ORE1 balances leaf senescence against maintenance by antagonizing G2-like-mediated transcription

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Leaf senescence is a key physiological process in all plants. Its onset is tightly controlled by transcription factors, of which NAC factor ORE1 (ANAC092) is crucial in *Arabidopsis thaliana*. Enhanced expression of *ORE1* triggers early senescence by controlling a downstream gene network that includes various senescence-associated genes. Here, we report that unexpectedly ORE1 interacts with the G2-like transcription factors GLK1 and GLK2, which are important for chloroplast development and maintenance, and thereby for leaf maintenance. ORE1 antagonizes GLK transcriptional activity, shifting the balance from chloroplast maintenance towards deterioration. Our finding identifies a new mechanism important for the control of senescence by ORE1.

Keywords: transcription factor; senescence; chloroplast; protein–protein interaction

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INTRODUCTION

Leaf senescence is a developmentally controlled process that involves extensive reprogramming and modulation of gene expression to maximize plant fitness by remobilizing nutrients from deteriorating leaves to newly growing vegetative and reproductive organs. Early and noticeable features of leaf senescence are Rubisco and chlorophyll degradation, and a decline of photosynthetic activity owing to chloroplast

dismantling [1,2]. Transcription factors (TFs) have important roles in coordinating the gene regulatory networks that underlie the senescence process [3,4]. One of the key senescence-control TF in *A. thaliana* is the NAC protein ORE1 (ANAC092; At5g39610). Overexpression of *ORE1* in transgenic plants triggers early senescence, while its inhibition retards senescence [5,6]. ORE1 exerts its regulatory function by controlling the expression of various known senescence-associated genes (SAGs) [5]. Expression of *ORE1* itself is controlled by at least two molecular mechanisms, one that involves currently unknown upstream TFs that determine leaf age- and abiotic stress-dependent *ORE1* transcriptional activity [5], and a second one that leads to *ORE1* messenger RNA degradation by transacting *miR164* [6]. Both processes contribute to establishing a coordinated expression of *ORE1*, which is low in young, but high in aging leaves.

In an aging leaf, the onset of senescence is counterbalanced by still vaguely defined chloroplast maintenance mechanisms. Key elements in this process are the Golden2-like TFs that act as nuclear regulators of chloroplast development and maintenance by coordinating the expression of genes-encoding proteins of the photosynthetic apparatus in various plant species, including *A. thaliana*, *Zea mays* and the moss *Physcomitrella patens* [7,8]. In *Arabidopsis*, *GLK* genes exist as a pair of homologous genes, *GLK1* and *GLK2*, and they have been shown to be functionally redundant such that only *glk1/glk2* double mutants show a clear phenotype [7,8].

Herein, we report the unexpected finding that ORE1 interacts with GLK TFs at the protein level. Elevated expression of *ORE1* in the presence of *GLK* expression strongly reduces the capacity of GLK to activate its target genes. Our data suggest a model by which ORE1 counteracts the chloroplast maintenance function of GLKs, thereby adding a new mechanism to the finely tuned control network underlying senescence.

RESULTS AND DISCUSSION

ORE1 interacts with GLK1 and GLK2 in yeast

To identify proteins interacting with ORE1, we performed a yeast two-hybrid screen using bait construct pLexPD-ORE1 and a

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Fig 1 | ORE1 interacts with GLK1 and GLK2 *in vitro* and *in vivo*. (A) Yeast two-hybrid. First row, negative control with EVs, pLex-PD and pACT2-Gal4. Rows two, three and four: results from auto-activation tests of ORE1, GLK2 and GLK1 individually fused either with LexA-DBD or Gal4-AD, co-expressed with the respective EV. Auto-activation of ORE1 (not shown) is repressed on medium supplemented with 20 mM 3AT (SDIV + 3AT). The last three rows show interaction tests of ORE1 with GLK2 or GLK1, where the growth test on selection media (SDIV + 3AT) and X-gal assay (SDII + X-gal) indicate an interaction of ORE1 with GLK2 or GLK1. Note, that no growth test was conducted for EV combinations on SDII + X-gal plates. (B) Western blot of *in vitro* pulled-down HIS::GLK2 recombinant fusion protein detected by anti-HIS monoclonal antibody (representative of three repeats). (C) Bimolecular fluorescence complementation of YFP confirming interaction between ORE1 and GLK2 in *Arabidopsis* mesophyll cell protoplasts prepared from rosette leaves of 4-week-old plants. Rows 1–3, negative controls. Row 1: *pSPYNE-nYFP* and *pSPYCE-cYFP* empty vectors. Row 2: *pSPYNE-ORE1::nYFP* and *pSPYCE-cYFP* empty vector. Row 3: *pSPYCE-GLK2::cYFP* and *pSPYNE-nYFP* empty vector. Row 4: mesophyll cell protoplast co-transformed with the *pSPYNE-ORE1::nYFP* and *pSPYCE-GLK2::cYFP* vectors, showing YFP signal in the nucleus (arrows). Data are representatives of four repeats. aa, amino acids; AD, activation domain; DBD, DNA-binding domain; EV, empty vector; SD, selective dropout medium; X-gal, 5-bromo-4-chloro-indolyl- β -D-galactopyranoside; 3AT, 3-amino-1,2,4-triazole.

prey complementary DNA library derived from *Arabidopsis* seedlings [9]. To increase screening specificity, we only included the region downstream of the highly conserved DNA-binding domain of ORE1. We identified GLK2 as an ORE1-interacting protein (Fig 1A).

GLK1 and GLK2 share ~50% amino-acid sequence identity. The similarity is higher, 90% and 79%, respectively, when the two conserved regions, GARP and GCT domains, are compared [10]. This high sequence similarity prompted us to test whether GLK1 would similarly interact with ORE1, which was indeed the case when a GLK1–GAL4 activation domain fusion was employed as prey (Fig 1A).

The NAC TF ORS1 (ORE1 SISTER1; ANAC059; At3g29035) is a paralog of ORE1 [11,12]. Within their NAM domains, both TFs share an overall amino-acid identity of 94%. We tested by two-hybrid assay for interactions of ORS1 with GLK1 or GLK2, using pLexPD-ORS1 as bait, and GLK1–GAL4 or GLK2–GAL4 as prey clones. Neither GLK1 nor GLK2 interacted with ORS1 (supplementary Fig S1 online), indicating specificity of the interaction of ORE1 with the two GLKs. Furthermore, an *in vitro* pull-down assay revealed that immobilized GST–ORE1 fusion

protein precipitated histidine-tagged GLK2 (HIS–GLK2) protein (Fig 1B; supplementary Fig S2 online) confirming the interaction of ORE1 and GLK2.

ORE1 and GLK2 interact *in planta*

We next used bimolecular fluorescence complementation to test for interaction of ORE1 and GLK2 in plant cells. Yellow fluorescence was exclusively detected in nuclei of mesophyll cell protoplasts co-expressing ORE1::nYFP and GLK2::cYFP (Fig 1C).

To test the effect of ORE1 on the subcellular localization of GLK2, we introduced *35S:GLK2::GFP* plasmid into mesophyll cell protoplasts prepared from *VGE:ORE1::HA* plants, which express haemagglutinin (HA)-tagged ORE1 protein from a methoxyfenozide (MOF)-inducible promoter (see supplementary Fig S3 online for induction of ORE1 protein after MOF induction). Confocal imaging revealed green fluorescent protein (GFP) signal in the nucleus of both MOF- and mock-treated cells (Fig 2A–D). This result clearly indicates that GLK2 is a nuclear-localized TF and that ORE1 interacts with GLK2 in the nucleus.

We further confirmed ORE1–GLK2 interaction by co-immunoprecipitation assay. To this end, *VGE:ORE1::HA* mesophyll cell

Fig 2 | Interaction of ORE1 and GLK2 in planta. Mesophyll cell protoplasts were prepared from rosette leaves of 4-week-old plants. (A) Non-induced, non-transformed *VGE:ORE1::HA* protoplast (first negative control). (B) *VGE:ORE1::HA* protoplast induced with MOF (80 μ M) and transformed with *35S::GFP* plasmid (second negative control). (C) Non-induced *VGE:ORE1::HA* protoplast transformed with *35S::GLK2::GFP* plasmid; note GFP signal in the nucleus (arrows). (D) MOF-induced *VGE:ORE1::HA* protoplast transformed with *35S::GLK2::GFP* plasmid; nuclear GFP signal is visible (arrows). The mat green signals in chloroplasts likely arise from fluorescence of undefined metabolites. (E) Co-immunoprecipitation of ORE1::HA/GLK2::GFP protein complex from *VGE:ORE1::HA* protoplasts transformed either with *35S::GLK2::GFP* or *35S::GFP* construct; ORE1::HA expression was induced by MOF treatment for 24 h. Anti-GFP and anti-HA monoclonal antibodies were used for immunoprecipitation and immunoblot analysis of interacting protein, respectively. Protein A/G agarose beads alone were used as negative control in a Co-IP assay. Data are representatives of three repeats. GFP, green fluorescent protein; HA, haemagglutinin; IB, immunoblotting; IP, immunoprecipitation; MOF, methoxyfenozide.

protoplasts transformed either with *35S::GLK2::GFP* or *35S::GFP* constructs were induced with MOF for 24 h. As shown in Fig 2E, immunoblot analysis detected an interaction between ORE1-HA and GLK2-GFP proteins. Thus, our data provide strong evidence for an interaction of ORE1 and GLK2 in *Arabidopsis* cells.

Functional consequence of ORE1 and GLK interaction

ORE1 is a key regulator of leaf senescence; its overexpression accelerates senescence while loss-of-function *ore1* mutants show delayed senescence and flowering [5,6]. In contrast, GLK1 and GLK2 regulate chloroplast development. Under long-day condition, *glk1* and *glk2* mutants produced fewer vegetative leaves than wild type but flowered within a similar time frame [7]. However, whether GLKs affect the timing or progression of leaf senescence has not been investigated previously. To elucidate the potential biological relevance of the ORE1–GLK interaction for senescence, we first analysed *GLK* transgenic lines and observed a delay of senescence, compared with wild type, in *35S::GLK1* and *35S::GLK2* overexpressors (Fig 3A), while *glk1* and *glk2* single

mutants did not show a striking alteration in their senescence behaviour (supplementary Fig S4 online). The pale-green phenotype of the *glk1/2* double mutant did not seem to alter age-dependent senescence. Delayed leaf yellowing observed in *GLK* overexpression plants was further confirmed by measuring senescence-related physiological and molecular parameters. Total chlorophyll content was significantly higher in *GLK1* and *GLK2* overexpressors than in wild-type and *glk1/2* double-mutant plants 8 weeks after sowing (Fig 3B). In accordance with this, ion leakage was less prominent in *GLK* overexpressors than wild-type and mutant plants (Fig 3C). Expression of early- and late-senescence marker genes, *SAG13* and *SAG12*, was down-regulated, whereas expression of photosynthesis-related genes (*CAB2*, *LHCB2.1*, *CAB4* and *LHCB4.1*) was enhanced in *GLK* overexpression plants compared with wild type, in accordance with their known molecular functions and the delayed senescence phenotype (Fig 3D,E).

We next generated *ORE1/GLK2* double overexpressors by crossing *35S::ORE1* and *35S::GLK2* lines (supplementary Fig S4D

Fig 3 | Characterization of transgenic plants. (A) Phenotype of WT, 35S:ORE1/35S:GLK2 double overexpressor, 35S:ORE1, 35S:GLK1, 35S:GLK2, *glk1/2* double-mutant and *ore1-1* mutant plants at 29 and 54 DAS. (B) Chlorophyll content and (C) ion leakage measured 4, 6 and 8 weeks after sowing. Bars represent the mean $\pm$ s.d., $n = 3$. (D) Transcript abundance of *SAG13* and *SAG12* genes measured by qRT-PCR 6 and 7 weeks after sowing. (E) Transcript abundance of *CAB2*, *LHC2.1*, *CAB4* and *LHC4.1* measured 7 weeks after sowing. Heat maps indicate log₂ fold-change expression ratio of selected genes in all lines compared with WT. DAS, days after sowing; qRT-PCR, quantitative reverse-transcriptase PCR; WT, wild type.

online). Under long-day condition, 35S:ORE1/35S:GLK2 plants bolted considerably earlier (5 days) and showed earlier senescence than wild-type controls (Fig 3A; supplementary Fig S4E online). Total chlorophyll was significantly lower, whereas ion leakage was higher in leaves of 35S:ORE1/35S:GLK2 lines compared with wild type (Fig 3B,C; supplementary Fig S4F,G online). Expression of *SAG12* and *SAG13* was higher in double overexpressors, while expression of photosynthesis-related genes was significantly reduced (Fig 3D,E). Taken together, these data demonstrate accelerated bolting and leaf senescence in transgenic plants overexpressing both *ORE1* and *GLK2*, resembling the typical phenotype of *ORE1* overexpression plants [5,6].

This observation indicates that the interaction of *ORE1* with *GLKs* antagonizes *GLK* function suggesting a negative impact of *ORE1* on photosynthetic apparatus maintenance and chloroplast integrity.

Effect of ORE1/GLK2 interaction on target genes

The fact that 35S:ORE1/35S:GLK2 double overexpressors highly resemble 35S:ORE1 plants with respect to bolting and senescence suggests a possible negative influence of *ORE1/GLK2* heteromerisation on the expression of *GLK* target genes. *GLK* target genes were previously reported [8]. We tested the expression of *ORE1* and *GLK* target genes by quantitative reverse-transcriptase PCR in

Fig 4 | Effect of ORE1 and GLK2 on transcriptional activation of downstream target genes. (A) Expression of *ORE1* and its target genes *RNS3*, *SINA1*, *BFN1*, *SAG29* and *VNI2*, and expression of *GLKs* and *GLK2* target genes *LHCB3*, *LHCB2.2*, *LHCB4.2*, *LHCB2.4*, *MRU1* and *Hydrolase* in 7-week-old wild-type (WT), *35S:ORE1/35S:GLK2*, *35S:ORE1*, *35S:GLK1*, *35S:GLK2*, *glk1/2* and *ore1-1* plants (leaf number 11). Heat map indicates expression level (log2 scale) relative to WT. (B) Effect of *GLK2* on *ORE1* transcriptional activity towards *ORE1* target genes in Col-0 protoplasts. Note that *GLK2* did not affect the transcriptional activation activity of *ORE1* towards its target genes. (C) Effect of *ORE1* on *GLK2* transcriptional activity towards *GLK* target genes in Col-0 protoplasts. Note that *ORE1* strongly reduces the transcriptional activation capacity of *GLK2* towards its photosynthesis-related target genes, while it did not affect expression of its non-photosynthetic targets (*MRU1* and *Hydrolase*). Protoplasts were prepared from rosette leaves of 4-week-old plants. *35S:GLK2* and *35S:ORE1* effector constructs were omitted in control experiments. Luciferase signal was determined 24 h after transfection. Error bars $\pm$ s.d. are given ($n = 3$). WT, wild type.

35S:GLK1, *35S:GLK2*, *glk1/2*, *35S:ORE1/35S:GLK2*, *35S:ORE1*, *ore1-1* and wild-type plants. Expression of the *ORE1* targets *RNS3*, *SINA1*, *BFN1*, *SAG29* and *VNI2* was higher in *35S:ORE1/35S:GLK2* than wild-type plants, similar to *35S:ORE1* plants. These genes did not show any significant difference in the expression in *glk1/2*, *35S:GLK1* and *35S:GLK2* plants compared with wild-type plants, except *VNI2* that showed a slight downregulation in both overexpression lines (Fig 4A). The lack of repression of *ORE1* target genes in the *GLK* overexpressors is fully consistent with the fact that *ORE1* expression itself is not largely affected at the developmental stage analysed (Fig 4A, middle).

On the other hand, expression of all six tested *GLK1* and *GLK2* target genes was highly upregulated in *35S:GLK1* and *35S:GLK2* plants, and strongly downregulated in the *glk1/2* double mutant compared with wild type, except for *MRU1* that showed no change in expression in *35S:GLK2* and *glk1/2* plants. Notably, none of the *GLK* targets was induced in the *35S:ORE1/35S:GLK2*

double overexpressor even in the presence of high transcript abundance of *GLK2*. This expression pattern was similar to the expression in *35S:ORE1* plants, which showed either no change in expression for some of the genes, such as *LHCB4.2*, *MRU1* and *LHCB3*, or downregulation of others, such as *Hydrolase*, *LHCB2.2* and *LHCB2.4*. Interestingly, expression of these genes was upregulated in the *ore1-1* mutant, except for *MRU1*, *LHCB3* and *Hydrolase*, which showed unaltered expression (Fig 4A). Collectively, our data demonstrate that *ORE1* attenuates the transcriptional activity of *GLK2*.

Transactivation assays

To further analyse the effect of *ORE1/GLK2* heteromers on the expression of downstream target genes, we performed luciferase-based transactivation assays using *Arabidopsis* mesophyll cell protoplasts. To this end, we fused the promoters of *ORE1* target genes to the firefly luciferase reporter. *ORE1* significantly transactivated all target gene promoters in protoplasts

Fig 5 | Model of GLK-ORE1 action. Expression of GLK target genes (green) is strongly hindered in the presence of ORE1 protein, which leads to an age-dependent decline in the expression of photosynthesis-related genes (GLK targets), while expression of ORE1 target genes (yellow) is stimulated leading to the onset and subsequent progression of senescence. *ORE1* transcript abundance is regulated by developmental and stress-related parameters through currently unknown upstream TFs, as well as by *miR164*, which is under negative control of ethylene signalling involving *EIN2* protein. TFs upstream of GLKs are currently unknown as well. TF, transcription factor.

co-transfected with a *35S:ORE1* effector plasmid and the reporter constructs (*proORE1-fLUC*, *proRNS3-fLUC*, *proSINA1-fLUC*, *proBFN1-fLUC*, *proSAG29-fLUC* and *proVNI2-fLUC*) (Fig 4B). *ORE1*-mediated transcriptional activation was not altered when *35S:ORE1* was co-transfected with *35S:GLK2* effector plasmid (Fig 4B). Thus, the results obtained from luciferase-based transactivation assays further support the conclusion that *ORE1/GLK2* heteromerisation does not affect the expressional regulation of *ORE1* target genes.

Next, we performed transactivation assays with reporter constructs harbouring GLK target gene promoters. As shown in Fig 4C, *GLK2* significantly transactivated all its target promoters (*proLHCB3-fLUC*, *proLHCB2.2-fLUC*, *proLHCB4.2-fLUC*, *proLHCB2.4-fLUC*, *proMRU1-fLUC* and *proHydrolase-fLUC*). This transcriptional activation by *GLK2* significantly decreased for all photosynthetic target genes when the *35S:ORE1* effector plasmid was transfected in addition to *35S:GLK2*, while the expression of non-photosynthetic GLK targets, that is, *MRU1* and *Hydrolase*, was not affected by *ORE1* (Fig 4C). This observation clearly indicates that *ORE1* has an inhibitory effect on the transcriptional activity of *GLK2*.

Model of GLK-ORE1 action

On the basis of the experimental data obtained herein, we propose a model that can explain how *ORE1* and *GLKs* coordinate the age-dependent alteration of the two antagonistic activities: chloroplast maintenance and initiation/progression of leaf senescence (Fig 5). During early leaf development, *ORE1* transcript level is low owing to transcriptional regulation [3,5] and post-transcriptional control by *miR164* [6], whereas *GLK1* and *GLK2* expression is high,

which transcriptionally activates genes of photosynthetic proteins involved in chloroplast development and maintenance [7,8]. At later stages of leaf development, accumulation of *ORE1* and its interaction with *GLKs* render inactive heterodimers unable to activate photosynthetic *GLK* target genes, while activation of *ORE1* targets is favoured. Thus, the senescence-triggering role of *ORE1* is dominant over the photosynthetic apparatus maintenance role of *GLKs*. Thus, *A. thaliana*, and possibly other plants, seem to use an intriguing and multilevel control mechanism to balance chloroplast maintenance against dismantling.

METHODS

Detailed experimental procedures are described in the supplementary information online.

Plant growth conditions. *A. thaliana* ecotype Col-0 was used. Seedlings were grown in soil (Einheitserde GS90; Gebrüder Patzer) in a climate-controlled chamber with 16-h day length provided by fluorescent light at $100\mu\text{mol m}^{-2}\text{s}^{-1}$ and a day/night temperature of 22/16 °C and a relative humidity of 60/75%.

Molecular and biochemical methods. Total RNA extraction, synthesis of cDNA and quantitative reverse-transcriptase PCR were performed as described [4,13]. cDNA quality was tested using *GAPDH* primers; genomic DNA contamination was checked using primers annealing to an intron of gene *AGL68*. *ACTIN2* served as an internal control for normalization of expression data. Yeast two-hybrid screens were performed using yeast strain *L40ccxU* as reported [14]. The growth tests were performed on selective dropout nutrient medium SDIV lacking Leu, Trp, His and Ura; SDII medium lacking Trp and Leu was used as control. Bimolecular fluorescence complementation was performed as described [15] using *Arabidopsis* mesophyll cell protoplasts. DNA cloning was done using standard procedures [16]. Details on the construction of the plasmids used here are given in the supplementary information online. Primer sequences are given in supplementary Table S1 online. Expression of GST1-*ORE1* and HIS-*GLK2* fusion proteins in *Escherichia coli* strain Rosetta (DE3) pLysS (Merck), protein purification and *in vitro* pull-down assays for immunological detection of purified GST-*ORE1* and HIS-*GLK2* fusion proteins were performed as described [17,18]. For co-immunoprecipitation of interacting protein complexes, protein was extracted from mesophyll cell protoplasts obtained from the *VGE:ORE1::HA* line transfected with the *35S:GFP* or the *35S:GLK2::GFP* construct. Anti-GFP antibody was used for immunoprecipitation. A mouse monoclonal anti-HA antibody (Santa Cruz Biotechnology) and IRDye800CW-conjugated goat anti-mouse secondary antibody (LI-COR, Bad Homburg, Germany) were used for immune blotting.

Dual-luciferase transactivation assay. *Arabidopsis* mesophyll cell protoplasts were prepared from rosette leaves of 4-week-old plants as described [19]. *35S:ORE1* and/or *35S:GLK2* were used as effector constructs. Reporter constructs of *ORE1* target genes were individually transformed in the absence of *35S:ORE1* effector, or together with *35S:ORE1* and/or *35S:GLK2*. Likewise, reporter constructs of *GLK2* target genes were individually transformed in the absence of *35S:GLK2* effector, or together with *35S:GLK2* and/or *35S:ORE1*. The *35S:RLuc* vector [20] was used for normalization against transformation efficiency. Firefly and *Renilla* luciferase (RLuc) activity were assayed using the Dual Luciferase Reporter Assay System (Promega) as described [21,22].

AGI codes. *ORE1* (At5g39610); *ORS1* (At3g29035); *RNS3* (At1g26820); *SINA1* (At3g13672); *BFN1* (At1g11190); *SAG29* (At5g13170); *VNI2* (At5g13180); *GLK1* (At2g20570); *GLK2* (At5g44190); *Hydrolase* (At1g80280); *LHCB2.2* (At2g05070); *LHCB4.2* (At3g08940); *LHCB2.4* (At3g27690); *MRU1* (At5g35490); *LHCB3* (At5g54270); *SAG13* (At2g29350); *SAG12* (At5g45890); *CAB2* (At1g29920); *CAB4* (At3g47470); *LHCB2.1* (At2g05100); *LHCB4.1* (At5g01530); *AGL68* (At5g65080); *ACTIN2* (At3g18780); and *GAPDH* (At3g26650).

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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