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The GLK1 'regulon' encodes disease defense related proteins and confers resistance to *Fusarium graminearum* in *Arabidopsis*

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Abstract

Overexpression (OE) was used to study the role of the Arabidopsis Golden2-like (GLK1) transcriptional activator in regulating gene expression. Affymetrix Gene Chip and RT-PCR analyses indicated that GLK1 OE in *Arabidopsis* reprogrammed gene expression networks to enhance a high constitutive expression of genes encoding disease defense related proteins. These include PR10, isochorismate synthase, antimicrobial peptides, glycosyl hydrolases, MATE efflux and other genes associated with pathogen response and detoxification. However, PR1, an indicator of systemic acquired resistance (SAR), was downregulated in GLK1 OE. GLK1 OE in *Arabidopsis* confers resistance to *Fusarium graminearum*, a broad host pathogen responsible for major losses in cereal crops. This is the first identification of the GLK1 'regulon' and a novel role for GLK1 in plant defense, suggesting its potential use for providing disease resistance in crop plants.

Keywords: GLK1; Golden2-like; Regulon; Disease resistance; *Fusarium graminearum*; Transcription factor; *Arabidopsis*; Transcriptional network

The maize Golden2-like or GLK genes encode proteins belonging to a class of GARP domain transcriptional activators [1–4]. GARP domain transcriptional activators are involved in plant specific processes such as GLK in chloroplast development [5,6], ARR-Bs in cytokinin signaling [7–9], PSR1 in phosphorus metabolism [10], KANADI in regulation of organ polarity [11] in b-ZIP G-box binding [12] and GLK1,2 in photosynthetic adaptation to cold stress [13]. While GLKs have been shown to transactivate in GAL4-BD reporter assays [3], the *in planta* targets of GLK regulated transcription have not been identified. In this paper, we use GLK1 OE to study reprogramming of gene expression networks in *Arabidopsis* and to identify an associated phenotype.

Materials and methods

Growth of plant material. *Arabidopsis thaliana* (ecotype Columbia) seeds were germinated in 5 cm soil pots. Seedlings were grown in Conviron E15 chambers at 22 °C, 16 h daylight at 150 μE m^{-2} and fertilized with NPK 20:20:20 once weekly.

RNA isolation. One gram quantities of rosette leaf (2.5–3 cm long) tissues were collected 4 h after the onset of light period and frozen in liquid N_2. Total RNA was isolated using Trizol™, P/N 15596-018 (Invitrogen, Burlington, ON, Canada) according to manufacturer's instructions.

Cloning of AtGLK1 (AT2G20570) and transformation. First strand synthesis reactions were carried out on 1 μg RNA with oligo dT (T_{20}VN) and Superscript III (Invitrogen 18080-044). GLK1 5′ XbaI-tatatttggatctagaagtgaag and GLK1 3′ KpnI-ttggttctcgggtacctacgg primers were used to amplify the resultant cDNA fragment. The PCR fragment was subcloned into pGEM-t-easy (Promega, Madison Wisconsin) and transformed into *Escherichia coli* DH5α. The sequenced Xba/KpnI fragment was excised and ligated into the pHS723 cassette containing the GUS-NPTII fusion and 70S-nos-terminator [14] to form pHS723-GLK1. pHS723-GLK1 was introduced into *Agrobacterium tumafaciens* (strain GV3109pmp90) to infect *Arabidopsis* by the floral dip method [15]. The plants were selected on 50 μg/ml kanamycin. Plantlets were transferred to soil and further selected with GUS to obtain single insertion homozygous plants.

Microarray analyses. Hybridizations and analyses of the Affymetrix Arabidopsis ATH1-121501 24K Gene Chip were carried as per manufacturer's protocols at the McGill University and Genome Quebec Innovation Centre (Montreal). Ten micrograms of total RNA per replicate were used. The data were MAS5.1 mean scale normalized.

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Validation of microarray data for selected genes was carried out by RT-PCR with the primers shown in Supplementary Material Table 1.

Assessment of susceptibility to Fusarium graminearum. *Arabidopsis* leaves were challenged with *F. graminearum* strain DOAM233423 as described by Makandar et al. [16] with the following modifications. Inocula containing 50,000 spores per ml were infiltrated into the abaxial surface of 2 cm leaves from 4-week-old *Arabidopsis* seedlings with a needle-less syringe. The pathogen-inoculated plants were kept humid in a clear plastic dome. Disease spread was recorded 2–6 days post inoculation (DPI) by Trypan-blue stain.

Results

GLK1 OE upregulates defense related genes

Two independent homozygous Arabidopsis GLK1 OE lines (2–6, 5–7) were identified. Seedlings (T5) of both GLK1 OE lines were approximately 5 days slower in growth than the wild type under conditions specified in Materials and methods. No other distinct visible phenotypes were observed (Fig. 1). Microarray analyses of GLK1 OE indicated that 194 and 146 genes were significantly up- and downregulated more than twofold, respectively (Supplementary Material Table 2). Defense related genes as well as genes of enzymatic function implicated in biotic stress responses were observed to be most highly upregulated. The upregulation of a subset of these genes was confirmed by RT-PCR (Fig. 2). PR1, an indicator of SAR activation [17], was downregulated as indicated in both microarray and RT-PCR analyses.

GLK1 OE results in resistance to F. graminearum, a non-host pathogen in Arabidopsis

Leaves of GLK1 OE lines were challenged with *F. graminearum*, a broad host pathogen that causes *Fusarium* Head Blight (FHB) in wheat and Giberella ear mold in corn. Fig. 3 shows comparisons between NahG, an SA impaired line that is more susceptible to infection than the wild type [18], the wild type (WT) and the GLK1 OE (5–7) lines. At 4 DPI, the presence of the highest density of hyphae and condiphores was observed on the NahG line

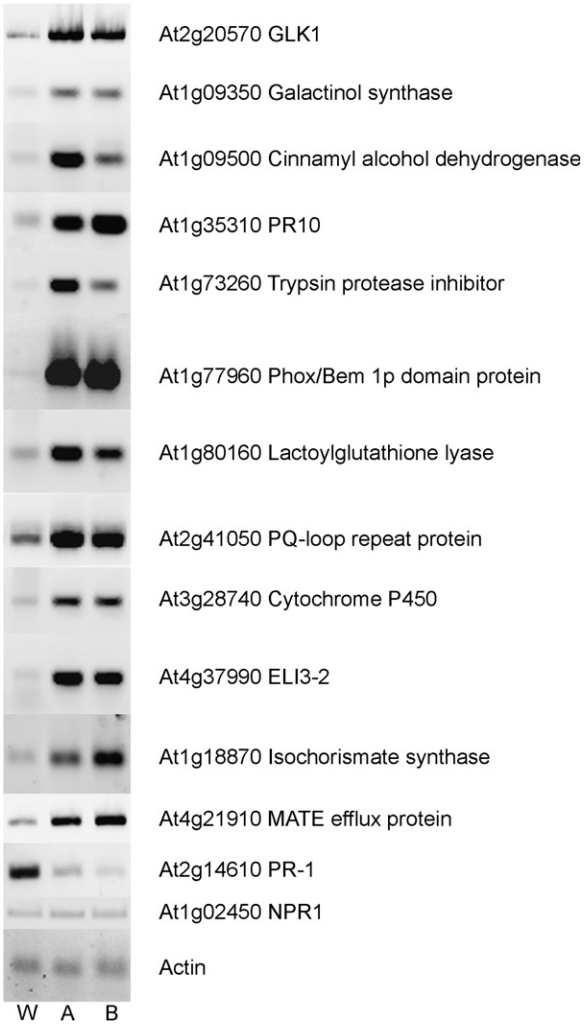

Fig. 2. RT-PCR confirmation of a subset of highly regulated genes identified by Affymetrix analyses. Lanes W: wild type; A: GLK1 OE line 2–6; B: GLK1 OE line 5–7.

Fig. 1. Comparison of growth rates between wild type and *Arabidopsis* overexpressing GLK1. Left: wild type; middle: GLK1 OE lines 5–7; right: GLK1 OE lines 2–6. Plants were grown for 24 days under conditions described in Materials and methods.

Fig. 3. Comparison of susceptibility to *Fusarium graminearum* infection between leaves of GLK1 OE lines and wild type. All panels are leaves recorded four days post inoculation. Top panel: NahG line; middle panel: wild type; bottom panel: GLK1 OE line 5–7.

followed by the WT. No mycelial growth was observed on leaves of the GLK1 OE line. Similar results were obtained with the GLK1 OE 2–7 lines (not shown). The NahG phenotype was included to ensure that differences in susceptibility were detected by the assay.

Discussion

The GLK1 regulon encodes defense related genes

We showed that the Arabidopsis GLK1 'regulon' encode genes associated with disease defenses and that GLK1 OE reprogramming resulted in the development of non-host resistance to *F. graminearum*. This is the first report of such a role for GLK1. In *Arabidopsis*, GLK occur as GLK1 and GLK2 gene pairs. GLK1 and GLK2 are differentially regulated and show overlapping yet distinct differential tissue expression patterns [2]. The study of the GLK2 regulon is in progress.

Genes encoding disease defense proteins in the GLK1 regulon include MATE efflux protein, a family of multidrug and toxin extrusion efflux transporters critical for disease resistance [19–21]. Its upregulation is consistent with resistance to *F. graminearum,* which uses the mycotoxin deoxnivalenol (DON) as a major virulence factor [22,23]. Another upregulated gene, PR10, has been observed to be upregulated in response to pathogens [24,25], and possesses antimicrobial [26] and steroid binding activities [27]. One of the most highly accumulating transcripts encodes a Phox/Bem 1 (PBI) domain protein. PBI domains are associated with protein–protein interactions and are involved in the activation of NADPH oxidase in responses to pathogen, ROS and suppression of cell death [28–30]. Another highly accumulating gene transcript is At2g41050, a PQ-loop cystinosin-like repeat protein. Cystinosin (CLT) is a lysosomal cystine transporter. While the role of LCTs is not well studied in plants, cystinosin mutations in human results in cystinosis and decreased levels of glutathione [31]. The importance of GSH in disease resistance in *Arabidopsis* is documented [32]. Other defense related genes in the GLK1 regulon include cinnamyl alcohol dehydrogenase (CAD) and ELI3-2, an aromatic alcohol dehydrogenase. Both of these genes are upregulated in response to pathogen and are implicated in defense mechanisms [33–37].

The GLK1 regulon may provide a state of defense readiness in Arabidopsis

GLK1 OE upregulates isochorismate synthase (At1g18870). Isochorismate synthase (ICS1), a key enzyme in salicylic acid (SA) biosynthesis in chloroplast, is induced in response to pathogen and induction of SAR in *Arabidopsis* [38]. Although ICS1 operates upstream of SA, we observed that there is no concurrent transcript accumulation of PR1, a positive marker for the induction of SAR. This suggests that GLK1 may operate independently of SA. Furthermore, transcriptome analyses have not indicated positive responses of GLK1 to pathogenesis [39]. Thus, *F. graminearum* resistance in GLK1 OE may result from priming of the plant for pathogen invasion by transactivation of defense related genes. Priming is a phenomenon that preconditions the plant to stress [40].

The mechanism by which GLK1 transactivates target genes is not known. ARR1 and ARR10-B GARP domain proteins have been observed to bind to an AGATT sequence [7,8]. The 1 kb regulatory regions of the genes in Fig. 2 all contain at least one AGATT or AGAT sequence although it is not known if GLK1 will bind to the same sequence. Identification of GLK1 target genes will facilitate gaining insights into GLK1 transactivation and its role in disease resistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.05.084.

References

[1] L.N. Hall, L. Rossini, L. Cribb, J.A. Langdale, GOLDEN 2: a novel transcriptional regulator of cellular differentiation in the maize leaf, Plant Cell 10 (1998) 925–936.

[2] W. Fitter, D.J. Martin, M.J. Copley, R.W. Scotland, J.A. Langdale, GLK gene pairs regulate chloroplast development in diverse plant species, Plant J. 31 (2002) 713–727.

[3] D.L. Rossini, L. Cribb, D.J. Martin, J.A. Langdale, The maize Golden2 gene defines a novel class of transcriptional regulators in plants, Plant Cell 13 (2001) 1231–1244.

[4] J.L. Riechmann, J. Heard, G. Martin, L. Reuber, C.-Z. Jiang, J. Keddie, L. Adam, O. Pineda, O.J. Ratcliffe, R.R. Samaha, R. Creelman, M. Pilgrim, P. Broun, J.Z. Zhang, D. Ghandehari, B.K. Sherman, G.-L. Yu, *Arabidopsis* transcription factors: genome-wide comparative analysis among eukaryotes, Science 290 (2000) 2105–2110.

[5] L. Cribb, L.N. Hall, J.A. Langdale, Four mutant alleles elucidate the role of the G2 protein in the development of C(4) and C(3) photosynthesizing maize tissues, Genetics 159 (2001) 787–797.

[6] Y. Yasumura, E.C. Moylan, J.A. Langdale, A conserved transcription factor mediates nuclear control of organelle biogenesis in anciently diverged land plants, Plant Cell 17 (2005) 1894–1907.

[7] H. Sakai, T. Honma, T. Aoyama, S. Sato, T. Kato, S. Tabata, A. Oka, ARR1, a transcription factor for genes immediately responsive to cytokinins, Science 294 (2001) 1519–1521.

[8] K. Hosoda, A. Imamura, E. Katoh, T. Hatta, M. Tachiki, H. Yamada, T. Mizuno, T. Yamazaki, Molecular structure of the GARP family of plant Myb-related DNA binding motifs of the *Arabidopsis* response regulators, Plant Cell 14 (2002) 2015–2029.

[9] M.G. Mason, D.E. Mathews, D.A. Argyros, B.B. Maxwell, J.J. Kieber, J.M. Alonso, J.R. Ecker, G.E. Schallera, Multiple type-B response regulators mediate cytokinin signal transduction in *Arabidopsis*, Plant Cell 17 (2005) 3007–3018.

[10] D.D. Wykoff, A.R. Grossman, D.P. Weeks, H. Usuda, K. Shimogawara, Psr1, a nuclear localized protein that regulates phosphorus metabolism in Chlamydomonas, Proc. Natl. Acad. Sci. USA 96 (1999) 15336–15341.

[11] R.A. Kerstetter, K. Bollman, R.A. Taylor, K. Bomblies, R.S. Poethig, KANADI regulates organ polarity in *Arabidopsis*, Nature 411 (2001) 706–709.

[12] H. Tamai, M. Iwabuchi, T. Meshi, *Arabidopsis* GARP transcriptional activators interact with the pro-rich activation domain shared by G-Box-binding bZIP factors, Plant Cell Physiol. 43 (2002) 99–107.

[13] L.V. Savitch, G. Allard, M. Seki, L.S. Robert, N.A. Tinker, N.P.A. Huner, K. Shinozaki, J. Singh, The effect of overexpression of two Brassica CBF/DREB1-like transcription factors on photosynthetic capacity and freezing tolerance in *Brassica napus*, Plant Cell Physiol. 46 (2005) 1525–1539.

[14] R.B. Nair, R.W. Joy, E. Kurylo, X. Shi, J. Schnaider, R.S. Datla, W.A. Keller, G. Selvaraj, Protein identification of a CYP84 family of cytochrome P450-dependent mono-oxygenase genes in *Brassica napus* and perturbation of their expression for engineering sinapine reduction in the seeds, Plant Physiol. 123 (2000) 1623–1634.

[15] S.J. Clough, A.F. Bent, Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*, Plant J. 16 (1998) 735–743.

[16] R. Makandar, J.S. Essig, M.A. Schapaugh, H.N. Trick, J. Shah, Genetically engineered resistance to *Fusarium* head blight in wheat by expression of *Arabidopsis* NPR1, Mol. Plant Microbe Interact. 19 (2006) 123–129.

[17] S. Uknes, B. Mauch-Mani, M. Moyer, S. Williams, S. Dincher, D. Chandler, S. Potter, A. Slusarenko, E. Ward, J. Ryals, Acquired resistance in *Arabidopsis*, Plant Cell 4 (1992) 645–656.

[18] T. Gaffney, L. Freidrich, B. Vernooij, D. Negrotti, G. Nye, S. Uknes, E. Ward, H. Kessmann, J. Ryals, Requirement of salicylic acid for the induction of systemic acquired resistance, Science 261 (1993) 754–756.

[19] M.H. Brown, I.T. Paulsen, R.A. Skurray, The multidrug efflux protein NorM is a prototype of a new family of transporters, Mol. Microbiol. 31 (1998) 393–395.

[20] A.C. Diener, R.A. Gaxiola, G.R. Fink, ALF5, a multidrug efflux transporter gene family member, confers resistance to toxins in *Arabidopsis*, Plant Cell 13 (2001) 1625–1637.

[21] C. Nawrath, S. Heck, N. Parinthawong, J.-P. Métraux, EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in *Arabidopsis*, is a member of the MATE transporter family, Plant Cell 14 (2002) 275–286.

[22] R.H. Proctor, T.M. Hohn, S.P. McCormick, Reduced virulence of Gibberella zeae caused by disruption of a trichothecene toxin biosynthetic gene, Mol. Plant Microbe Interact. 8 (1995) 593–601.

[23] L.J. Harris, S.C. Gleddie, A modified Rpl3 gene from rice confers tolerance of the *Fusarium graminearum* mycotoxin deoxynivalenol to transgenic tobacco, Physiol. Mol. Plant Pathol. 58 (2001) 173–181.

[24] G.P. Moiseyev, J.J. Beintema, L.I. Federeyeva, G.I. Yakolev, High sequence similarity between a ribonuclease from gingseng calluses and fungus-elicited proteins from parsley indicates that intracellular pathogenesis-related proteins are ribonucleases, Planta 193 (1994) 470–472.

[25] C. Despres, R. Subramaniam, D. Matton, N. Brisson, The activation of the potato PR-10a gene requires the phosphorylation of the nuclear factor PBF-1, Plant Cell 7 (1995) 589–598.

[26] C.-J. Park, K.-J. Kim, R. Shin, J.M. Park, Y.-C. Shin, K.-H. Paek, Pathogenesis-related protein 10 from hot pepper functions as a ribonuclease in an antiviral pathway, Plant J. 37 (2004) 186–198.

[27] Z. Markovic-Housley, M. Degano, D. Lamba, E. von Roepenack-Lahaye, S. Clemens, M. Susani, F. Ferreira, O. Scheiner, H. Breitender, Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier, J. Mol. Biol. 325 (2003) 123–133.

[28] F. Simon-Plas, T. Elmayan, J.-P. Blein, The plasma membrane oxidase NtrbohD is responsible for AOS production in elicited tobacco cells, Plant J. 31 (2002) 137–147.

[29] M.A. Torres, J.D.G. Jones, J.L. Dangl, Pathogen induced, NADPH oxidase derived reactive oxygen intermediates suppress spread of cell death in *Arabidopsis*, Nat. Genet. 37 (2005) 1130–1134.

[30] Y. Narusaka, M. Narusaka, P. Park, Y. Kubo, T. Hirayama, M. Seki, T. Shiraishi, J. Ishida, M. Nakashima, A. Enju, T. Sakurai, M. Satou, M. Kobayashi, K. Shinozaki, RCH1, a locus in *Arabidopsis* that confers resistance to the hemibiotrophic fungal pathogen *Colletotrichum higginsianum*, Mol. Plant Microbe Interact. 17 (2004) 749–762.

[31] E. Levtchenko, A. de Graaf-Hess, M. Wilner, L. van den Heuvel, Altered status of glutathione and its metabolites in cystinotic cells, Nephrol. Dial. Transplant. 20 (2005) 1828–1832.

[32] V. Parisy, B. Poinssot, L. Owsianowski, A. Buchala, J. Glazebrook, F. Mauch, Identification of *PAD2* as a γ-glutamylcysteine synthetase

highlights the importance of glutathione in disease resistance in *Arabidopsis*, Plant J. 49 (2007) 159–172.
[33] M.H. Walter, J. Grima-Pettenatit, C. Grand, A.M. Boudett, C.J. Lamb, Cinnamyl-alcohol dehydrogenase, a molecular marker specific for lignin synthesis: cDNA cloning and mRNA induction by fungal elicitor, Proc. Natl. Acad. Sci. USA 85 (1988) 5546–5550.
[34] H.J. Mitchell, J. Hall, M.S. Barber, Elicitor-induced cinnamyl alcohol dehydrogenase activity in lignifying wheat (Triticum aestivum 1) leaves, Plant Physiol. 104 (1994) 551–556.
[35] E. Schmelzer, S. Kruger-Lebus, K. Hahlbrock, Temporal and spatial patterns of gene expression around sites of attempted fungal infection in parsley leaves, Plant Cell 1 (1989) 993–1001.
[36] I.E. Somssich, P. Wernert, S. Kiedrowski, K. Hahlbrock, *Arabidopsis thaliana* defense-related protein ELI3 is an aromatic alcohol:NADP+ dehydrogenase, Proc. Natl. Acad. Sci. 93 (1996) 14199–14203.
[37] S. Kiedrowski, P. Kawalleck, K. Hahlbrock, I.E. Somssich, J.L. Dangl, Rapid activation of a novel plant defense gene is strictly dependent on the Arabidopsis RPM1 disease resistance locus, EMBO J. 11 (1992) 4677–4684.
[38] M.C. Wildermuth, J.D.G. Wu, F.M. Ausubel, Isochorismate synthase is required to synthesize salicylic acid for plant defence, Nature 414 (2001) 562–571.
[39] L. Bülow, M. Schindler, R. Hehl, PathoPlant®: a platform for microarray expression data to analyze co-regulated genes involved in plant defense responses, Nucleic Acids Res. 35 (2007) D841–D845.
[40] Prime-A-Plant Group: U. Conrath, G.J.M. Beckers, V. Flors, P. García-Agustín, G. Jakab, F. Mauch, M.-A. Newman, C.M.J. Pieterse, B. Poinssot, M.J. Pozo, A. Pugin, U. Schaffrath, J. Ton, D. Wendehenne, L. Zimmerli, B. Mauch-Mani, Priming: getting ready for battle, Mol. Plant Microbe Interact. 19 (2006).