

[Type text]

1 **Short title:** The role of *RGO* in plant development

2 **Corresponding author:** Jas Singh

3

4 **Title:** The Arabidopsis gene *RGO* mediates cytokinin responses and increases seed yield

5

6 Jhadeswar Murmu<sup>1</sup>, Ghislaine Allard<sup>2</sup>, Denise Chabot<sup>1</sup>, Eiji Nambara<sup>3</sup>, Raju Datla<sup>4</sup>, Shelley  
7 Hepworth<sup>5</sup>, Rajagopal Subramaniam<sup>1</sup>, and Jas Singh<sup>1</sup>

8

9 <sup>1</sup>Ottawa Research and Development Centre, Agriculture and Agri-Food Canada, Ottawa, Ontario,  
10 K1A 0C6, Canada

11 <sup>2</sup>Public Health Agency of Canada, Ottawa, Ontario, K1A 0K9, Canada

12 <sup>3</sup>Department of Cell and Systems Biology, University of Toronto, Toronto, Ontario M5S 3B2,  
13 Canada

14 <sup>4</sup>Global Institute for Food Security, 110 Gymnasium Place Saskatoon, SK S7N 0W9

15 <sup>5</sup>Department of Biology and Institute of Biochemistry, Carleton University, Ottawa, Ontario, K1S  
16 5B6, Canada

17

18 **One-sentence summary:** *RGO*, a novel gene from Arabidopsis, is essential for plant development,  
19 mediates CK signaling and increases seed yield in Arabidopsis and rapeseed when overexpressed.

20 **Author contributions:** JM and JS designed the experiments; JM, GA, and DC performed the  
21 experiments; EJ quantified cytokinins; JM, JS and RS analyzed the data, and JM, RD, SRH, RS,  
22 JS wrote the manuscript. RS and JS contributed equally to this research. JS agrees to serve as author  
23 responsible for contact and ensures communication.

24 **Funding information:** This research was supported by the Genomics Research and Development  
25 Initiative (GRDI) of Agriculture and Agri-Food Canada to JS and RS

26 Author of contact: [jas.singh@canada.ca](mailto:jas.singh@canada.ca)

27

[Type text]

1 **Abstract**

2 A novel gene, *At1g77960*, from *Arabidopsis thaliana* was characterized. *At1g77960* transcripts  
3 accumulate to very high levels in plants ectopically overexpressing the *Golden2-like1 (GLK1)*  
4 transcription factor and is designated as a *Response to GLK1 Overexpression (RGO)* gene. *RGO*  
5 encodes a protein with domains of tandem QH and QN repeats. Transcripts and promoter GUS  
6 reporter analyses indicated that *RGO* is expressed in roots, leaves, stems, floral and siliques tissues  
7 but not in seeds. Expression of the *RGO:YFP* fusion protein demonstrated that *RGO* is localized  
8 to the endoplasmic reticulum. MicroRNA mediated silencing of *RGO* resulted in severe reductions  
9 in vegetative and root growth, delayed flowering and reduced seed yield and viability, suggesting  
10 that *RGO* is essential for plant development. Conversely, ectopic overexpression of *RGO* resulted  
11 in enhanced vegetative growth including increased axillary bud formation and a 20% higher seed  
12 yield. Stable overexpression of *RGO* in *Brassica napus* also produced a similar increase in seed  
13 yield. Cytokinin (CK) response assays including root growth, green calli formation from excised  
14 hypocotyls and chlorophyll retention during dark-induced senescence suggest that one role of *RGO*  
15 is to mediate CK responses in plant development. These results suggest that *RGO* could be a target  
16 gene for increasing crop seed yields.

17 **Keywords**

18 *Arabidopsis thaliana*, *At1g77960*, *GLK1* overexpression, cytokinin response, seed yield

19

[Type text]

## 1 **Introduction**

2 Significant progress have been made in our understanding of the role of cytokinins (CK) in plant  
3 growth, and development. Through interactions with other hormonal pathways and transcriptional  
4 factors, CKs plays an important role in a variety of plant processes including greening (Cortleven  
5 and Schmölling, 2015), etioplast-to-chloroplast transition (Cortleven et al., 2016), root greening  
6 (Kobayashi et al., 2012; 2017), shoot and shoot development (Howell et al., 2003; Kieber and  
7 Schaller, 2014; Raines et al., 2016) and delay in leaf senescence (Gan and Amasino, 1995; Kim et  
8 al., 2006; Kieber and Schaller, 2014; Raines et al., 2016; Talla et al., 2016). The current knowledge  
9 of CK signalling involves a multistep signal transduction system that begins with the perception of  
10 CK by the Arabidopsis Histidine Kinases (*AHK2*, *AHK3*, *CRE1/AHK4*) in the endoplasmic  
11 reticulum (ER) phosphate transfer via the Arabidopsis Histidine Phosphotransfer proteins (AHPs).  
12 The information is transmitted to the nucleus where the transcription factors such as the type-B  
13 Arabidopsis Response Regulators (ARRs) affect gene expression (Kieber and Schaller, 2014;  
14 Romanov et al., 2018).

15 The roles of CK in chloroplast development are mediated by core components of the CK signalling  
16 pathway which includes the CK receptors *AHK2* and *AHK3* and the response regulators *ARR1*,  
17 *ARR10* and *ARR12* (Cortleven and Schmölling, 2015). Chloroplast development and maintenance  
18 in leaves also requires functional *Golden2-like (GLKs)* transcription factors (Fitter et al., 2002;  
19 Yasumura et al., 2005; Waters et al., 2008; Waters et al., 2009) which are members of the Myb  
20 superfamily of transcription factors containing a DNA binding “GARP” domain named after the  
21 maize *Golden2*, the type-B *Arabidopsis Response Regulators (ARR)*, and the *Chlamydomonas*  
22 *Phosphate starvation response (PSR1)* transcription factors (Reichmann et al., 2000). In  
23 Arabidopsis leaves, *GLK1* and *GLK2* operate in redundant fashion as only the *glk1 glk2* double  
24 mutant showed impaired chloroplast development to display a distinct pale green phenotype (Fitter  
25 et al., 2002). The role of *GLKs* in chloroplast maintenance is also linked to leaf senescence,  
26 underscored by the observation that elevated expression of the transcription factor *ORE1* triggered  
27 early senescence in Arabidopsis through down regulation of *GLKs* and it has been observed that  
28 overexpression of *GLK1* delays leaf senescence (Rauf et al., 2013; Garapati et al., 2015). The role  
29 of *GLKs* in leaf senescence is also reflected in the observation that overexpression of *GLK1*  
30 resulted in the reduction of infection of the necrotrophic pathogen *Botrytis cinerea* (Murmu et al.,  
31 2014) as it is known that delay of senescence is a key factor in resistance to this pathogen  
32 (Swartzberg et al., 2008; Lai et al., 2011; Wang et al., 2013; Haffner et al., 2015).

[Type text]

1  
2 A direct link between *GLKs* and CK signalling however, is lacking. There is evidence to suggest  
3 that type B *ARRs* coordinate the expression of *HY5* (elongated hypocotyl 5), which is required by  
4 *GLK2* for maximal (although not essential) greening in roots (Kobayashi et al., 2012).  
5 Overexpression of *GLK2*, *GNC* (*GATA*, *NITRATE-INDUCIBLE*, *CARBON-METABOLISM-*  
6 *INVOLVED*) and *CGA1* (*CYTOKININ-RESPONSIVE GATA 1/GNC-LIKE*) result in root greening,  
7 however, neither *GLK1* nor *GLK2* are required for the CK-dependent root greening phenotype  
8 (Kobayashi et al., 2012). In tomato, overexpression of *GLK2* in the fruit leads to enhanced CK  
9 responsiveness and delayed ripening, thus differentiating function between the two *GLKs* in  
10 development programs such as fruit ripening (Lupi et al., 2019). However, the role of *GLKs* in CK  
11 response is lacking in leaves. There is no evidence to suggest that *GLKs* can participate in the  
12 phosphor-relay step and in this regard, are similar to the CK response regulator *ARR21C*, a  
13 truncated form of *ARR21* where the phosphor-relay N-terminus domain is removed (Kiba et al.,  
14 2005). Ectopic overexpression of *ARR21C* resulted in altered CK signalling, affecting plant  
15 development. *ARR21* has been shown to function in CK signalling as it can complement *arr1* and  
16 *arr12* mutants, although its precise role in CK signalling remains to be established (Hill et al.,  
17 2013). With the loss of the phospho-regulatory domain, ectopic overexpression of *ARR21C* in  
18 Arabidopsis produced a range of highly abnormal phenotypes with hypersensitivity to exogenous  
19 applications of very low levels of CK (Tajima et al., 2004; Kiba et al., 2005). To explore if an  
20 association exists between *GLK1* overexpression and CK signalling, we focussed on a previously  
21 uncharacterized Arabidopsis gene *At1g77960*, which was highly upregulated by ectopic  
22 overexpression of *GLK1* (Savitch et al., 2007). An examination of the microarray data from CK  
23 hypersensitive plants overexpressing *ARR21C* (Goda et al., 2008) also indicated that this gene was  
24 also substantially overexpressed.

25 Few other studies have implicated *At1g77960* in different plant processes. The *At1g77960* gene  
26 transcripts was upregulated in the *esr1-1* (enhanced stress response 1) mutant with increased  
27 resistance to the fungal pathogen *Fusarium oxysporum* (Thatcher et al., 2015) and was  
28 downregulated after infection with the bacterial pathogen *Pseudomonas syringae* pv tomato  
29 Dc3000 (Lewis et al., 2015). Despite the correlation between levels of gene expression in various  
30 plant processes, detailed characterization of *At1g77960* is lacking. Therefore, in this study we  
31 designated *At1g77960* gene as *Response to GLK1 Overexpression (RGO)*, and investigated its

[Type text]

1 potential role in modulation of CK response and its significance in plant development,  
2 performance and seed yields.

3

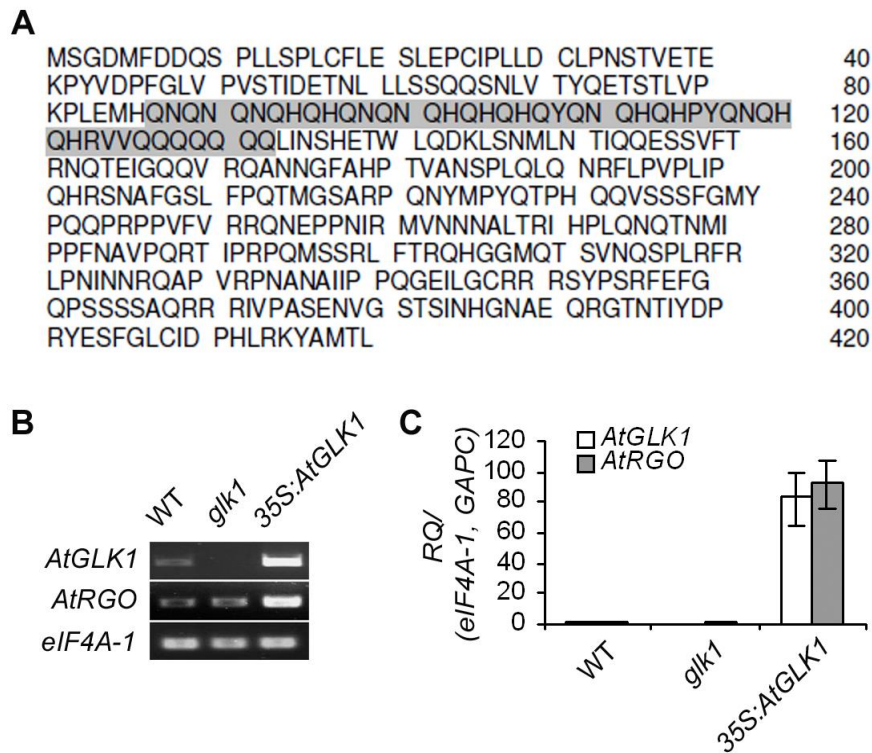
## 4 **Results**

### 5 ***Arabidopsis RGO* is a novel gene discovered from ectopic overexpression of *GLK1***

6

7 Transcripts of *AtRGO* were observed to be highly accumulated in *Arabidopsis* plants ectopically  
8 overexpressing *GLK1* (Savitch et al., 2007). A detailed RT-PCR and qPCR analyses demonstrated  
9 that *RGO* transcripts are highly upregulated in plants constitutively overexpressing *GLK1* (Fig. 1,  
10 B and C). Transcript levels in the *glk1* mutant remained unchanged when compared to the WT  
11 (Fig. 1, B and C) suggesting that *RGO* is not directly regulated by *GLK1*. An examination of the  
12 microarray data from CK hypersensitive plants overexpressing *ARR21C* also upregulated *RGO*  
13 transcripts by nine fold (Supplementary Table. S1). To further substantiate the observation of  
14 *GLK1* in the activation of *RGO* overexpression, we cloned and overexpressed *TaGLK1* from bread  
15 wheat (NCBI Accession EF105406) in *Arabidopsis*. *Arabidopsis* plants overexpressing *TaGLK1*  
16 also showed a high upregulation of *RGO* (Supplemental Fig. S1). *RGO* is a single copy gene  
17 encoding a 48 kilo Dalton (kDa) glutamine (Q) rich (14%) protein of 420 amino acids. The N-  
18 terminus of the deduced protein contains a hydrophobic region (45% hydrophobic residues)  
19 followed by tandem repeats of Q with either asparagine (Q/N) or histidine (Q/H) followed by a 7  
20 amino acid Q repeat (Fig. 1A). QQ repeats are also observed throughout the *RGO* protein sequence.  
21 *RGO* has been erroneously annotated as repressor *ROX1-like* in TAIR (The *Arabidopsis*  
22 Information Resource) database; *ROX1* encodes a DNA-binding protein that represses the  
23 expression of hypoxia genes in yeast (Balasubramanian et al., 1993; Deckert et al., 1995). Protein  
24 sequence alignments between *RGO* and the yeast repressor ROX1 showed no homology. As well,  
25 *RGO* does not contain a high-mobility group (HMG) DNA binding domain that is characteristic of  
26 repressor ROX1 proteins. Similarly, several genes with deduced protein sequence homology to  
27 *RGO* in *Camelina sativa*, *Brassica rapa* and *Brassica napus* (Supplemental Fig. S2) have also been  
28 incorrectly annotated as repressor ROX1 proteins. Proteins with homology to *RGO* have yet to be  
29 identified in the sequence databases of plants outside of the *Cruciferae* species.

[Type text]



1

## 2 **Figure 1**

3 **Deduced amino acid sequence and upregulation of *Arabidopsis* Response to *GLK1***  
 4 **Overexpression (*RGO*, *At1g77960*).** (A) amino acid sequence of *AtRGO* in single-letter code and  
 5 the glutamine rich region is highlighted in gray. Numbering of amino acids are on the right. (B)  
 6 Transcripts accumulation of *AtGLK1* and *AtRGO* in wild-type (WT), *glk1* mutant, and  
 7 *35S:AtGLK1* plants with RT-PCR compared to *eIF4A-1*, a housekeeping gene transcripts; (C)  
 8 *AtGLK1* and *AtRGO* transcripts with qPCR relative to *eIF4A-1* and *GAPC*, two housekeeping gene  
 9 transcripts. Relative transcripts represent as mean fold change values  $\pm$  standard error of mean  
 10 (SEM) from three biological replicates.

11

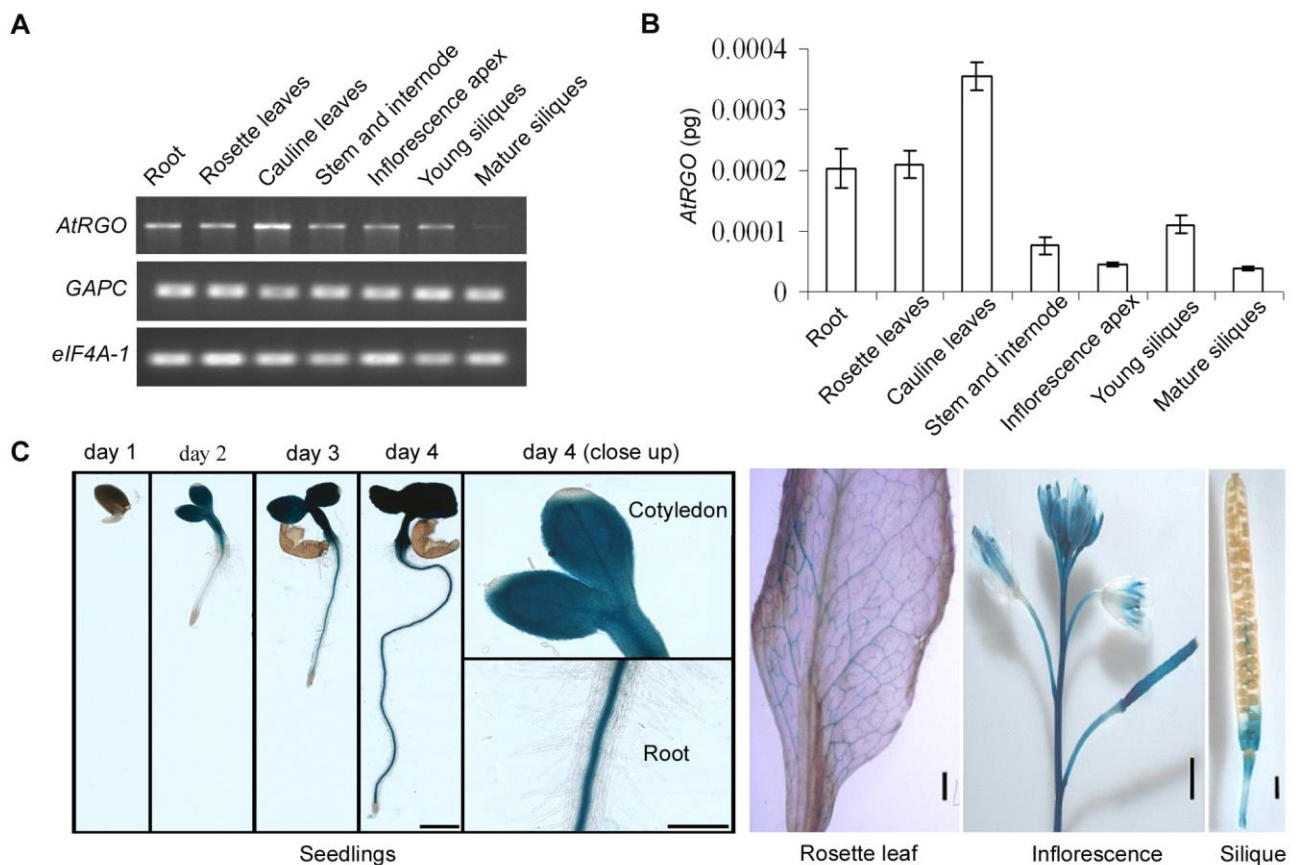
## 12 ***RGO* expression is ubiquitous in *Arabidopsis* vegetative and floral tissues**

13

14 *RGO* transcripts were expressed ubiquitously in *Arabidopsis* plant tissues such as roots, leaves,  
 15 stem and internodes, inflorescence apex, and siliques as demonstrated by semi-quantitative RT-  
 16 PCR (Fig. 2A). *RGO* transcripts were relatively abundant in cauline leaves and scarce in mature  
 17 siliques as validated by qPCR experiments (Fig. 2B). We also generated transgenic lines  
 18 expressing a 1.9 kb *RGO* promoter fragment fused to GUS reporter gene in the WT (Col-0)  
 19 background to localize expression of this gene. Gus expression was most intense in early seed  
 20 germination, starting one day after germination, and in cotyledons and slowly progressed to

[Type text]

1 vascular tissues in the root (Fig. 2C). In later stages of plant development, expressions were  
2 mainly confined to veins of rosette and cauline leaves. In inflorescence tissues, expressions were  
3 prominent in the stem, young apex, and young siliques. By contrast, both mature flowers and  
4 mature siliques displayed reduced GUS accumulation and no expression was detected in seeds.  
5 The *RGO* expression is also diurnally regulated (Supplemental Fig. S3); *RGO* transcripts began  
6 to accumulate early in the light cycle and peaked during the middle of the light cycle, then  
7 gradually diminished towards the end of the light-cycle and remain unchanged during the dark  
8 cycle (Supplemental Fig. S3). This data suggest that *RGO* expression is light or photoperiod  
9 sensitive.  
10



11  
12  
13

14 **Figure 2**  
15 **Expression pattern of *AtRGO*.** (A) *AtRGO* transcripts accumulation in wild-type (WT) Col-0  
16 with RT-PCR compared to *eIF4A-1* and *GAPC*, two housekeeping control transcripts; (B)

[Type text]

1 Absolute quantification of *AtRGO* transcripts with qPCR. Transcripts represented are mean fold  
2 change values  $\pm$  standard error of mean (SEM) from three biological replicates; (C) *AtRGO*  
3 promoter driven reporter GUS reporter gene expression in WT plants. GUS expression was  
4 observed starting one day after germination; GUS expression was intense in cotyledons and  
5 slowly progressed to vascular tissues in the root. Scale bar, 200  $\mu$ m in seedlings, and bar, 1 cm in  
6 mature tissues.

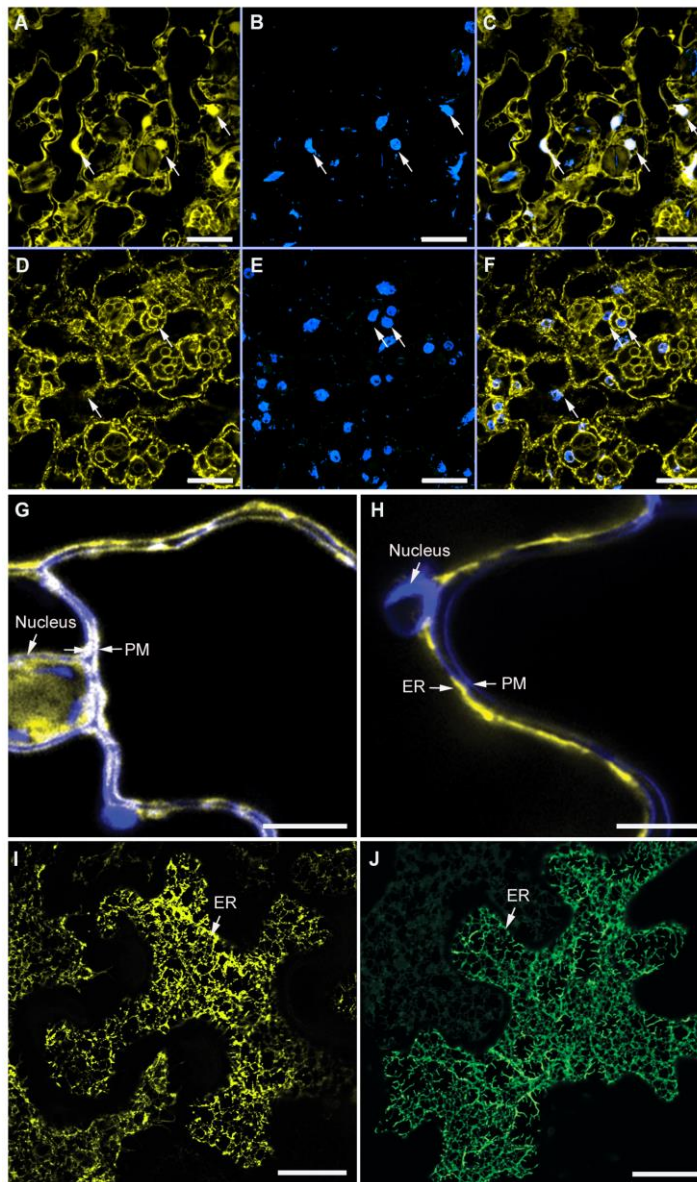
7  
8

## 9 **RGO is localized to the Endoplasmic Reticulum**

10 The presence of hydrophobic amino acid residues at the N-terminus of the RGO protein suggested  
11 that it is most likely membrane localized. As the RGO protein sequence did not reveal any  
12 functional domains and organelle localization signals, we undertook cellular localization studies  
13 to provide insights into the potential function of RGO. A C-terminal translational fusion of *RGO*  
14 with YFP (yellow fluorescent protein) under the control of the Cauliflower Mosaic virus (CaMV)  
15 35S promoter was constructed and stable transgenic Arabidopsis plants were generated. Transgenic  
16 plants expressing 35S:YFP were used as a control. As displayed, free YFP fluorescence were  
17 observed to be evenly distributed in the cytoplasm and nucleus of leaves of 35S:YFP transgenic  
18 plants, (arrows, Fig. 3, A and C). The nuclei were identified by DAPI staining in Fig. 7B. In  
19 epidermal cells of leaves expressing 35S:*RGO*:YFP, YFP fluorescence was observed in distinct  
20 locations in the cytoplasm and surrounding the nucleus (Fig. 3, D and F). Unlike the control  
21 35S:YFP leaf tissues, no fluorescence was detected in the nucleus (Fig. 3F). The nuclei were  
22 identified by DAPI staining in Fig. 7E. This pattern of fluorescence is consistent with proteins  
23 localizing to the endoplasmic reticulum (ER) (Nelson et al., 2007; Wulfetange et al., 2011). The  
24 presence of the hydrophobic N-terminus of RGO likely enables it to be localized to the membranes.  
25 To confirm these findings, FM4-64FX, a plasma membrane specific fluorescent dye was used in  
26 combination with YFP fluorescence to demonstrate that 35S:*RGO*-YFP fluorescence (yellow) was  
27 distinctly separated from the FM4-64FX (blue) plasma membrane stain (Fig. 3H), whereas, free  
28 fluorescence in the control leaf tissues (35S:YFP) overlapped (white colour) with the plasma  
29 membrane as well as the nucleus (Fig. 3G). These fluorescence patterns were indicative of ER  
30 localization and suggest that RGO is associated with the ER and not the plasma membrane. To  
31 further confirm the ER localization of RGO protein, we compared transient overexpression of an  
32 ER marker, 35S:GFP-HDEL (Batoko et al., 2000) to the expression of 35S:*RGO*-YFP in tobacco  
33 leaves. The expression pattern of RGO (Fig. 3I) is identical to the ER expression pattern of  
34 35S:GFP-HDEL (Fig. 3J) and confirms that RGO is localized to the ER.



[Type text]



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15

**Fig. 3**

**Subcellular localization of *AtRGO*-YFP fusion protein by confocal microscopy in transgenic**

***Arabidopsis* leaves and tobacco leaves. Plants expressing 35S:YFP were used as positive**

control (A-C) for comparing *AtRGO*-YFP fusion expression (D-F). (A and D), YFP signal in

yellow; (B and E), DAPI stained nuclei in blue; (C and F) merged. Arrow head shows the

nucleus and endoplasmic reticulum (ER). Zoom in view of 35S:YFP (G) and *AtRGO*:YFP (H)

with FM4-64FX stain. YFP fluorescence in yellow and plasma membrane stain in blue, a false

colour for contrast is shown in G-H and arrow head shows the nucleus and plasma membrane

(PM) in G-H. Transient expression of *AtRGO*-YFP (I) and GFP:HDEL, an ER marker (J) in

tobacco leaves. Arrow head shows the ER in I-J. Scale bar, 20 μm in A-F and 5 μm in G-J.

[Type text]

## 1 ***RGO* is required for root development, normal growth and flowering**

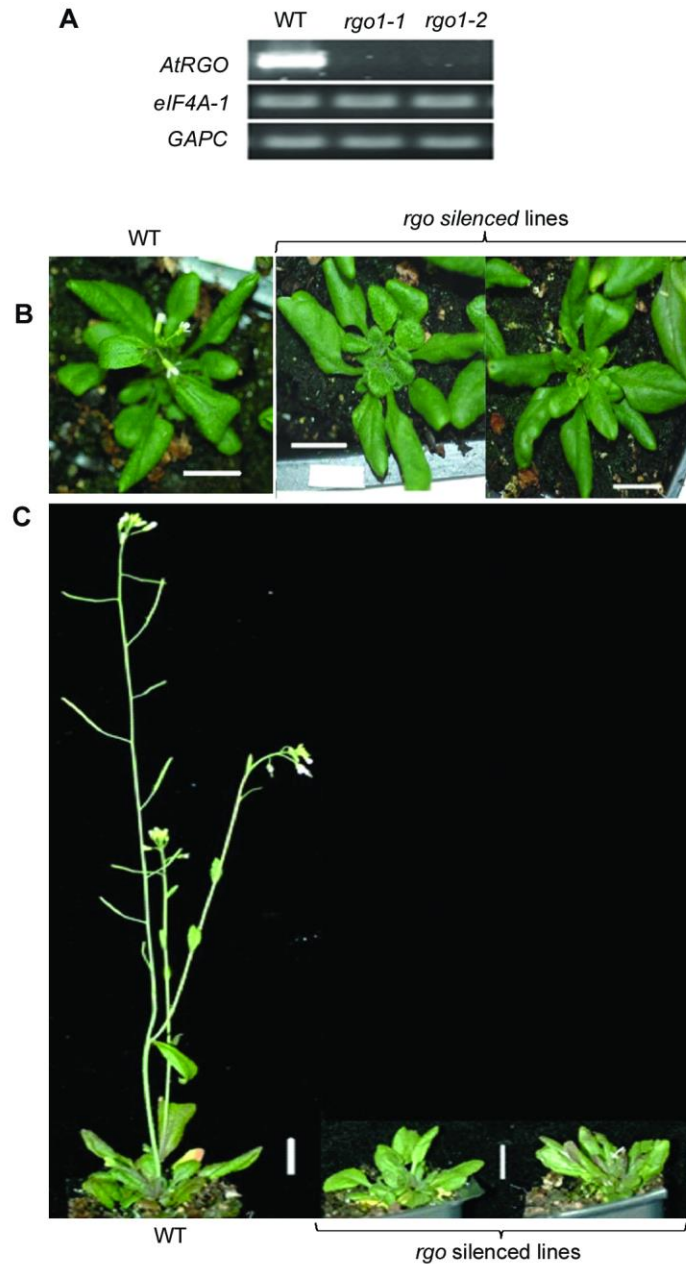
2 Phenotypic studies were undertaken to further elucidate the function of *RGO*. We obtained three  
3 T-DNA insertion lines (Salk\_025545, Salk\_109614, and Salk\_109424) for *RGO* from the  
4 Arabidopsis Biological Resource Center (ABRC), Ohio State University, USA. Arabidopsis T-  
5 DNA design tool (<http://signal.salk.edu/tdnaprimers.2.html>) indicated that Salk-109424 has a  
6 second T-DNA insertion in gene *At5g33175*. We could not detect T-DNA insertions in the *RGO*  
7 gene in the Salk\_109424 line (data not shown). However, we identified a T-DNA insertion in the  
8 first exon of *RGO* at 33 bp down stream of the transcription start site in the Salk\_109614 plants  
9 (Supplemental Fig. S4A). Similarly, we identified a T-DNA insertion in Salk\_025545 in the fifth  
10 intron at 1170 bp down stream of transcription start site (Supplemental Fig. S4A). Both  
11 Salk\_109614 and Salk\_025545 T-DNA insertion lines produced uninterrupted full length *RGO*  
12 transcripts (Supplemental Fig. S4D), which was verified by sequencing the *RGO* gene transcripts  
13 from both Salk\_109614 and Salk\_109424 plants and showed that the transcripts were identical to  
14 the WT.

15 As we could not isolate homozygous T-DNA insertional knockout lines for *RGO*, we generated  
16 artificial micro RNA (amiR) silencing lines for the gene. We screened 72 Basta resistant primary  
17 transformed plants and identified two lines (*rgo-1* and *rgo-2*) and transcripts analyses of T2  
18 generations of by RT-PCR as well as qPCR revealed that these two lines had significant reductions  
19 in transcript accumulation of *RGO* (Fig. 4 A). Plants from both *rgo* lines exhibited a minimum of  
20 two-week delay in bolting compared to the WT (Fig. 4, B and C). These plants were able to set  
21 seeds albeit to much lesser degrees than WT plants and with the phenotypes being stable in  
22 subsequent generations. Seeds from subsequent generations (T4) showed substantial decreased  
23 germination rates (70-80%) compared to the WT (Supplemental Fig. S5A). To validate the  
24 phenotypes observed in the *RGO* silenced (*rgo*) lines, we generated Arabidopsis lines that  
25 constitutively expressed *RGO* (35S:*RGO*). Section and staining of *rgo* seeds showed impaired  
26 embryo development compared to the WT and to the 35S:*RGO* plants. In the *rgo* lines, the majority  
27 of embryos were immature, which corroborated with the lethality of seeds and consequently  
28 decreases in seed germination. (Supplemental Fig. S5B). In light of these findings that linked *RGO*  
29 with plant development in vegetative tissues, we examined the effect of *RGO* silencing on root  
30 growth. Compared to the WT seedlings, *rgo* seedlings grew significantly slower and with a 67%  
31 decrease in root length compared to the WT, whereas the root length of 35S:*RGO* seedlings were

[Type text]

1 similar to the WT (Fig. 5, A and B). Collectively, these results suggest that *RGO* is required for  
2 normal growth as well as viable seed development.

3



4

5

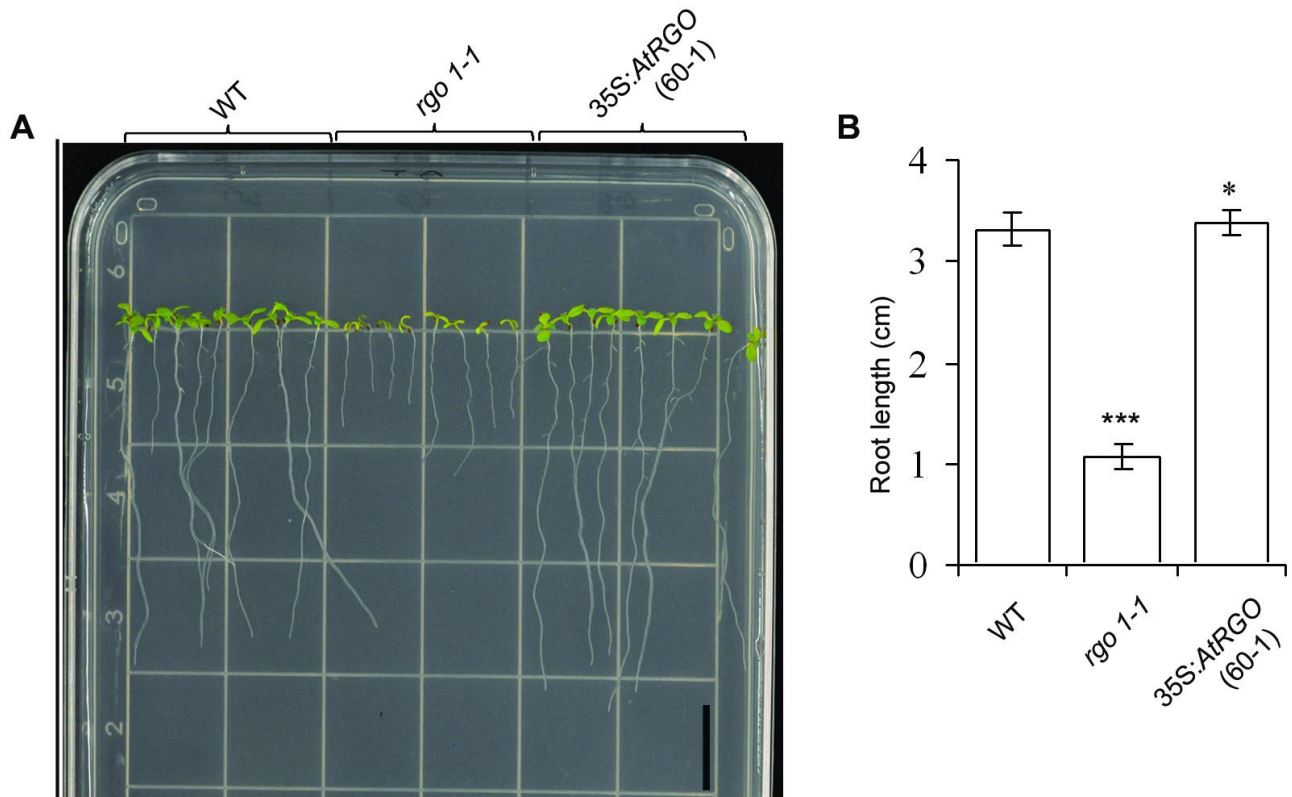
#### 6 **Figure 4**

7 **Phenotype of *rgo* plants compared to WT (Col-0).** (A) A RT-PCR of full length *AtRGO*  
8 transcripts in WT and *rgo* plants compared to *eIF4A-1* and *GAPC* transcripts; (B) qPCR of  
9 *AtRGO* transcripts in WT and *rgo* plants; (C) phenotype at four-week age; (D) phenotype at six-  
10 week age. Scale bar, 1 cm in C-D.

11

[Type text]

1  
2



3  
4  
5

## 6 **Figure 5**

### 7 **Root growth phenotype of WT, *rgo* and 35S: *AtRGO* lines at seven days post germination.**

8 (A) representative images on minimal media (MM), (B) corresponding root length. Error bar =  
9 standard error of mean. Scale bar, 1 cm in A. Statistical significance of root lengths were  
10 analyzed using student's t-test: Two sample assuming unequal variance at 95% confidence level.  
11 \*\*\* represents the p value < 0.00000001, and \* represents p value > 0.1.

12

### 13 **Constitutive expression of *RGO* increases growth and seed yield**

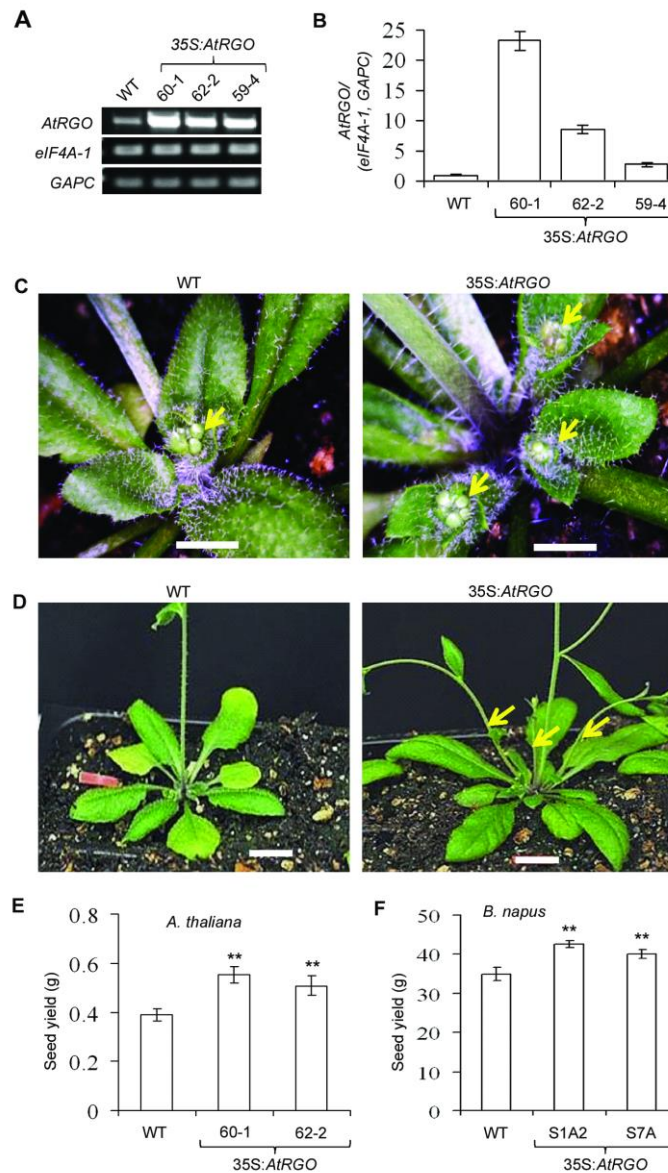
14

15 Analyses of T3 generations of three independent 35S:*RGO* lines indicated a significant  
16 accumulation of *RGO* transcripts, with line 60-1 exhibiting a 20-fold increase in transcript  
17 accumulation compared to the WT (Fig. 6, A and B). All the lines overexpressing *RGO* showed an  
18 acceleration of flowering by one-week, when compared to the WT under long-day growth  
19 conditions (Fig. 6C). Additionally, aerial vegetative growth were observed 2-3 weeks earlier

[Type text]

1 compared to the WT and by week four, the transgenic plants showed increased numbers of axillary  
2 vegetative buds per rosette compared to the WT resulting in increased number of shoots per rosette  
3 (Fig. 6D). We also observed that the length of the rosette leaves in *RGO* transgenic plants were  
4 considerably longer than that of the WT (Supplemental Fig. S6). Given the significant increase in  
5 biomass and shoots, we assessed seed yield in these transgenic plants overexpressing *RGO*. On  
6 average, there was a 20 % higher seed yield than the WT under identical growth conditions (Fig.  
7 6E). We were interested to know if the seed yield phenotype can be recapitulated in related Brassica  
8 species. We generated two lines of *Brassica napus* overexpressing *AtRGO* (35S:*RGO*:). Stable  
9 transgenic *B. napus* (T3) lines overexpressing *RGO* showed similar increased vegetative branching  
10 compared to the WT (Supplemental Fig. S7) with an average 18 % increase in seed yields (Fig.  
11 6F) when grown under controlled environmental conditions.

[Type text]



1

## 2 **Figure 6**

3 **Phenotype of 35S:AtRGO plants compared to WT.** (A) A RT-PCR of full length *AtRGO*  
4 transcripts compared to *eIF4A-1* and *GAPC* transcripts in WT and three independent lines of  
5 *35S:AtRGO* plants; (B) qPCR of *AtRGO* transcripts in WT and *AtRGO* overexpressing lines; (C)  
6 Four-week old plants; (D) Five-week old plants. Emergence of inflorescence buds are shown  
7 with arrow head in C and axillary buds in D; (E) Average seed yield from 10 *Arabidopsis* plants  
8 of WT and *35S:AtRGO* from a long-day photoperiod growth conditions; (F) seed yield from  
9 individual plants of *B. napus* in WT and *35S:AtRGO* (two lines). Scale bar, 1cm in C-D.  
10 Statistical significance was analysed using student's t-test: Two-sample assuming unequal  
11 variances at 95% confidence level compared with empty vector. The p value for each analysis is  
12 shown as asterisk, where p value  $\leq 0.001$  (\*\*). Error bar = standard error of mean.

13

14

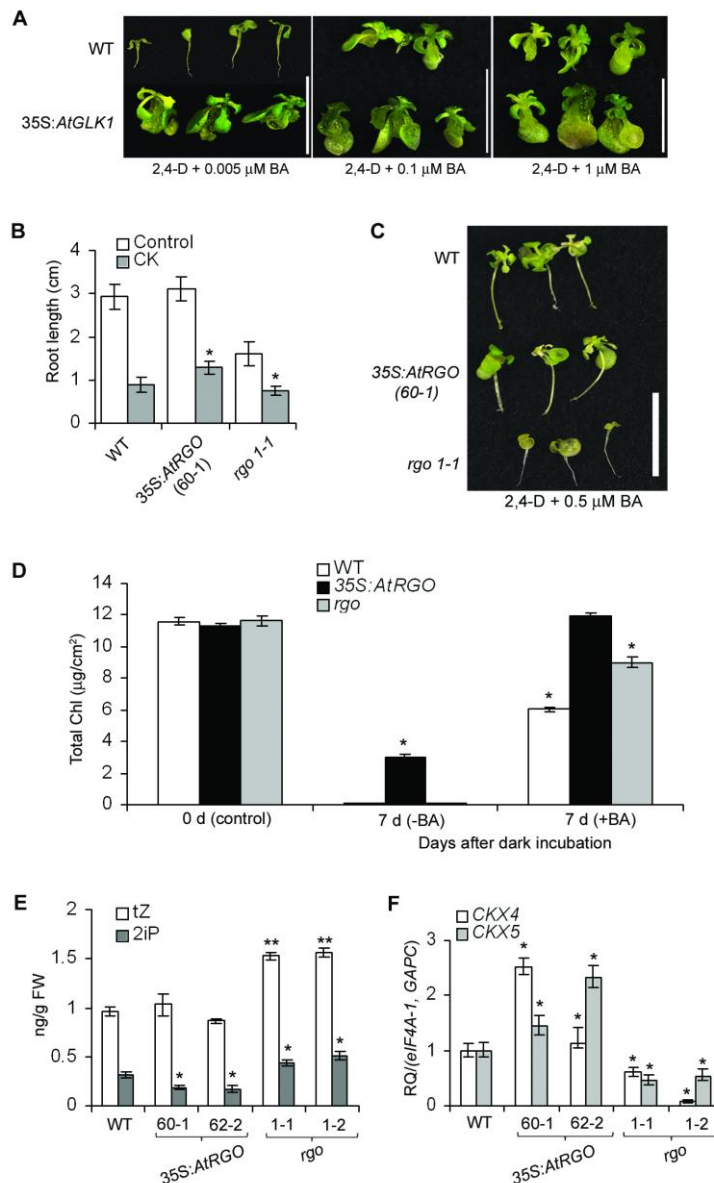
[Type text]

1 ***RGO* mediates CK responses to regulate endogenous CK levels and safeguard the positive**  
2 **effects of CK in plant development**

3  
4 To explore if a relationship exists between *GLK1* overexpression, *RGO* upregulation and CK  
5 responsiveness, we first examined if ectopic overexpression of *GLK1* has an effect on sensitivity  
6 to CK signalling using the green calli/shoot regeneration assay (Kiba et al., 2004). Robust green  
7 calli formation was observed for external application of 0.005  $\mu$ M BA for *GLK1* overexpressing  
8 plants compared to 0.1  $\mu$ M BA for the WT (Fig.7A). This observation prompted us to use *RGO*  
9 silencing and overexpression to determine if *RGO* can, in part, be a factor in CK response. One of  
10 the well-documented effects of exogenous CK on plants is shortened root growth (Skoog and  
11 Miller, 1957) and that CK levels regulate root development (Werner et al., 2001; Werner and  
12 Schmülling, 2009). As observed, *rgo* lines display shorter root growth compared to the WT and  
13 *RGO* overexpressor seedlings (Fig. 5A). Therefore, we compared the root growths of the WT,  
14 *35S:AtRGO* and *rgo* seedlings in the presence of 5  $\mu$ m Zeatin, a synthetic CK. The effect on root  
15 growth inhibition by Zeatin was partially mitigated in *RGO* overexpressing seedlings compared to  
16 the WT and *rgo* seedlings suggesting that overexpression of *RGO* can potentially dampen the  
17 inhibitory effects of CK on root development (Fig. 7B). In addition to the effects on root growth,  
18 it is known that external applications of CK also facilitate green calli and shoot regeneration. Green  
19 calli formation from excised hypocotyl was evaluated to measure CK responses (Kiba et al., 2005).  
20 The results showed that while the *RGO* overexpressing and WT plants showed minimal differences  
21 in their response to exogenous applications of BA, the *rgo* plants were unable to respond to BA  
22 and did not induce green calli formation (Fig. 7B). External applications of CK has also been shown  
23 to improve chlorophyll retention and delay dark-induced senescence in leaves (Gan and Amasino  
24 1995; Kim et al 2006; Talla et al 2016). As well, increased endogenous levels of CKs have been  
25 shown to be able to delay senescence in plants (Richmond and Lang, 1957; Lin et al., 2002; Ma  
26 and Liu, 2009; Zhang et al., 2010; Liu et al., 2012). We assessed chlorophyll retention in detached  
27 leaves after dark-induced senescence in the absence or presence of BA. After seven days of dark  
28 incubation in the absence of BA (7d-BA), neither the WT nor the *rgo* plants retained any  
29 chlorophyll (Chl) (Fig. 7D). In contrast, *35S:AtRGO* leaves retained 26% of the Chl content (Fig.  
30 7D). In the presence of BA (7d +BA), *35S:AtRGO* plants did not lose any Chl (Fig. 7D).  
31 Interestingly, in the 7d+BA treatment, the *rgo1-1* silenced line also retained significantly more Chl  
32 than the WT (Fig. 7D). This retention could result from increased CK accumulation in the silenced

[Type text]

1 line facilitating some signalling even though *RGO* is knocked down. Analyses of bioactive CK  
 2 levels confirmed that that both trans-zeatin (tZ) and 2-isopentenyladenine (2iP) were significantly  
 3 elevated in the *rgo* plants compared to the WT (Fig. 7E). There was a modest but significant  
 4 decrease in 2iP levels in *RGO* overexpressing plants (Fig. 7E). This increase in endogenous CK  
 5 levels in *rgo* plants (Fig. 7E) is reflected in lower transcript levels encoding CK degrading enzymes  
 6 CK oxidases *CKX4* and 5, (Fig. 7F). Taken together, these results suggest that *RGO* can respond  
 7 to modulate CK levels in a fashion that facilitates or promotes favorable plant processes. It can  
 8 attenuate the negative effect of external CK application on root growth and conversely, positively  
 9 facilitate CK induced delay of senescence in excised leaves.  
 10



11

16



[Type text]

## 1 **Figure 7**

### 2 **Cytokinin (CK) response, CK levels in 35S:*AtRGO* and *rgo* silenced plants. (A)**

3 Hypersensitive response of 35S:*AtGLK1* plants to BA, a synthetic cytokinin (CK), in green calli  
4 formation. WT and 35S:*AtGLK1* seeds were germinated in dark for five days on half strength MS  
5 agar plates then elongated hypocotyls were excised out and grown on half strength MS agar  
6 plates supplemented with 0.005  $\mu$ M 2,4-D plus varying concentration (0.005 to 1  $\mu$ M) of BA  
7 under long-day photoperiod conditions. After 30 days representative callus were photographed.  
8 Scale bar = 1 cm. (B) Root length of seedlings at seven days post germination on minimal agar  
9 media in absence or presence of 5  $\mu$ M CK, \*represents the p value < 0.001; (C) Green callus  
10 formation from excised hypocotyl in presence of 0.005  $\mu$ M 2,4-D plus 0.5  $\mu$ M BA (CK) on MS  
11 agar plates of WT, 35S:*AtRGO* and *rgo*. Scale bar = 1 cm. (D) Effect of exogenous application of  
12 CK on leaf chlorophyll (Chl) retention during dark-induced senescence; (E) Bioactive CK [zeatin  
13 (tZ, open bar) and 2-isopentenyladenine (2iP, grey bar)] level in four-week old rosette leaves. p  
14 value < 0.001 (\*\*) and p value < 0.01 (\*). Error bars = SE (n=3). \* denotes the significant  
15 difference between -BA and +BA treatment (p<0.001, Student's t-test); (F) Relative transcripts  
16 of *CKX4* and *CKX5* quantified with qPCR in four week old leaves of wild-type (WT) Col-0,  
17 35S:*AtRGO*, and *rgo*, compared to *eIF4A-1* and *GAPC*, two housekeeping control transcripts.  
18 Relative transcripts (RQ) represent as mean fold change values  $\pm$  standard error of mean (SEM)  
19 from three biological replicates. Error bars = SE (n=3). \* denotes the statistical significant  
20 (p<0.001, Student's t-test).

21  
22

## 23 **Discussion**

24

25 *RGO* (*At1g77960*) is a single copy gene in Arabidopsis and encodes a protein of unknown function.  
26 *RGO* transcript levels increased highly in Arabidopsis plants ectopically overexpressing either the  
27 Arabidopsis *GLK1* or wheat *TaGLK1* transcription factor. *RGO* expression however did not seem  
28 to be regulated directly by *GLK1* as *glk1* plants did not show any down regulation of *RGO*. To  
29 explore if *RGO* expression was related to requirement for CK levels rather than directly to *GLK1*  
30 overexpression, we measured *GLK1* and *RGO* transcript levels in *atipt1 3 5 7*, a quadruple CK  
31 biosynthetic isopentyltransferases mutant severely deficient in endogenous iP- and tZ-type  
32 cytokinins (Miyawaki et al., 2006). Interestingly, *GLK1* levels were increased over 30-fold in this  
33 mutant, but increases in *RGO* levels were not observed (Supplemental Fig.S8). This indicated that  
34 increased transcript levels of *RGO* and *GLK1* does not necessarily occur in tandem and suggests  
35 that *GLK1* does not directly regulate *RGO*. It is not known why *GLK1* is so highly upregulated in  
36 this mutant. It is well established, however, that *GLK1* gene transcription and protein accumulation  
37 is regulated by plastid retrograde signals (Martin et al., 2016; Tokumaru et al., 2017) and it is  
38 possible to consider that endogenous CK levels may have an effect on this signalling.  
39 Nevertheless, this result further underscores the requirement of *RGO* in CK response. The genes

[Type text]

1 directly regulating transcription of *RGO* is not yet known. The elucidation of the transcriptional  
2 elements directly regulating the expression of *RGO* will have to await identification of binding  
3 domains in the promoter region of *RGO* to enable yeast-one-hybrid screens. Unlike *GLK*, *RGO* is  
4 not directly involved in the regulation of chloroplast development as *RGO* silenced plants did not  
5 show a pale green phenotype (Fig. 4, C and D) observed in the *glk1 glk2* plants, which are impaired  
6 in chloroplast development (Yasumura et al., 2005; Waters et al., 2008). Moreover, expression of  
7 *RGO* (Fig. 3) in all tissue types including photosynthetic tissues also argues against a role critical  
8 to chloroplast development. Ectopic overexpression of *RGO* in the pale green *glk1 glk2* background  
9 did not rescue chloroplast development, suggesting that it does not act upstream of the *GLKs*  
10 (unpublished). More likely, upregulation of *RGO* expression is a response to ectopic  
11 overexpression of *GLK1* or type-B ARR<sub>s</sub> without a phosphor-relay regulatory capacity such as  
12 *ARR21C* (Tajima et al., 2004; Kiba et al., 2005) in plants with normal endogenous CK levels.

13

#### 14 ***RGO* is essential for normal plant development.**

15

16 *RGO* is a single copy gene and the inability to acquire homozygous *RGO* knockout lines without  
17 *RGO* transcripts is suggestive that it is essential in plant development. We used amiR silencing as  
18 well as ectopic overexpression of *RGO* in Arabidopsis to gain insights into its role in plant  
19 development. Observations of reduced transcripts in the *RGO* downregulated (*rgo*) plants were  
20 correlated to displays of alterations in normal vegetative and root developments including growth  
21 retardation, low seed yields and seed quality leading to lower germination rates in successive  
22 generations (Fig. 4, Fig. 5, Supplemental Fig. S5). Conversely, Arabidopsis plants ectopically  
23 overexpressing *RGO* consistently produced higher seed yields and vegetative shoots.

24 We hypothesized that at least one explanation for the phenotypes produced by *RGO* overexpression  
25 and *RGO* knockdowns could be the result of the modulations of CK response by *RGO*. To this end,  
26 we carried out experiments to test specifically whether *RGO* is involved in attenuating or  
27 facilitating CK responses. We provide several lines of evidence to support this hypothesis. Firstly,  
28 overexpression of *RGO* dampened the effects of external application of CK on root development  
29 (Fig. 7A). Conversely, *RGO* silenced (*rgo*) plants showed retarded root growth even in the absence  
30 of external CK application (Fig. 5, A and B). Secondly, excised hypocotyls from *RGO* silenced  
31 plants were unable to produce green calli/shoot formation in external application of CK (Fig. 7B).  
32 Thirdly, increased expression of CK oxidase genes, *CKX4* and *CKX5* in leaves of *RGO*

[Type text]

1 overexpressing plants (Fig. 7E) suggested that one role of the involvement of *RGO* in CK response  
2 is to monitor and respond to endogenous levels of CK through *CKX* expression. Elevated CK levels  
3 can be attributed to modulation of *CKX* that will prevent the degradation of CKs (Mok and Mok,  
4 2001; Werner et al., 2001; 2003). Conversely, knockdown of *RGO* produced significant increases  
5 in bioactive CK concomitant with significant decreases of *CKX4* and *CKX5* expression in leaves  
6 of the *rgo* plants (Fig. 7D,E). *AtRGO* was identified as a candidate in a GWAS (genome wide  
7 association study) study to elucidate genes in shade avoidance  
8 (<https://explorations.ucdavis.edu/docs/2015/choi.pdf>). There was however, no indication the report  
9 was peer reviewed and that whether the knockouts of *AtRGO* were genotyped to assure  
10 homozygosity as well as absence of *AtRGO* gene transcripts. Nevertheless, it is known that shade  
11 avoidance is in part hormonally regulated and involves auxin and cytokinin levels mediated by leaf  
12 *CKXs* (Wu et al., 2017; Yang and Li, 2017). It is conceivable therefore that *AtRGO* could play a  
13 role in shade avoidance and should offer opportunities for future studies.

14

#### 15 **Modulation of CK response by *RGO* leads to increased seed yield**

16

17 Ectopic overexpression of *RGO* significantly increased seed yield in both *Arabidopsis* and *B.*  
18 *napus*. It was observed that the *Arabidopsis cckx3 cckx5* double mutant had increased CK levels  
19 resulting in increased floral meristems leading to increased seed yield (Bartrina et al., 2011). It is  
20 known that tissue specific expression of *CKXs* influence CK levels in those tissues (Mok and Mok,  
21 2001; Werner et al., 2001; 2003). Similarly, silencing of *OSCKX2* in rice increased CK  
22 accumulation in inflorescence meristems and increased tiller number resulting in enhanced grain  
23 yield (Ashikari et al., 2005; Yeh et al., 2015). Grain weight in wheat is also associated with  
24 *TaCKX6-D1*, an orthologue of *OSCKX2* (Zhang et al., 2012). Silencing of barley *HvCKX* genes  
25 has also been observed to increase seed yield (Zalewski et al., 2010, 2012, 2014). It is conceivable  
26 that increased seed yields in *RGO* overexpressing plants are, in part, a result of enhanced CK  
27 signaling. The reduction of *CKX3* and *CKX5* transcript levels in inflorescence buds of *RGO*  
28 overexpressing plants (Supplemental Table S4) supports the view that enhanced effects of CK  
29 responses are influenced by *RGO*.

30 The mechanism by which *RGO* facilitates CK responses or regulate plant development remains to  
31 be elucidated. *RGO* encodes a glutamine (Q) rich protein containing domains of Qs and tandem  
32 QNs or QHs. Other than these features, and a hydrophobic N-terminal, *RGO* contains no known

[Type text]

1 identifiable protein domains. The absence of localization of RGO to the nucleus (Fig. 3) precludes  
2 direct functional interactions with transcription factors. Proteins with high QN contents and poly-  
3 Q stretches have been associated with neurodegenerative disorders in humans through protein  
4 aggregation (Perutz, 1994; Guo et al., 2007; Kuiper et al., 2017) and expansion of Q stretches has  
5 been suggested to even modulate the aggregative properties of flanking amino acids (Kuiper et al.,  
6 2017). Little is known however of the functional roles of high Q containing proteins in plant  
7 development, although Q and HQ domains have been implicated in protein binding/aggregation  
8 (Guan et al., 2017) and in RNA binding (Muthuramalingam et al., 2016) in plants. As both poly Q  
9 and poly HQ domains are prominent in the RGO protein (Fig. 1A) and as the RGO protein is  
10 localized to the ER., we speculate that RGO may interact with CK signaling components as CK  
11 signaling components reside in the ER (Caesar et al., 2011; Lomin et al., 2011; Wulfetange et al.,  
12 2011; Lomin et al., 2018; Romanov et al., 2018) as well as mediating CK homeostasis by CKXs  
13 which takes place in the ER (Schmülling et al., 2003; Werner et al., 2003). Mechanistic aspect of  
14 RGO at protein level is under investigation. Identification of transcription factors that directly  
15 regulate *RGO* transcription as well as RGO protein/protein interaction partners will aid in  
16 elucidation of a mechanism of RGO in plant development.

## 17 **Materials and Methods**

### 18 **Plant material**

19

20 For routine propagation of *Arabidopsis thaliana*, seeds from wild-type (Col-0), T-DNA lines (Salk-  
21 025545, Salk-109614, Salk-109424), *RGO* silenced lines, 35S:*RGO*, *glk1* and the *glk1 glk2* double-  
22 knockout lines (N9806, N9807, respectively, obtained from the Nottingham Arabidopsis Stock  
23 Centre (NASC), Nottingham, UK.), the 35S:*GLK1* and 35S:*TaGLK1* lines were surface sterilized  
24 with 2% bleach (v/v) plus 0.001% tween-20, washed five times with sterile water and stratified for  
25 two days at 4°C. Seeds were grown in 48 cell trays on sterilised PRO-MIX MPV potting mixture  
26 (Premier Tech Horticulture, Rivière-du-Loup, QC, Canada) and grown in a Conviron PGC 20 CMP  
27 model 6050 cabinets (Winnipeg, Manitoba, Canada) at 21-22 °C, 40-60 % humidity, under long-  
28 day conditions (16-h-light/8-h-dark cycle) and a fluorescent light intensity of 100-120 μmol  
29 photons m<sup>-2</sup> s<sup>-1</sup>. Plants were fertilized once a week using 1g per litre solution of 20-20-20 fertilizer  
30 (Master Plant-Prod Inc. Brampton, ON, Canada). Similarly, sterilized seeds of *Brassica napus cv*  
31 Westar (wild-type) or *RGO* overexpressing transgenic plants were grown in six inch round pots

[Type text]

1 with sterilized mixture of 50% Promix, 25% soil and 25% sand in a Conviron as described above  
2 under long-day conditions with a light intensity of 750  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . A solution of two  
3 grams per litre 20-20-20 fertilizer was given once every third day for the first two weeks and then  
4 once a week until seed set. Ten plants each from *Brassica napus* wild-type and two-independent  
5 transgenic lines were grown in growth cabinets with identical growth conditions. Seeds were  
6 collected from each individual plant and weighed.

7

## 8 **Promoter GUS construct and analyses**

9

10 A 1.9 kb upstream of the transcription start site ATG of the *Atlg77960* gene was PCR amplified  
11 with RGO-promo-F and RGO-Promo-R primer pairs using Phusion® High-Fidelity DNA  
12 Polymerase (New England Biolabs, Whitby, ON, Canada) with Arabidopsis genomic DNA as the  
13 template and was verified by DNA sequencing (Eurofins Genomics, Louisville, KY, USA). The  
14 resulting PCR fragment was gel purified and cloned into the gateway entry vector pENTR®/D-  
15 TOPO® (Thermo Fisher Scientific, Waltham, MA, USA). The *RGO* promoter: pENTR®/D-TOPO®  
16 plasmid was transferred to the binary vector, pMDC162 (Curtis and Grossniklaus, 2003) using LR  
17 clonase II (Thermo Fisher Scientific, Waltham, MA, USA) to generate transcriptional fusion with  
18 GUS reporter gene. *Agrobacterium tumefaciens* strain GV3101 -pMP90RK containing the *RGO*  
19 promoter: pMDC162 plasmid was transformed to Arabidopsis wild-type (Col-0) plants by floral  
20 dipping (Clough and Bent, 1998). Primary transgenic plants ( $T_0$ ) were selected on Murashige and  
21 Skoog (MS) basal media plus agar plates in presence of 25  $\mu\text{g ml}^{-1}$  hygromycin. At least 10  
22 independent transformed lines from  $T_1$  through  $T_3$  generation were chosen for GUS analyses.  
23 GUS staining was performed as previously described (Murmu et al., 2010). Representative images  
24 from three independent lines were presented in figure. All the cloning primers are found in  
25 Supplemental Table S2.

26

## 27 **Constructs for subcellular localization of full length *RGO***

28

29 An overlap extension approach was used to create a C-terminal translational fusion of *RGO* with  
30 YFP. The *RGO* coding sequence (CDS) without the stop codon was PCR amplified from wild-  
31 type (WT) leaf cDNA with RGO-cDNA-F plus YFP-RGO cDNA-R primer pairs that generated a  
32 1284 bp PCR product with an overlap of 20 bp with the N-terminal of YFP. Next, *YFP* was PCR

[Type text]

1 amplified using RGO-YFP-F plus YFP-R primer pairs that generated a 743 bp PCR product with  
2 an overlap of 20 bp at the C-terminal of *RGO* CDS. The above two-PCR fragments were then used  
3 as templates for PCR amplification with RGO-cDNA-F plus YFP-R primer pairs to generate a  
4 1987 bp *RGO-YFP* translational fusion construct. The resulting PCR product was cloned into the  
5 Gateway entry vector pENTR/D-TOPO. Similarly, the YFP CDS was PCR amplified using YFP-  
6 F4-Topo plus YFP-R primer pairs and cloned into pENTR/D-TOPO for positive control. All  
7 constructs were verified by DNA sequencing. The *RGO-YFP* construct was then transferred to the  
8 pK7GW2D binary vector downstream of the Cauliflower Mosaic Virus (CaMV) 35S promoter  
9 (35S), using LR clonase II (Thermo Fisher Scientific, Waltham, MA, USA). Similarly, the YFP  
10 construct was transferred to the pMDC32 binary vector downstream of the CaMV 35S promoter  
11 (Curtis and Grossniklaus, 2003). The resulting 35S:*RGO-YFP*:pK7GW2D and 35S:YFP:pMDC32  
12 plasmids were introduced into *Agrobacterium tumefaciens* and were used to transform WT (Col-  
13 0) *Arabidopsis* plants by floral dipping as described in the previous section. Primary transgenic  
14 plants were selected on MS agar plates in the presence of 25  $\mu\text{g ml}^{-1}$  hygromycin for the construct  
15 in pMDC32 or in the presence of 50  $\mu\text{g ml}^{-1}$  kanamycin for the construct in pK7GW2D.

16

### 17 **Construction of *RGO* for overexpression studies**

18

19 A full length CDS of *RGO* was PCR amplified from WT leaf cDNA with Xba1- *RGO*-F and Kpn1-  
20 *RGO*-R primer pairs. The resulting 1.3 kb DNA fragment was fused to the CaMV 35S promoter at  
21 the Xba1 and Kpn1 sites in the pHS723 binary vector that also constitutively express the GUS  
22 reporter gene (Nair et al., 2000). The 35S:*RGO*:pHS723 plasmid was transformed into *A.*  
23 *tumefaciens* as described in the previous section. Primary transgenic plants were selected on MS  
24 agar plates in the presence of 50  $\mu\text{g/ml}$  kanamycin and highly expressing transgenic plants were  
25 further confirmed by GUS staining. *B. napus* cv Westar transformation with the 35S:*RGO*:pHS723  
26 construct was carried out as previously described (Savitch et al., 2005).

27

### 28 **Construction of artificial microRNA (amiR) to silence *RGO***

29

30 Artificial microRNA (amiRNA) gene silencing has been efficiently characterized in *Arabidopsis*  
31 (Ossowski et al., 2008). The detail of primer designing and step wise procedure for generating  
32 amiRNA constructs can be found in Web MicroRNA Designer (<http://wmd3.weigelworld.org>).

[Type text]

1 The Web MicroRNA Designer was used to generate a 21mer amiRNA  
2 (TAACGGAATACACGGTTGCGG) sequence found within the CDS of *RGO* between 68-84 bp  
3 to silence *RGO*. Based on this 21mer amiRNA sequence, four primers: [I: microRNA forward (I  
4 miR-S68), II: microRNA reverse (II miR-A68), III: microRNA\* forward (III miR\* S68), IV:  
5 microRNA\* reverse (IV mir\*A68)] were used to engineer the 21mer amiRNA by site-directed  
6 mutagenesis into the Arabidopsis endogenous plant microRNA, miR319a. The engineered  
7 miRNA319a targets the *RGO* gene for silencing. The following steps were used to engineer the  
8 amiRNA and amiRNA\* sequences into an endogenous miR319a precursor. First, three PCR  
9 fragments using the pRS300 plasmid as template that harbour the miRNA19a were generated. The  
10 first PCR amplified a 271 bp fragment with pRS300 A plus IV mir\*A68 primer set that generated  
11 overlap with one end of the pRS300 plasmid and mir\*A68. The second PCR amplified a 170 bp  
12 product with III miR\* S68 plus II miR-A68 primer set that generated overlap with mir\*A68 and  
13 miR-A68. The third PCR amplified a 290 bp product with I miR-S68 plus pRS300 B primer set  
14 that generated overlap with miR-A68 and other end of pRS300 plasmid. Finally, an overlap PCR  
15 was performed using the above three PCR fragments as templates with pRS300 A plus pRS300 B  
16 primer set that generated a 699 bp fragment of engineered miRNA19a with overlap of pRS300  
17 destined for subsequent cloning. The engineered miRNA19a was cloned into pJET1.2 (Thermo  
18 Fisher Scientific, Waltham, MA, USA) and verified by sequencing (Eurofins Genomics,  
19 Louisville, KY, USA). The engineered miRNA19a was excised from pJET1.2 with BamH1 and  
20 EcoR1, and ligated into the binary vector pBAR1 under the CaMV 35S promoter  
21 (35S:amiRNA19a:pBAR1). The construct was transformed to Arabidopsis WT plants via *A.*  
22 *tumefaciens* as described in the previous section. Basta-resistant transformed plants on soil were  
23 selected using Glufosinate ammonium (AgrEvo). Plants with most reduction of *RGO* transcripts  
24 were chosen as silenced lines.

25

## 26 **Semi-quantitative RT-PCR and quantitative RT-PCR (qPCR)**

27

28 Total RNA were isolated from rosette leaves, cauline leaves, stem and internode, and inflorescence  
29 apex tissues six-week old Arabidopsis WT plants grown under long-day conditions using Trizol  
30 reagent (Ambion, Thermo Fisher Scientific, Waltham, MA, USA). Total RNA from two-week old  
31 roots was isolated using RNAqueous® according to the manufacturer's instructions (Ambion,  
32 Thermo Fisher Scientific, Waltham, MA, USA). Total RNA from young and mature siliques were

[Type text]

1 isolated using a method described earlier (Murmu et al., 2010). RNA samples were treated with  
2 TURBO DNase<sup>TM</sup> (Ambion) prior to cDNA synthesis. Total cDNA from each RNA sample was  
3 synthesized from 2 µg of RNA template in a 20 µl reaction using Multiscribe reverse transcriptase  
4 (Applied Biosystems, Burlington, ON, Canada). The synthesized cDNA samples were diluted 1:5  
5 with diethylpyrocarbonate (DEPC)-treated water (Ambion). Semi-quantitative RT-PCR was  
6 performed using 2 µl of diluted cDNA as template and gene-specific primers (Supplemental Table  
7 S3). Similarly, quantitative RT-PCR (qPCR) reactions were performed using 2 µl of diluted cDNA  
8 as the template and gene-specific primers in triplicate by Power SYBR Green Kit and in a StepOne  
9 Plus Real-Time PCR System according to manufacturer's instructions (Applied Biosystems) as  
10 described earlier (Murmu et al., 2014). For absolute quantification of *RGO* transcripts in different  
11 tissues by qPCR, a standard curve method was used where the *RGO* standard curve was created  
12 using the *RGO*:pENTR-D-Topo plasmid, and *RGO* transcripts were calculated accordingly.  
13 Comparative  $C_T$  ( $\Delta\Delta C_T$ ) was used for relative quantification of transcripts of *GLK1* with GLK1-  
14 F2 plus GLK1-R2 primer set; and *RGO* with RGO-QF3 plus RGO-QR3 primer set and normalized  
15 using two endogenous control genes, *eIF4A-1* (*At3g13920*), and *GAPC* (*At3g04120*). The data  
16 represents three biological replicates with three technical replicates of each. All the qPCR data  
17 were analysed with  $P < 0.05$  as statistically significant value using the StepOne 2.1 software  
18 (Applied Biosystems). A list of the genes and primers used in RT-PCR and qPCR is found in  
19 Supplemental Table S2.

20

### 21 **Green callus formation assay**

22

23 Surface sterilized and stratified seeds were grown in square petri dish (VWR International, Ltd,  
24 Quebec, Canada) on half MS salt plus 0.7 % (w/v) Agar plates in the dark at room temperature  
25 for five days. After five days, root portion was excised out with a razor blade in a sterile laminar  
26 flow hood and immediately transferred onto half MS plus agar plate supplemented with 0.005  
27 µM 2,4-D (2,4-Dichlorophenoxyacetic acid), a synthetic auxin, plus varying concentrations  
28 (0.005 to 0.5 µM) of BA (6-Benzylaminopurine) according to a procedure described earlier (Kiba  
29 et al., 2005). Plates were grown for an additional 30 days under long-day photoperiod conditions  
30 for generation of green calli. Green calli images were photographed with a Nikon D90 DSRL.

31

### 32 **Root growth assays**



[Type text]

1  
2 Sterilized seeds were placed on square petri plates (VWR International, Ltd, Quebec, Canada)  
3 containing minimal agar media (Haughn and Somerville, 1986) alone or supplemented either with  
4 1% sucrose or 5  $\mu\text{M}$  Kinetin (a synthetic CK). Plates were sealed with two layers of parafilm and  
5 grown vertically under a long-day growth cabinet for seven-day. At least 10 seeds from each  
6 genotype were placed on each plate with five to six replicates of the plates. The root growth assay  
7 was repeated three times with similar results. Roots were photographed with a Nikon D90 DSRL  
8 camera and root lengths were measured from the digital photographs with ImageJ software  
9 (<https://imagej.nih.gov/ij/>).

10

### 11 **Subcellular localization studies**

12

13 For subcellular localization, leaf discs were prepared from three-week old soil grown Arabidopsis  
14 plants and stained for 1 hour with 10  $\mu\text{M}$  DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)  
15 in the dark at room temperature. After 1 hour the leaf discs were washed three times with sterile  
16 water and then mounted in Fluoromount-G<sup>TM</sup> (Electron microscopy Sciences, Hatfield, PA, USA).  
17 Leaf discs were imaged with a Zeiss LSM800 Airyscan laser scanning confocal microscope (Carl  
18 Zeiss MicroImaging, Göttingen, Germany). For visualisation of DAPI and YFP, excitation lasers  
19 at 405 nm, 488 nm respectively were used and emission was monitored between 400 nm to 580  
20 nm, 490 nm to 585 nm were acquired using the GaAsP detector and a Plan-Apochromat 63X/1.46  
21 objective lens. For plasma membrane visualisation, leaf discs were stained with the lipophilic styryl  
22 fluorescent dye FM4-64FX (Molecular Probes, Eugene, OR, USA) at a concentration of 0.01  
23  $\mu\text{g}/\text{mL}$  in 1xPBS (Phosphate buffer saline) pH 7.4 and were incubated for 2 hours at room  
24 temperature in the dark. Subsequently, leaf discs were mounted in fresh dye and imaged with a  
25 Plan-Apochromat 63x/1.4 objective lens and the high resolution Airyscan detector using excitation  
26 laser line 488 nm and emission bands 490 nm to 580 nm, and 635 nm to 700 nm for YFP and  
27 plasma membrane, respectively. For endoplasmic reticulum (ER) localization of 35S:*GFP:HDEL*,  
28 an ER marker (Batoko et al., 2000) and 35S:*RGO:YFP* were infiltrated into four-week old  
29 *Nicotiana benthamiana* leaves via *Agrobacterium tumefaciens* according to a procedure previously  
30 described (Kosma et al., 2014). GFP and YFP fluorescence was monitored three days post  
31 infiltration. Essentially, leaf discs were mounted in Fluoromount-G<sup>TM</sup> and YFP fluorescence was  
32 captured as described above. Similarly, GFP fluorescence was detected with excitation laser 488

[Type text]

1 nm and emission was monitored between 490 nm to 585 nm using the GaAsP detector and a Plan-  
2 Apochromat 63X/1.46 objective lens. For ER localization, similar plane and cell-types were  
3 imaged. All the confocal images were processed with ZEN 2.1 (Zeiss MicroImaging, Göttingen,  
4 Germany) and Adobe Photoshop CS6 (<http://www.adobe.com/>).

5

## 6 **Chlorophyll retention of dark-induced senescence with BA (6-Benzylaminopurine)** 7 **treatment**

8

9 Rosette leaves number 4 and 5 from four-week old plants of WT, 35S:*RGO*, and *rgo* plants grown  
10 in a long-day photoperiod conditions were used for this assay. Two set of six leaves from each  
11 genotypes were floated in a volume of 20 ml sterile water with 0.01N NaOH (-BA) or with 5  $\mu$ M  
12 BA in 0.01N NaOH (+BA) in a petri dish, wrapped with aluminum foil and incubated in dark for  
13 seven days according to a procedure described earlier (Vercruyssen et al., 2015). After seven days  
14 of dark treatments (-BA, +BA), the leaves were imaged with a Nikon D90 digital SLR camera. The  
15 second set of leaves with similar treatments were used for chlorophyll (Chl: Chl a + Chl b)  
16 measurements. Two of 0.5 cm<sup>2</sup> leaf disc were resuspended in 1ml DMF (*N,N*-Dimethylformamide)  
17 in the dark at 4<sup>0</sup> C overnight for total Chl extraction and Chl was measured with UV-visible  
18 spectrophotometer (Thermo Scientific) at 647 nm and 664.5 nm in 1 mm cuvettes. Total Chl was  
19 calculated per cm<sup>2</sup> according to a procedure described earlier (Inskeep and Bloom, 1985). The  
20 experiment was repeated three times.

## 21 **ACKNOWLEDGEMENTS**

22 We are grateful to Professor Tatsuo Kakimoto (Precursory Research for Embryonic Science and  
23 Technology, Japan Science and Technology Agency, Kawaguchi, Japan) for generously  
24 providing seeds of the *Arabidopsis ipt1 3 5 7* quadruple mutant and Professor Owen Rowland,  
25 Carleton University, for providing the GFP-HDEL construct.

26

## 27 **LITERATURE CITED**

28

[Type text]

- 1 Ashikari M, Sakakibara H, Lin S, Yamamoto T, Takashi T, Nishimura A, Angeles ER, Qian Q,  
2 Kitano H, Matsuoka M (2005) Cytokinin oxidase regulates rice grain production. *Science* 309:  
3 741-745
- 4 Balasubramanian B, Lowry CV, Zitomer RS (1993) The Rox1 repressor of the *Saccharomyces*  
5 *cerevisiae* hypoxic genes is a specific DNA-binding protein with a high-mobility-group motif.  
6 *Mol Cell Biol* 13: 6071-6078
- 7 Bartrina I, Otto E, Strnad M, Werner T, Schmülling T (2011) Cytokinin regulates the activity of  
8 reproductive meristems, flower organ size, ovule formation, and thus seed yield in *Arabidopsis*  
9 *thaliana*. *Plant Cell* 23: 69-80
- 10 Batoko H, Zheng HQ, Hawes C, Moore I (2000) A Rab1GTPase is required for transport between  
11 the endoplasmic reticulum and Golgi apparatus and for normal Golgi movement in plants. *Plant*  
12 *Cell* 12: 2201-2218
- 13 Caesar K, Thamm AMK, Witthöft J, Elgass K, Huppenberger P, Grefen C, Horak J, Harter K  
14 (2011) Evidence for the localization of the *Arabidopsis* cytokinin receptors AHK3 and AHK4  
15 in the endoplasmic reticulum. *J Exp Bot* 62: 5571-5580
- 16 Clough SJ, Bent AF (1998) Floral dip: a simplified method for agrobacterium-mediated  
17 transformation of *Arabidopsis thaliana*. *Plant J* 16: 735-743
- 18 Cortleven A, Marg I, Yamburenko MV, Schlicke H, Hill K, Grimm B, Schaller GE, Schmülling T  
19 (2016) Cytokinin regulates the etioplast-chloroplast transition through the two-component  
20 signaling system and activation of chloroplast-related genes. *Plant Physiol* 172: 464-478
- 21 Cortleven A, Schmülling T (2015) Regulation of chloroplast development and function by  
22 cytokinin. *J Exp Bot* 66: 4999-5013
- 23 Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional  
24 analysis of genes in planta. *Plant Physiol* 133: 462-469
- 25 Deckert J, Rodriguez Torres AM, Simon JT, Zitomer RS (1995) Mutational analysis of Rox1, a  
26 DNA-bending repressor of hypoxic genes in *Saccharomyces cerevisiae*. *Mol Cell Biol* 15:  
27 6109-6117
- 28 Fitter DW, Martin DJ, Copley MJ, Scotland RW, Langdale JA (2002) GLK gene pairs regulate  
29 chloroplast development in diverse plants. *Plant J* 31:713-727
- 30 Gan S, Amasino RM (1995) Inhibition of leaf senescence by auto-regulated production of  
31 cytokinin. *Science* 270: 1986-1988

[Type text]

- 1 Garapati P, Xue G-P, Munné-Bosch A, Balazadeh S (2015) Transcription factor ATAF1 in  
2 Arabidopsis promotes senescence by direct regulation of key chloroplast maintenance and  
3 senescence transcriptional cascades. *Plant Physiol* 168: 1122-1139
- 4 Goda H, Sasaki E, Akiyama K, Maruyama-Nakashita, Nakayabashi K, Li W, Ogawa, M,  
5 Yamaguchi Y, Preston J, Aoiki K et al., (2008) The ATGenExpress hormone and chemical  
6 treatment data set: experimental design, data evaluation, model data analysis and data access.  
7 *Plant J* 55: 526-542
- 8 Guan P, Ripoll JJ, Wang R, Vuong L, Bailey-Steinitz LJ, Ye D, Crawford NM (2017) Interacting  
9 TCP and NLP transcription factors control plant responses to nitrate availability. *Proc Natl*  
10 *Acad Sci USA* 114: 2419-2424
- 11 Guo L, Han A, Bates DL, Cao J, Chen L (2007) Crystal structure of a conserved N-terminal domain  
12 of histone deacetylase 4 reveals functional insights into glutamine-rich domains. *Proc Natl*  
13 *Acad Sci USA* 104: 4297-4302
- 14 Häffner E, Konietzki S, Diederichsen E (2015) Keeping Control: The Role of Senescence and  
15 Development in Plant Pathogenesis and Defense. *Plants (Basel)* 4: 449-488.
- 16 Haughn GH, Somerville C (1986) Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. *Mol Gen*  
17 *Genet* 204: 430-434
- 18 Hill K, Mathews DE, Kim HJ, Street IH, Wildes SL, Chiang YH, Mason MG, Alonso JM, Ecker  
19 JR, Kieber JJ, Schaller GE (2013) Functional characterization of type-B response regulators in  
20 the *Arabidopsis* cytokinin response. *Plant Physiol* 162: 212-224
- 21 Howell SH, Lall S, Che P (2003) Cytokinins and shoot development. *Trends Plant Sci* 8: 453-459
- 22 Inskip WP, Bloom PR (1985) Extinction coefficients of chlorophyll a and B in N,N-  
23 dimethylformamide and 80% acetone. *Plant Physiol* 77: 483-485
- 24 Kiba T, Naitou T, Koizumi N, Yamashino T, Sakakibara H, Mizuno T (2005) Combinatorial  
25 microarray analysis revealing *Arabidopsis* genes implicated in cytokinin responses through the  
26 His-Asp phosphorelay circuitry. *Plant Cell Physiol* 46: 339-355
- 27 Kieber JJ, Schaller GE (2014) Cytokinins. *The Arabidopsis Book* e0168
- 28 Kim HJ, Ryu H, Hong SH, Woo HR, Lim PO, Lee IC, Sheen J, Nam HG, Hwang I (2006)  
29 Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in  
30 *Arabidopsis*. *Proc Natl Acad Sci USA* 103: 814-819

[Type text]

- 1 Kobayashi K, Baba S, Obayashi T, Sato M, Toyooka K, Keränen M, Aro EM, Fukaki H, Ohta H,  
2 Sugimoto K, Masuda T (2012) Regulation of root greening by light and auxin/cytokinin  
3 signaling in Arabidopsis. *Plant Cell* 24: 1081-1095
- 4 Kobayashi K, Ohnishi A, Sasaki D, Fujii S, Iwase A, Sugimoto K, Masuda T, Wada H (2017)  
5 Shoot removal induces chloroplast development in roots via cytokinin signaling. *Plant Physiol*  
6 173: 2340-2355
- 7 Kosma DK, Murmu J, Razeq FM, Santos P, Bourgault R, Molina I, Rowland O (2014) AtMYB41  
8 activates ectopic suberin synthesis and assembly in multiple plant species and cell types. *Plant*  
9 *J* 80: 216-229
- 10 Kuiper EFE, de Mattos EP, Jardim LB, Kampinga HH, Bergink S (2017) Chaperones in  
11 polyglutamine aggregation: Beyond the Q-stretch. *Front Neurosci* 11: 145
- 12 Lai Z, Wang F, Zheng Z, Fan B, Chen Z (2011) A critical role of autophagy in plant resistance to  
13 necrotrophic fungal pathogens. *Plant J* 66: 953-968
- 14 Lewis LA, Polanski K, de Torres-Zabala M, Jayaraman S, Bowden L, Moore J, et al (2015)  
15 Transcriptional dynamics driving MAMP-triggered immunity and pathogen effector-mediated  
16 immunosuppression in Arabidopsis leaves following infection with *Pseudomonas syringae* pv  
17 tomato DC3000. *Plant Cell* 27: 3038-3064
- 18 Lin YJ, Cao ML, Xu CG, Chen H, Wei J, Zhang QF (2002) Cultivating rice with delaying led-  
19 senescence by P-SAG12-IPT gene transformation. *Act Bot Sin* 44: 1333-1338
- 20 Liu YD, Yin ZJ, Yu JW, Li J, Wei HL, Han XL, Shen FF (2012) Improved salt tolerance and  
21 delayed leaf senescence in transgenic cotton expressing the *Agrobacterium* IPT gene. *Biol*  
22 *Plant* 56: 237-246
- 23 Lomin SN, Myakushina YA, Arkhipov DV, Leonova OG, Popenko VI, Schmülling T, Romanov  
24 GA (2018) Studies of cytokinin receptor-phosphotransmitter interaction provide evidences for  
25 the initiation of cytokinin signalling in the endoplasmic reticulum. *Funct Plant Biol* 45: 192-  
26 202
- 27 Lomin SN, Yonekura-Sakakibara K, Romanov GA, Sakakibara H (2011) Ligand-binding  
28 properties and subcellular localization of maize cytokinin receptors. *J Exp Bot* 62: 5149-5159
- 29 Lupi ACD, Lira BS, Gramegna G, Trench B, Alves FRR, Demarco D, Peres LEP, Purgatto E,  
30 Freschi L, Rossi M (2019) *Solanum lycopersicum* GOLDEN 2-LIKE 2 transcription factor  
31 affects fruit quality in a light- and auxin-dependent manner. *PLoS One* 14: e0212224

[Type text]

- 1 Ma QH, Liu YC (2009) Expression of isopentenyl transferase gene (*ipt*) in leaf and stem delayed  
2 leaf senescence without affecting root growth. *Plant Cell Rep* 28: 1759-1765
- 3 Martín G, Leivar P, Ludevid D, Tepperman JM, Quail PH, Monte E (2016) Phytochrome and  
4 retrograde signalling pathways converge to antagonistically regulate a light-induced  
5 transcriptional network. *Nat Commun* 7: 11431
- 6 Miyawaki K, Tarkowski P, Matsumoto-Kitano M, Kato T, Sato S, Tarkowska D, Tabata S,  
7 Sandberg G, Kakimoto T (2006) Roles of Arabidopsis ATP/ADP isopentenyltransferases and  
8 tRNA isopentenyltransferases in cytokinin biosynthesis. *Proc Natl Acad Sci USA* 103: 16598-  
9 16603
- 10 Mok DW, Mok MC (2001) Cytokinin metabolism and action. *Annu Rev Plant Physiol Plant Mol*  
11 *Biol* 52: 89-118
- 12 Murmu J, Bush MJ, DeLong C, Li S, Xu M, Khan M, Malcolmson C, Fobert PR, Zachgo S,  
13 Hepworth SR (2010) Arabidopsis basic leucine-zipper transcription factors TGA9 and TGA10  
14 interact with floral glutaredoxins ROXY1 and ROXY2 and are redundantly required for anther  
15 development. *Plant Physiol* 154: 1492-1504
- 16 Murmu J, Wilton M, Allard G, Pandeya R, Desveaux D, Singh J, Subramaniam R (2014)  
17 Arabidopsis Golden2-like transcription factors (GLK) activate JA-dependent disease  
18 susceptibility against the biotrophic pathogen *Hyaloperonospora arabidopsidis* as well as JA-  
19 independent plant immunity against the necrotrophic pathogen *Botrytis cinerea*. *Mol Plant*  
20 *Pathol* 15: 174-184
- 21 Muthuramalingam M, Wang Y, Li Y, Mahalingam R (2017) Interacting protein partners of  
22 Arabidopsis RNA-binding protein AtRBP45b. *Plant Biol (Stuttg)* 19: 327-334
- 23 Nair RB, Joy RW<sup>4th</sup>, Kurylo E, Shi X, Schnaider J, Datla RS, Keller WA, Selvaraj G (2000)  
24 Identification of a CYP84 family of cytochrome P450-dependent mono-oxygenase genes in  
25 *Brassica napus* and perturbation of their expression for engineering sinapine reduction in the  
26 seeds. *Plant Physiol* 123: 1623-1634
- 27 Nelson BK, Cai X, Nebenführ A (2007) A multicolored set of in vivo organelle markers for co-  
28 localization studies in Arabidopsis and other plants. *Plant J* 51: 1126-1136
- 29 Ossowski S, Schwab R, Weigel D (2008) Gene silencing in plants using artificial microRNAs and  
30 other small RNAs. *Plant J* 53:674-690
- 31 Perutz MF, Johnson T, Suzuki M, Finch JT (1994) Glutamine repeats as polar zippers: their  
32 possible role in inherited neurodegenerative diseases. *Proc Natl Acad Sci USA* 91: 5355-5358

[Type text]

- 1 Raines T, Shanks C, Cheng CY, McPherson D, Argueso CT, Kim HJ, Franco-Zorrilla JM, López-  
2 Vidriero I, Solano R, Vaňková R, Schaller GE, Kieber JJ (2016) The cytokinin response factors  
3 modulate root and shoot growth and promote leaf senescence in Arabidopsis. *Plant J* 85: 134-  
4 147
- 5 Rauf M, Arif M, Dortay H, Matallana-Ramírez LP, Waters MT, Gil Nam H, Lim PO, Mueller-  
6 Roeber B, Balazadeh S (2013) ORE1 balances leaf senescence against maintenance by  
7 antagonizing G2-like-mediated transcription. *EMBO Rep* 14: 382-388
- 8 Reichmann JL, Heard J, Martin G, Reuber L, Jiang CZ, Keddie J, Adam L, Pineda O, Ratcliffe OJ,  
9 Samaha RR, Creelman R, Pilgrim M, Broun P, Zhang JZ, Ghandehari D, Sherman BK, Yu GL  
10 (2000) Arabidopsis transcription factors: genome-wide comparative analysis among  
11 eukaryotes. *Science* 290: 2105-2110
- 12 Richmond AE, Lang A (1957) Effect of kinetin on protein content and survival of detached  
13 Xanthium leaves. *Science* 125: 650-651
- 14 Romanov GA, Lomin SN, Schmülling T (2018) Cytokinin signaling: from the ER or from the PM?  
15 That is the question! *New Phytol* 218: 41-53
- 16 Savitch LV, Allard G, Seki M, Robert LS, Tinker NA, Huner NP, Shinozaki K, Singh J (2005) The  
17 effect of overexpression of two Brassica CBF/DREB1-like transcription factors on  
18 photosynthetic capacity and freezing tolerance in Brassica napus. *Plant Cell Physiol* 46: 1525-  
19 1539
- 20 Savitch LV, Subramaniam R, Allard G, Singh J (2007) The GLK1 ‘regulon’ encodes disease  
21 defense related proteins and confers resistance to *Fusarium graminearum* in Arabidopsis.  
22 *Biochem Biophys Res Commun* 359: 234-238
- 23 Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissue  
24 cultured. *In Vitro Symp Soc Exp Biol* 11: 118-131
- 25 Schmülling T, Werner T, Riefler M, Krupkov E, Bartrina y Manns I (2003) Structure and function  
26 of cytokinin oxidase/dehydrogenase genes of maize, rice, Arabidopsis and other species. *J Plant*  
27 *Res* 116: 241-252
- 28 Swartzberg D, Kirshner B, Rav-David D, Elad Y, Granot D (2008) *Botrytis cinerea* induces  
29 senescence and is inhibited by autoregulated expression of the *IPT* gene. *Eur J Plant Pathol*  
30 120: 289-297

[Type text]

- 1 Tajima Y, Imamura A, Kiba T, Amano Y, Yamashino T, Mizuno T (2004) Comparative studies on  
2 the type-B response regulators revealing their distinctive properties in the His-to-Asp  
3 phosphorelay signal transduction of *Arabidopsis thaliana*. *Plant Cell Physiol* 45: 28-39
- 4 Talla SK, Panigrahy M, Kappara S, Nirosha P, Neelamraju S, Ramanan R (2016) Cytokinin delays  
5 dark-induced senescence in rice by maintaining the chlorophyll cycle and photosynthetic  
6 complexes. *J Exp Bot* 67: 1839-1851
- 7 Thatcher LF, Kamphuis LG, Hane JK, Oñate-Sánchez L, Singh KB (2015) The *Arabidopsis* KH-  
8 domain RNA-binding protein ESR1 functions in components of jasmonate signalling,  
9 unlinking growth restraint and resistance to stress. *PLoS One* 10: e0126978
- 10 Tokumaru M, Adachi F, Toda M, Ito-Inaba Y, Yazu F, Hirose Y, Sakakibara Y, Suiko M,  
11 Kakizaki T, Inaba T (2017) Ubiquitin-proteasome dependent regulation of the GOLDEN2  
12 LIKE 1 transcription factor in response to plastid signals. *Plant Physiol* 173: 524–535
- 13 Vercruyssen L, Tognetti VB, Gonzalez N, Van Dingenen J, De Milde L, Bielach A, De Rycke R,  
14 Van Breusegem F, Inzé D (2015) GROWTH REGULATING FACTOR5 stimulates  
15 *Arabidopsis* chloroplast division, photosynthesis, and leaf longevity. *Plant Physiol* 167: 817-  
16 832
- 17 Wang H, Liu G, Li C, Powell AL, Reid MS, Zhang Z, Jiang CZ (2013) Defence responses regulated  
18 by jasmonate and delayed senescence caused by ethylene receptor mutation contribute to the  
19 tolerance of petunia to *Botrytis cinerea*. *Mol Plant Pathol* 14: 453-469
- 20 Waters MT, Moylan EC, Langdale JA (2008) GLK transcription factors regulate chloroplast  
21 development in a cell-autonomous manner. *Plant J* 56: 432-444
- 22 Waters MT, Wang P, Korkaric M, Capper RG, Saunders NJ, Langdale JA (2009) GLK  
23 transcription factors coordinate expression of the photosynthetic apparatus in *Arabidopsis*.  
24 *Plant Cell* 21: 1109-1128
- 25 Werner T, Motyka V, Strnad M, Schmülling T (2001) Regulation of plant growth by cytokinin.  
26 *Proc Natl Acad Sci USA* 98: 10487-10492
- 27 Werner T, Motyka V, Laucou V, Smets R, Van Onckelen H, Schmülling T (2003) Cytokinin-  
28 deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating  
29 opposite functions of cytokinins in the regulation of shoot and root meristem activity. *Plant*  
30 *Cell* 15: 2532-2550
- 31 Werner T, Schmülling T (2009) Cytokinin action in plant development. *Curr Opin Plant Biol* 12:  
32 527-538



[Type text]

- 1 Wu Y, Gong W, Yang W (2017) Shade Inhibits Leaf Size by Controlling Cell Proliferation  
2 and Enlargement in Soybean. *Sci Rep* 7: 9259  
3
- 4 Wulfetange K, Lomin SN, Romanov GA, Stolz A, Heyl A, Schmülling T (2011) The cytokinin  
5 receptors of Arabidopsis are located mainly to the endoplasmic reticulum. *Plant Physiol* 156:  
6 1808-1818
- 7 Yang C, Li L (2017) Hormonal Regulation in Shade Avoidance. *Front Plant Sci* 8: 1527.
- 8 Yasumura Y, Moylan EC, Langdale JA (2005) A conserved transcription factor mediates nuclear  
9 control of organelle biogenesis in anciently diverged land plants. *Plant Cell* 17: 1894-1897
- 10 Yeh SY, Chen HW, Ng CY, Lin CY, Tseng TH, Li WH, Ku MS (2015) Down-regulation of  
11 cytokinin oxidase 2 expression increases tiller number and improves rice yield. *Rice* 8: 36
- 12 Zalewski W, Galuszka P, Gasparis S, Orczyk W, Nadolska-Orczyk A (2010) Silencing of the  
13 HvCKX1 gene decreases the cytokinin oxidase/dehydrogenase level in barley and leads to  
14 higher plant productivity. *J Exp Bot* 61: 1839-1851
- 15 Zalewski W, Gasparis S, Boczkowska M, Rajchel IK, Kała M, Orczyk W, Nadolska-Orczyk A  
16 (2014) Expression patterns of HvCKX genes indicate their role in growth and reproductive  
17 development of barley. *PLoS One* 9: e115729
- 18 Zalewski W, Orczyk W, Gasparis S, Nadolska-Orczyk A (2012) HvCKX2 gene silencing by  
19 biolistic or Agrobacterium-mediated transformation in barley leads to different phenotypes.  
20 *BMC Plant Biol* 12: 206
- 21 Zhang L, Zhao YL, Gao LF, Zhao GY, Zhou RH, Zhang BS, Jia JZ (2012) TaCKX6-D1, the  
22 ortholog of rice OsCKX2, is associated with grain weight in hexaploid wheat. *New Phytol* 195:  
23 574-584
- 24 Zhang P, Wang WQ, Zhang GL, Kaminek M, Dobrev P, Xu J, Gruissem W (2010) Senescence-  
25 inducible expression of isopentenyl transferase extends leaf life, increases drought stress  
26 resistance and alters cytokinin metabolism in cassava. *J Integr Plant Biol* 52: 653-669

27

## 28 **FIGURE LEGENDS**

29

### 30 **Figure 1**

31 **Deduced amino acid sequence and upregulation of Arabidopsis *Response to GLK1***  
32 ***Overexpression (RGO, At1g77960)***. (A) amino acid sequence of AtRGO in single-letter code and

[Type text]

1 the glutamine rich region is highlighted in gray. Numbering of amino acids are on the right. (B)  
2 Transcripts accumulation of *AtGLK1* and *AtRGO* in wild-type (WT), *glk1* mutant, and  
3 35S:*AtGLK1* plants with RT-PCR compared to *eIF4A-1*, a housekeeping gene transcripts; (C)  
4 *AtGLK1* and *AtRGO* transcripts with qPCR relative to *eIF4A-1* and *GAPC*, two housekeeping gene  
5 transcripts. Relative transcripts represent as mean fold change values  $\pm$  standard error of mean  
6 (SEM) from three biological replicates.

7

## 8 **Figure 2**

9 **Expression pattern of *AtRGO*.** (A) *AtRGO* transcripts accumulation in wild-type (WT) Col-0  
10 with RT-PCR compared to *eIF4A-1* and *GAPC*, two housekeeping control transcripts; (B)  
11 Absolute quantification of *AtRGO* transcripts with qPCR. Transcripts represented are mean fold  
12 change values  $\pm$  standard error of mean (SEM) from three biological replicates; (C) *AtRGO*  
13 promoter driven reporter GUS reporter gene expression in WT plants. GUS expression was  
14 observed starting one day after germination; GUS expression was intense in cotyledons and  
15 slowly progressed to vascular tissues in the root. Scale bar, 200  $\mu$ m in seedlings, and bar, 1 cm in  
16 mature tissues.

17

## 18 **Fig. 3**

19 **Subcellular localization of *AtRGO*-YFP fusion protein by confocal microscopy in transgenic**  
20 ***Arabidopsis* leaves and tobacco leaves.** Plants expressing 35S:YFP were used as positive control  
21 (A-C) for comparing *AtRGO*-YFP fusion expression (D-F). (A and D), YFP signal in yellow; (B  
22 and E), DAPI stained nuclei in blue; (C and F) merged. Arrow head shows the nucleus and  
23 endoplasmic reticulum (ER). Zoom in view of 35S:YFP (G) and *AtRGO*:YFP (H) with FM4-64FX  
24 stain. YFP fluorescence in yellow and plasma membrane stain in blue, a false colour for contrast  
25 is shown in G-H and arrow head shows the nucleus and plasma membrane (PM) in G-H. Transient  
26 expression of *AtRGO*-YFP (I) and GFP:HDEL, an ER marker (J) in tobacco leaves. Arrow head  
27 shows the ER in I-J. Scale bar, 20  $\mu$ m in A-F and 5  $\mu$ m in G-J.

28

## 29 **Figure 4**

30 **Phenotype of *rgo* plants compared to WT (Col-0).** (A) A RT-PCR of full length *AtRGO*  
31 transcripts in WT and *rgo* plants compared to *eIF4A-1* and *GAPC* transcripts; (B) qPCR of

[Type text]

1 *AtRGO* transcripts in WT and *rgo* plants; (C) phenotype at four-week age; (D) phenotype at six-  
2 week age. Scale bar, 1 cm in C-D.

3

#### 4 **Figure 5**

##### 5 **Root growth phenotype of WT, *rgo* and 35S:*AtRGO* lines at seven days post germination.**

6 (A) representative images on minimal media (MM), (B) corresponding root length. Error bar =  
7 standard error of mean. Scale bar, 1 cm in A. Statistical significance of root lengths were  
8 analyzed using student's t-test: Two sample assuming unequal variance at 95% confidence level.  
9 \*\*\* represents the p value < 0.00000001, and \* represents p value > 0.1.

10

#### 11 **Figure 6**

12 **Phenotype of 35S:*AtRGO* plants compared to WT.** (A) A RT-PCR of full length *AtRGO*  
13 transcripts compared to *eIF4A-1* and *GAPC* transcripts in WT and three independent lines of  
14 35S:*AtRGO* plants; (B) qPCR of *AtRGO* transcripts in WT and *AtRGO* overexpressing lines; (C)  
15 Four-week old plants; (D) Five-week old plants. Emergence of inflorescence buds are shown  
16 with arrow head in C and axillary buds in D; (E) Average seed yield from 10 Arabidopsis plants  
17 of WT and 35S:*AtRGO* from a long-day photoperiod growth conditions; (F) seed yield from  
18 individual plants of *B. napus* in WT and 35S:*AtRGO* (two lines). Scale bar, 1cm in C-D.  
19 Statistical significance was analysed using student's t-test: Two-sample assuming unequal  
20 variances at 95% confidence level compared with empty vector. The p value for each analysis is  
21 shown as asterisk, where p value ≤ 0.001 (\*\*). Error bar = standard error of mean.

22

#### 23 **Figure 7**

##### 24 **Cytokinin (CK) response, CK levels in 35S:*AtRGO* and *rgo* silenced plants.** (A)

25 Hypersensitive response of 35S:*AtGLK1* plants to BA, a synthetic cytokinin (CK), in green calli  
26 formation. WT and 35S:*AtGLK1* seeds were germinated in dark for five days on half strength MS  
27 agar plates then elongated hypocotyls were excised out and grown on half strength MS agar  
28 plates supplemented with 0.005 μM 2,4-D plus varying concentration (0.005 to 1 μM) of BA  
29 under long-day photoperiod conditions. After 30 days representative callus were photographed.  
30 Scale bar = 1 cm. (B) Root length of seedlings at seven days post germination on minimal agar  
31 media in absence or presence of 5 μM CK, \*represents the p value < 0.001; (C) Green callus  
32 formation from excised hypocotyl in presence of 0.005 μM 2,4-D plus 0.5 μM BA (CK) on MS

[Type text]

1 agar plates of WT, 35S:*AtRGO* and *rgo*. Scale bar = 1 cm. (D) Effect of exogenous application of  
2 CK on leaf chlorophyll (Chl) retention during dark-induced senescence; (E) Bioactive CK [zeatin  
3 (tZ, open bar) and 2-isopentenyladenine (2iP, grey bar)] level in four-week old rosette leaves. p  
4 value < 0.001 (\*\*) and p value <0.01 (\*). Error bars = SE (n=3). \* denotes the significant  
5 difference between -BA and +BA treatment (p<0.001, Student's t-test); (F) Relative transcripts  
6 of *CKX4* and *CKX5* quantified with qPCR in four week old leaves of wild-type (WT) Col-0,  
7 35S:*AtRGO*, and *rgo*, compared to *eIF4A-1* and *GAPC*, two housekeeping control transcripts.  
8 Relative transcripts (RQ) represent as mean fold change values  $\pm$  standard error of mean (SEM)  
9 from three biological replicates. Error bars = SE (n=3). \* denotes the statistical significant  
10 (p<0.001, Student's t-test).

11

12

13

14

15

16

17

18

19

20