 1.24   | 0.35    | 0.67            | 2.02 | 1.10    |
| <i>CRK8</i>  | 1.61  | 2.45 | 0.59    | 1.12        | 0.85 | 1.04    | 1.72        | 1.58 | 1.03    | 9.96        | 1.79   | 0.75    | 0.53            | 3.47 | 1.64    |
| <i>CRK10</i> | 1.26  | 0.72 | 0.80    | 1.46        | 0.28 | 0.70    | 2.30        | 1.00 | 0.66    | 7.20        | 0.47   | 0.65    | 0.98            | 0.64 | 1.00    |
| <i>CRK11</i> | 1.93  | 0.49 | 0.67    | 3.00        | 0.55 | 0.77    | 2.60        | 1.31 | 0.85    | 21.33       | 0.79   | 0.60    | 1.57            | 1.04 | 1.24    |
| <i>CRK13</i> | 0.66  | 1.73 | 1.64    | 0.80        | 0.72 | 0.85    | 1.43        | 1.33 | 1.48    | 9.12        | 0.88   | 1.12    | 0.33            | 0.90 | 1.20    |
| <i>CRK14</i> | 1.73  | 1.10 | 0.66    | 3.87        | 0.51 | 0.52    | 4.49        | 2.68 | 0.52    | 30.24       | 1.08   | 0.56    | 1.48            | 2.16 | 1.43    |
| <i>CRK15</i> | 0.98  | 0.90 | 0.57    | 1.81        | 0.27 | 0.36    | 2.44        | 0.76 | 0.39    | 11.28       | 0.64   | 0.52    | 0.85            | 1.33 | 0.95    |
| <i>CRK18</i> | 1.07  | 1.70 | 0.85    | 1.14        | 0.92 | 0.85    | 1.13        | 1.34 | 0.78    | 5.19        | 1.16 * | 0.86    | 0.02            | 1.69 | 1.52    |
| <i>CRK19</i> | 1.09  | 1.03 | 0.41    | 0.39        | 0.41 | 1.02    | 0.82        | 1.17 | 1.40    | 4.28        | 0.76   | 0.54    | 0.55            | 0.80 | 0.67    |
| <i>CRK20</i> | 0.58  | 1.47 | 0.42    | 0.55        | 0.20 | 0.37    | 0.55        | 0.48 | 0.62    | 5.09        | 0.30   | 0.52    | 0.00            | 0.28 | 0.54    |
| <i>CRK22</i> | 0.44  | 0.28 | 0.40    | 0.45        | 0.17 | 0.43    | 0.48        | 0.43 | 1.10    | 0.75        | 0.21   | 0.65    | 0.23            | 0.32 | 0.87    |
| <i>CRK27</i> | 1.46  | 1.18 | 1.54    | 1.08        | 0.53 | 0.33    | 0.57        | 1.02 | 1.88    | 1.88        | 1.28   | 0.24    | 0.68            | 2.19 | 0.71    |
| <i>CRK29</i> | 2.07  | 1.15 | 1.15    | 1.04        | 1.14 | 1.07    | 1.18        | 0.49 | 1.54    | 1.19        | 0.75   | 1.32    | 0.64            | 0.92 | 1.00    |
| <i>CRK31</i> | 1.29  | 1.27 | 0.82    | 1.29        | 0.79 | 0.77    | 0.67        | 0.55 | 0.72    | 2.14        | 1.23   | 0.65    | 0.67            | 1.26 | 0.64    |
| <i>CRK32</i> | 1.34  | 1.26 | 1.22    | 1.02        | 0.78 | 1.10    | 1.03        | 1.36 | 0.81    | 2.86        | 2.62   | 0.69    | 0.29            | 2.74 | 0.88    |
| <i>CRK36</i> | 0.83  | 2.08 | 0.61    | 1.33        | 1.18 | 0.67    | 2.64        | 1.00 | 0.45    | 37.33       | 1.37   | 0.58    | 1.01            | 2.61 | 1.45    |
| <i>CRK37</i> | 0.64  | 1.27 | 0.32    | 1.15        | 0.78 | 0.64    | 1.95        | 0.82 | 0.23    | 9.00        | 1.02   | 0.50    | 0.70            | 2.32 | 1.23    |
| <i>CRK39</i> | 0.44  | 1.03 | 1.32    | 1.02        | 0.88 | 0.97    | 2.17        | 1.21 | 0.22    | 16.34       | 0.63   | 0.54    | 0.72            | 1.00 | 0.85    |
| <i>CRK41</i> | 1.73  | 1.33 | 0.95    | 1.24        | 0.44 | 0.75    | 1.15        | 0.44 | 2.28    | 1.47        | 0.97   | 0.49    | 1.59            | 1.20 | 1.07    |
| <i>CRK45</i> | 1.06  | 0.86 | 0.48    | 2.25 *      | 0.45 | 0.25    | 0.56        | 0.45 | 1.08    | 3.09        | 1.08   | 0.21    | 1.15            | 2.56 | 1.13    |
| <i>CRK46</i> | 1.69  | 1.09 | 0.89    | 2.02        | 1.22 | 1.38    | 0.30        | 0.22 | 0.26    | 1.45        | 0.62   | 0.74    | 0.24            | 1.08 | 1.38    |

**Figure 8 Light stress response in hormone mutants.** The expression of 24 O<sub>3</sub>-inducible *CRKs* was analyzed by qPCR in Col-0 and *sid2*, *npr1*, *ein2* and *fad3/7/8* after 1 h and 2 h exposure to light stress conditions, and after 4 h light stress followed by 4 h recovery at normal growth light conditions. Transcript levels were calculated by comparison of light stress-treated plants with the corresponding control plants grown under normal light conditions. An expression level of one indicates no change of expression, increased expression is indicated by values larger than one while decreased expression is shown by values smaller than one. Increase in expression by 2-fold or higher is high-lighted in red and decrease in expression by 2-fold or more in green. NR - no reproducible data could be obtained for this gene. The experiment was repeated twice; fold change was calculated from the average normalized cycle difference of all biological repeats. Statistical significance (Benjamini-Hochberg FDR corrected p-value ≤ 0.1) is indicated with asterisks (see additional file 1).

was higher in *sid2* and *npr1* implying that SA acts as a negative regulator of the ROS signalling pathway. The O<sub>3</sub>-mediated transcriptional induction of *CRKs* was almost abolished in *fad3/7/8* and attenuated in *ein2*, suggesting that JA, and to a lesser extent ET are required for the proper transcriptional induction of *CRKs* in response to O<sub>3</sub>. This role for SA, JA and ET in O<sub>3</sub> signalling has been previously proposed based on the results from cDNA macroarray analysis [66]. The effect of light stress on the *CRK* expression in various mutant backgrounds was very different compared to the effect of the O<sub>3</sub> response. Whereas ET acts as positive regulator of *CRK* expression in the O<sub>3</sub> response, it appears to be a negative regulator in light stress since several *CRKs* displayed light stress-induced expression only in the *ein2* mutant (Figure 8). Under light stress conditions, the decreased expression of *CRKs* seen in wild type was even more pronounced in the SA mutants *sid2* and *npr1* and the JA mutant *fad3/7/8*.

*DND1* encodes CYCLIC NUCLEOTIDE GATED CHANNEL2 (CNGC2) which transports Ca<sup>2+</sup> into the cell and regulates nitric oxide production [67]. The complete lack of an effect of O<sub>3</sub> on *CRK* and marker gene expression in *dnd1* suggests an important role for CNGC2 in the O<sub>3</sub> response pathway, possibly by regulat-

ing Ca<sup>2+</sup> levels (Figure 6 and 7B). Previous studies have shown that O<sub>3</sub> rapidly invokes Ca<sup>2+</sup> transients [68,69] and blocking of Ca<sup>2+</sup> transport can prevent ROS-induced cell death [70]. The *dnd1* mutant also has several pleiotropic phenotypes which include elevated SA levels and constitutive defence responses [47]. Consequently, the lack of O<sub>3</sub> response in *dnd1* could be due to "dominance" of SA signaling over the ROS signalling pathway, and O<sub>3</sub> would have no effect when the SA pathway is fully stimulated. Previous reports have shown that several members of the *CRK* subfamily were transcriptionally induced through an external application of SA [19] or BTH (Figure 4). The response of *CRKs* to BTH was completely blocked in *npr1*, indicating that the SA pathway for regulating *CRKs* requires NPR1.

Intriguingly, different growth conditions had a strong impact on the expression of *CRKs* in various mutants. Several *CRKs* were expressed to higher levels in *ein2* and *fad3/7/8* in Weiss chamber-grown plants compared to Phytotron-grown plants. In contrast, the decreased expression of several *CRKs* in *sid2* and *npr1* was similar between two different growth conditions (Weiss chamber and Phytotron, Figure 5). A strong effect of environmental conditions on mutant phenotypes, transcript profiles

**Table 2: Motifs overrepresented in the promoters of the CRK family.**

| Promoter motifs |                           |        |                                     |         |                 |
|-----------------|---------------------------|--------|-------------------------------------|---------|-----------------|
| Promoter motifs |                           |        |                                     |         |                 |
| Number          | Subset                    | Region | Motif                               | q-value | Motif name      |
| 1               | CRKs O <sub>3</sub> up    | 500 bp | TTGAC(+)                            | 0.05    | W-box           |
| 2               | CRKs all                  | 500 bp | TTGAC(+/-)                          | 0.05    | W-box           |
| 3               | CRKs all                  | 500 bp | TTGAC(+)                            | 0.01    | W-box           |
| 4               | CRKs all                  | 500 bp | TTGAC(-)                            | 0.01    | W-box           |
| 5               | CRKs O <sub>3</sub> up up | 500 bp | ACACNNG(+/-) × TTGAC(+)             | 0.00    | DPBF1&2 × W-box |
| 6               | CRKs O <sub>3</sub> up    | 500 bp | ACACNNG(-) × ACTTTG(+)              | 0.05    | DPBF1&2 × T-box |
| 7               | CRKs O <sub>3</sub> up    | 500 bp | ACACNNG(-) × TTGAC(+)               | 0.05    | DPBF1&2 × W-box |
| 8               | CRKs O <sub>3</sub> up    | 500 bp | A [AC]C [AT]A [AC]C(-) × TTGAC(+)   | 0.05    | MYB4 × W-box    |
| 9               | CRKs O <sub>3</sub> up    | 500 bp | CAACA(-) × TTGAC(+)                 | 0.05    | RAV1-A × W-box  |
| 10              | CRKs O <sub>3</sub> up    | 500 bp | CAACA(-) × TTGAC(-)                 | 0.05    | RAV1-A × W-box  |
| 11              | CRKs O <sub>3</sub> up    | 500 bp | ACACNNG(-) × A [AC]C [AT]A [AC]C(-) | 0.05    | DPBF1&2 × MYB4  |
| 12              | CRKs O <sub>3</sub> up    | 500 bp | ACACNNG(-) × TTGAC(-)               | 0.05    | DPBF1&2 × W-box |
| 13              | CRKs O <sub>3</sub> up    | 500 bp | A [AC]C [AT]A [AC]C(-) × TTGAC(-)   | 0.05    | MYB4 × W-box    |
| 14              | CRKs all                  | 500 bp | ACACNNG(+/-) × TTGAC(+)             | 0.03    | DPBF1&2 × W-box |
| 15              | CRKs all                  | 500 bp | ACTTTG(+/-) × TTGAC(-)              | 0.04    | T-box × W-box   |
| 16              | CRKs all                  | 500 bp | ACACNNG(-) × TTGAC(+)               | 0.05    | DPBF1&2 × W-box |
| 17              | CRKs all                  | 500 bp | GATAAG(-) × AAATTAGT(+)             | 0.05    | lbox × BS2      |
| 18              | CRKs all                  | 500 bp | CAACA(-) × TTGAC(+)                 | 0.05    | RAV1-A × W-box  |
| 19              | CRKs all                  | 500 bp | CAACA(-) × TTGAC(-)                 | 0.01    | RAV1-A × W-box  |
| 20              | CRKs all                  | 500 bp | ACACNNG(-) × TTGAC(-)               | 0.03    | DPBF1&2 × W-box |
| 21              | CRKs all                  | 500 bp | GATAAG(-) × ACTAATTT(-)             | 0.03    | lbox × BS3      |
| 22              | CRKs all                  | 500 bp | A [AC]C [AT]A [AC]C(-) × TTGAC(-)   | 0.03    | MAB4 × W-box    |

The promoters of the CRK family were analyzed for enrichment of *Arabidopsis* verified promoter elements. Enrichment was calculated for single and double motifs in both plus and minus orientation. The CRKs were divided into three groups for the analysis: "CRKs all", "CRKs O<sub>3</sub> up" - O<sub>3</sub> increased expression and "CRKs O<sub>3</sub> down" - O<sub>3</sub> decreased expression. (+) motif on forward strand, (-) motif on reverse strand, (+/-) motif on either forward or reverse strand. The CRKs containing the respective motifs are shown in additional file

and other parameters are well known and a common problem when comparing results from different laboratories [71]. There could be several reasons for the differences in the expression levels of the *CRKs* between the Weiss chambers and the Phytotron growth conditions. Plants were tested at slightly different ages and grown in different soil (see materials and methods section). Illumination in the Weiss chambers was provided using fluorescent lamps while in lighting in the Phytotron was using metal halide lamps with different light spectra. Notably, the *CRKs* are responsive to UV-B [40]. This suggests that light conditions could have an effect on the expression profile of this RLK family. Another reason for this variation of gene expression could be that under control conditions most *CRKs* were expressed at very low levels; consequently, a minor perturbation either by genetic mutation or growth condition could lead to altered expression. Thus, expression of *CRKs* is very sensitive to the surrounding environment. Similar observations have been reported for the expression of the classical *PDF1.2* marker gene [49,50]. This gene has long been used to exemplify co-regulation by JA/ET. However, *PDF1.2* is only regulated by both hormones when plants are grown *in vitro* [49]. When plants are grown in soil, either hormone alone (JA or ET) is sufficient to induce expression. Thus, growth in soil is able to induce or prime defence signalling pathways.

## Conclusions

Based on the *CRK* expression patterns and integrating current knowledge of ROS signalling, PAMP perception and light responses [25,26,38,72], we propose a model for the regulation of increased expression of the *CRKs* (Figure 9): O<sub>3</sub> induces ROS production in the apoplast which is perceived by putative "ROS receptors" (or by other mechanisms) amplified by PLANT RESPIRATORY BURST OXIDASE HOMOLOG (RBOH)-mediated O<sub>2</sub><sup>-</sup> production, thus leading to activation of DND1/CNGC2. This activates further down-stream signalling events where JA and to a lesser extent ET act as positive regulators, and SA and NPR1 as negative regulators of *CRK* expression. Eventually, the signal reaches the nucleus where transcription factors bind to a "ROS" promoter element and activate transcription. In parallel, the genes are also regulated through a SA (synthesized by ICS1) and NPR1-dependent pathway converging on the W-box promoter element. Microbes and PAMPs could activate both pathways at different timing; a rapid pathway would act through a RBOH mediated ROS production and use the "ROS pathway", while a later "SA pathway" requires increased SA biosynthesis and NPR1. Further interconnections between the pathways are provided by the primary ET transcription factors ETHYLENE

INSENSITIVE 3 (EIN3) and ETHYLENE INSENSITIVE 3-LIKE (EIL1) which repress *SID2/ICS1* expression and thus decrease SA levels [73]. Light stress or chemical treatments that increase ROS in the chloroplast activate separate signalling pathway(s) mainly leading to repression of *CRK* expression, which could involve ABA and negative crosstalk with the SA pathway.

Is it possible to separate the roles of chloroplastic and apoplastic ROS in the regulation of *CRK* expression? Chloroplast-derived ROS production is known to be involved in the regulation of cell death during pathogen infection and in response to abiotic stress [74,75]. Specific removal of chloroplastic ROS prevents pathogen-induced cell death but has no impact on defence gene expression [75]. Furthermore, chloroplastic <sup>1</sup>O<sub>2</sub> regulates cell death dependent on EXECUTER1 [31]. In comparison, apoplastic ROS might be involved with intra- and intercellular signalling [76]. Thus, apoplastic ROS would have a role in regulating defence gene expression and chloroplastic ROS in regulation of cell death. In addition, there is crosstalk between apoplastic ROS and chloroplast ROS; rapid ROS production in the chloroplast can be detected in response to O<sub>3</sub> and blocking of ROS production in the chloroplast reduces O<sub>3</sub>-induced cell death [32,77]. Clearly, ROS regulation of defence signalling and/or cell death is very complex and several other regulatory components have been identified, including LESION SIMULATING DISEASE 1 (LSD1), ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and PHYTOALEXIN DEFICIENT 4 (PAD4), which are also involved in acclimation to light stress [42,78]. The only known phenotypes for *CRKs* have been obtained by ectopic overexpression, which induces HR-like cell death independent or dependent on SA signalling (depending on the specific *CRK*) [22,23]. How this induction of cell death might be achieved is still unclear since transcriptional regulation of *CRKs* occurs in response to apoplastic rather than chloroplastic ROS. Some members of the RLK family might participate in a positive feed-forward loop to regulate ROS production, defence gene expression, cell death and hormone signalling. This regulatory loop might be deregulated after overexpression of the *CRKs* leading to the observed cell death phenotypes. However, this will require experimental verification in the future.

What is the role of *CRKs* in plants and why are they regulated by PAMPs and O<sub>3</sub> treatment? The external domain of these RLKs could be the receptor for as yet uncharacterized PAMPs and they could be part of plant immune responses. An intriguing feature of the DUF26 domain is the presence of a conserved cysteine motif C-8X-C-2X-C. The configuration of cysteines is similar to the cysteine motif in the GRIM REAPER protein, which

has been shown to be involved in the regulation of ROS induced cell death [79]. Despite the ubiquitous role of ROS as signalling molecules in plants, no direct receptor for ROS has been described. Since cysteines are sensitive to redox modifications, could the DUF26 domain act as sensor of ROS in the apoplast and be the putative ROS sensor as depicted in Figure 9?

## Methods

### Plant growth conditions and treatments

#### Weiss chamber growth conditions

For exposure to O<sub>3</sub>, *Arabidopsis thaliana* Col-0 or mutant plants were grown in a peat/vermiculite (1:1) mixture for 21 days in Weiss 1300 growth cabinets (photon flux density 250 μmol m<sup>-2</sup> sec<sup>-1</sup>; tubular fluorescent lamps) under 12 hours day length (day: 23°C 70% relative humidity; night 18°C 90% relative humidity). Lights were switched on at 7 AM and off at 7 PM. O<sub>3</sub> treatments were started at 9 AM. 21-day old plants were used and exposed to 250 parts per billion (ppb) O<sub>3</sub> for 6 hours. Samples were harvested at the times indicated in the respective experiments after the onset of the O<sub>3</sub> treatment. Samples were taken in parallel from O<sub>3</sub> treated and clean air control plants and immediately shock-frozen in liquid nitrogen.

#### Phytotron growth conditions

For light stress treatments, plants were grown on a pre-fertilized garden soil/vermiculite (1:1) mixture for 28 days under 8 h/16 h light/dark at 22 or 20°C, respectively, and 50% humidity at a light intensity of 130 μmol m<sup>-2</sup> sec<sup>-1</sup> photon flux density (Metal halide lamps). For light stress treatment, plants were shifted to 1300 μmol m<sup>-2</sup> sec<sup>-1</sup> photon flux density for up to 4 hours. Subsequently, plants were returned to a light intensity of 130 μmol m<sup>-2</sup> sec<sup>-1</sup> photons. Controls were kept at 130 μmol photon flux density throughout the duration of the treatment and samples were taken in parallel with the light stress-treated plants. Samples were harvested at the times indicated in the respective experiments after the onset of the light stress treatment and immediately shock-frozen in liquid nitrogen.

For flg22 treatments, plants were grown on MS plates with Nitsch vitamins (MSN). After 7 days, seedlings were transferred to liquid MSN media and cultivated for 7 days. Before the flg22 treatment, fresh medium was added. After a 1 hour recovery period, the seedlings were treated with 100 nM flg22. Controls were treated with H<sub>2</sub>O. Samples were harvested at the times indicated in the respective experiments after the onset of the treatment and in parallel from corresponding controls and immediately shock-frozen in liquid nitrogen.

### RNA extraction and qPCR analysis

RNA was isolated as described [79]. 5 μg total RNA was DNaseI treated (Fermentas) and used for cDNA synthesis

with RevertAid Premium Reverse Transcriptase (Fermentas) and Ribolock RNase Inhibitor (Fermentas) according to manufacturers' instructions. The reaction was diluted to a final volume of 50 μl and 1 μl cDNA was used as template for PCR using LightCycler 480 SYBR Green I master mix (Roche Diagnostics) on a LightCycler 480 (Roche Diagnostics) in triplicate. Primer sequences and the primer amplification efficiency (E<sub>x</sub>; determined according to manufacturers instructions) are available in additional file 4.

For the normalization of the data several genes were evaluated to select a suitable gene for normalization based on the method of Vandesompele *et al.* [80]. *Actin-2* (*At3g18780*) was found to be stably expressed in control and ozone treated plants and was subsequently used for normalization. The raw Ct values were normalized to *Actin-2* and used to compare the results from untreated control samples with treated samples using the 2<sup>-ΔΔCt</sup> method. The resulting normalized cycle differences were used to calculate the average (μ) and standard deviation (σ) of the biological repeats and the p-value (using SPSS) based on [81]. The p-value was calculated using the one-sample t-test in SPSS and calibrated using the Benjamini-Hochberg false discovery rate (FDR) correction [82]. The 95% confidence intervals (CI<sub>±</sub>; lower and upper bound)

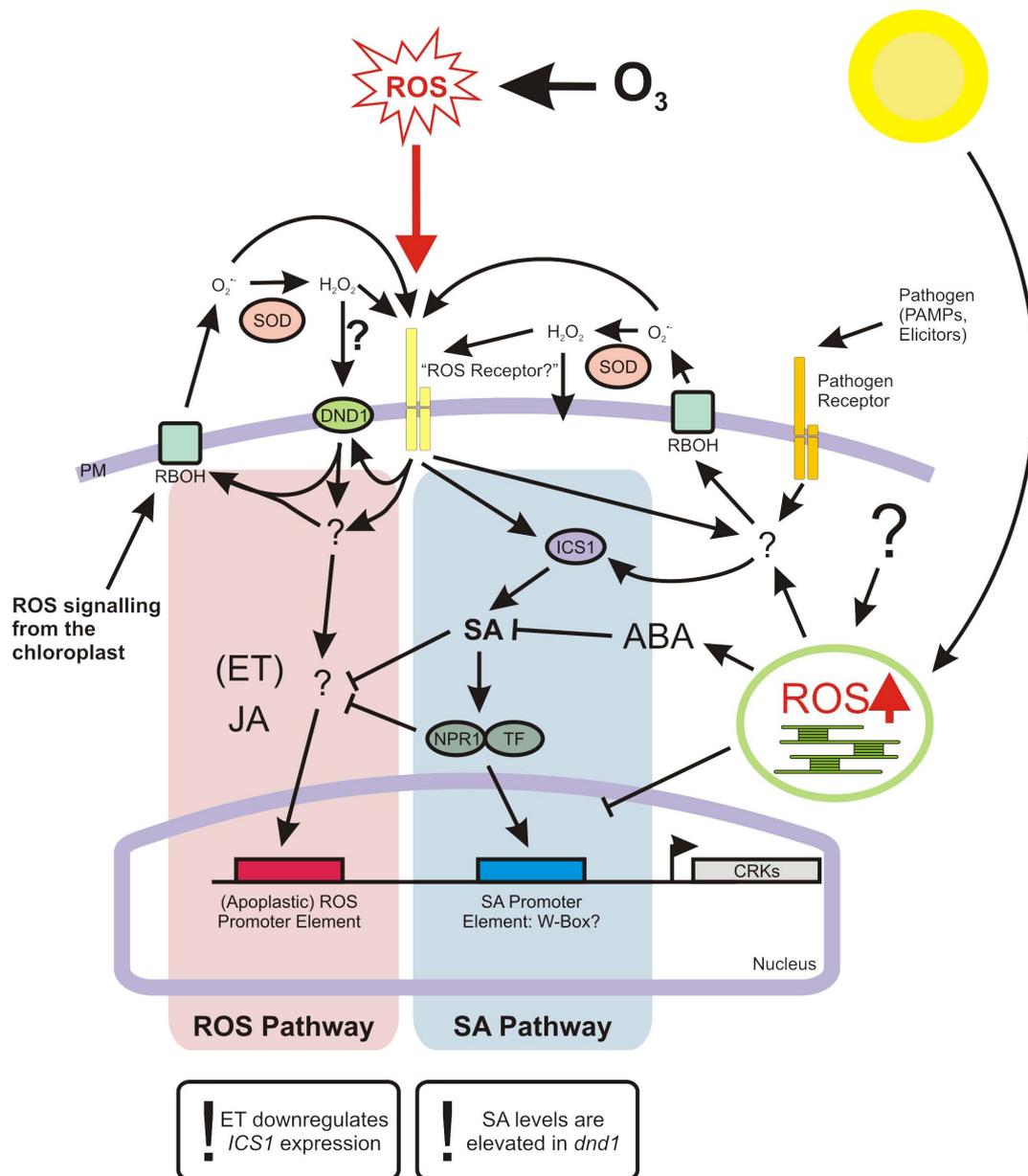
were calculated according to  $CI_{\pm} = E_x^{\mu \pm 1.96\sigma}$ , where E<sub>x</sub> is the efficiency of the reaction x. The μ, σ, CI and p-value for all qPCR experiments are shown in additional file 1. The mean μ of the normalized cycle difference was used to calculate the fold-change of expression using E<sub>x</sub> (Additional file 4).

### Phylogenetic analysis

RLK kinase domains were identified using PrositeScan <http://au.expasy.org/tools/scanprosite/>. Sequence alignments were performed using the ClustalW2 program [83]. Neighbour-joining trees were constructed with 1000 bootstrap sets using the Mega4 software package [84].

### Micro-array analysis

Affymetrix raw data was downloaded from NASCArrays <http://affymetrix.arabidopsis.info/narrays/experiment-browse.pl> (accession number NASCARRAYS-143, paraquat; NASCARRAYS-353, ZAT12; NASCARRAYS-176, ABA time course experiment 1; NASCARRAYS-192, Ibuprofen), ArrayExpress <http://www.ebi.ac.uk/microarray-as/ae/> (accession numbers E-GEOD-12856, *Blumeria graminis* sp. *hordei*; E-GEOD-5684, *Botrytis cinerea*; E-ATMX-13, Methyl Jasmonate; E-MEXP-739, Syringolin A; E-MEXP-1797, Rotenone), Gene Expression Omnibus <http://www.ncbi.nlm.nih.gov/geo/> (accession numbers GSE5615, Elicitors LPS, HrpZ, Flg22 and NPP1; GSE5685, Virulent and avirulent *Pseudomonas syringae*; GSE9955, BTH experiment 1, GDS417 *E. cichoracearum*;



**Figure 9 ROS, elicitor and hormone regulation of O<sub>3</sub>-induced CRKs.** O<sub>3</sub> enters the leaves through the stomata and immediately reacts with components of the cell wall to generate ROS. O<sub>3</sub> and the ROS induce an active production of ROS in the apoplast which is at least partly depending on membrane bound NADPH oxidases (RBOH), which produce O<sub>2</sub><sup>-</sup>. Similar ROS production in the apoplast takes place after infection of a plant with a pathogen or treatments with pathogen derived elicitors (PAMPs). ROS is hypothetically perceived *via* a "ROS receptor" which could sense ROS directly *via* protein modification, or *via* sensing of modified apoplastic proteins or other molecules that react with ROS. The perception of ROS initiates downstream signalling events. H<sub>2</sub>O<sub>2</sub> is also able to cross the plasma membrane and enter the cells. Inside the cell, the signalling pathway is split into two pathways. In the ROS pathway DND1/CNGC2 mediates a required step of the signalling pathway and JA and ET act as positive regulators, and SA and NPR1 are negative regulators. In the SA pathway ROS or pathogens activate SA biosynthesis *via* ICS1; and NPR1 is a required component. Since NPR1 is a positive regulator of the SA pathway and a negative regulator of the ROS pathway this implies that the separate signalling pathway use different transcription factors and promoter elements to regulate *CRK* expression, although it might be possible that two different transcription factors could converge on the same promoter element. In addition the pleiotropic nature of the *dnd1* mutant, including high SA-levels, could change the place of DND1/CNGC2 in the model - constitutive SA signalling in *dnd1* may limit the possibility for O<sub>3</sub> to activate the ROS pathway. Through the transcription factors EIN3 and EIL1 ET can repress SID2/ICS1 expression and SA levels. Increased ROS production in the chloroplast activates separate signalling pathway(s) leading to repression of *CRK* expression. One of these pathways could involve ABA and negative cross talk with the SA pathway.

GSE5530, H<sub>2</sub>O<sub>2</sub>; GSE5722, O<sub>3</sub>; GSE12887, Norflurazon; GSE10732, OPDA and Phytoprostane; GSE7112, ABA experiment 2) and The Integrated Microarray Database System <http://ausubellab.mgh.harvard.edu/imds> (Experiment name: BTH time course, BTH experiment 2).

The raw Affymetrix data was preprocessed with RMA using probe set annotations (custom cdf files) from <http://brainarray.mbni.med.umich.edu/>, version 11.0.1. Biological repeats of each experiment were combined by computing a mean of the measured gene expression. Gene expression was summarized by computing a log<sub>2</sub> ratio of the treatment and control expressions (differential expression, DE). A visualization of the DE values is shown in Figure 4. Variation of differential expression in an

experiment  $e$ ,  $\hat{\sigma}_e^2$ , was estimated by summing the variances of (logarithm of) treatment and control gene expressions.

Parametric bootstrapping was implemented by generating 1000 samples for each experiment and each gene from a Gaussian distribution with the estimated DE as

the mean and  $\hat{\sigma}_e^2$  as the variance.

Bootstrap samples were discretized to down regulated ( $\log_2 \text{DE} < -1$ ), no regulation ( $-1 < \log_2 \text{DE} < 1$ ), and up regulated ( $\log_2 \text{DE} > 1$ ) genes. Bayesian agglomerative hierarchical clustering algorithm was then applied to the discretized bootstrap data. The Bayesian hierarchical clustering algorithm computes the best number of clusters by Bayesian hypothesis testing. For each pair of genes (and experiments, depending on the clustering direction), the number of times they were assigned to the same cluster was computed. These gene (or experiment) similarities were then used as distances for computing the hierarchical clustering (ward method) shown in Figure 4.

### Promoter analysis

TAIR 9 version of promoter sequences of 500 bases and 1000 bases upstream of the *Arabidopsis* genes was downloaded from <http://www.arabidopsis.org/>. A list of verified *Arabidopsis* promoter elements was taken from <http://arabidopsis.med.ohio-state.edu/AtcisDB/binding-sites.html> [54]. The set of CRKs was divided into three groups (all, ozone up-regulated and ozone down-regulated) and the plus and minus strands of the promoters were searched for significant enrichment of single promoter elements or a combination of two promoter elements in either of the strands. Fisher exact test with false discovery rate correction (q-values; [55]) was used for measuring the significance of the enrichment; q-value of 0.05 was used as the threshold.

## Additional material

**Additional file 1 Lower and upper percentiles and p-values.** The raw normalized cycle differences ( $\Delta\Delta\text{Ct}$ ) for all experiments, their average, standard deviation, geometric mean, lower and upper percentile and the Benjamini-Hochberg False Discovery Rate-corrected p-value for all experiments is shown in the Excel File. Each Excel worksheet represents data for a Figure showing qPCR data.

**Additional file 2 Transcriptional regulation of the CRKs in response to flg22.** 14-day old *Arabidopsis* Col-0 were treated with 100 nM flg22 and samples taken after 30 and 60 minutes (water-treated control samples have been harvested at the same time points in parallel). Expression of several CRKs was analyzed by qPCR. Transcript levels were calculated by comparison with the corresponding control plants. An expression level of one indicates no change in expression, increased expression is indicated by values larger than one while decreased expression is shown by values smaller than one. Increase in expression by 2-fold or higher is high-lighted in red and decrease in expression by 2-fold or more in green.

**Additional file 3 List of CRKs for promoter motifs in table 2.** This file lists the AGI codes for the CRKs containing the promoter motif combinations shown in table 2.

**Additional file 4 Primer sequences for qPCR analysis.** All primer sequences used for qPCR analysis in the manuscript plus the experimentally determined primer amplification efficiencies  $E_x$  are listed.

### Authors' contributions

MW, MB, SR, SK, BK and JK designed research. MW, MB, JS, NI, SLK and SM carried out research. MW, MB, JS and JK analyzed the data. MW, MB and JK wrote the paper. All authors have read and approved the final manuscript.

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