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Specific control of *Arabidopsis* BAK1/SERK4-regulated cell death by protein glycosylation

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Abstract

Precise control of cell death is essential for the survival of all organisms. *Arabidopsis thaliana* BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 (BAK1) and somatic embryogenesis receptor kinase 4 (SERK4) redundantly and negatively regulate cell death through elusive mechanisms. By deploying a genetic screen for suppressors of cell death triggered by virus-induced gene silencing of *BAK1/SERK4* on *Arabidopsis* knockout collections, we identified STT3a, a protein involved in *N*-glycosylation modification, as an important regulator of *bak1/serk4* cell death. Systematic investigation of glycosylation pathway and endoplasmic reticulum (ER) quality control (ERQC) components revealed distinct and overlapping mechanisms of cell death regulated by BAK1/SERK4 and their interacting protein BIR1. Genomewide transcriptional

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Author contributions

M.V.V.O., G.X., B.L., L.S. and P.H. conceived and designed the experiments and wrote the manuscript with input from all co-authors. M.V.V.O., G.X., B.L., L.S.V., X.M., X.C., X.Y., S.A.S., A.C.I., A.M.M. and A.L.B performed the experiments; M.V.V.O., G.X., B.L., L.S.V., G.A.S.F., L.S. and P.H. analysed data; H.K. provided reagents.

Additional information

Supplementary information is available online.

Competing interests

The authors declare no competing financial interests.

analysis revealed the activation of members of cysteine-rich receptor-like kinase (*CRK*) genes in the *bak1/serk4* mutant. Ectopic expression of *CRK4* induced STT3a/*N*-glycosylation-dependent cell death in *Arabidopsis* and *Nicotiana benthamiana*. Therefore, *N*-glycosylation and specific ERQC components are essential to activate *bak1/serk4* cell death, and *CRK4* is likely to be among client proteins of protein glycosylation involved in BAK1/SERK4-regulated cell death.

Plant receptor-like kinases (RLKs) regulate diverse biological processes ranging from plant growth, development, symbiosis to immunity^{1,2}. BRI1 is a receptor for plant brassinosteroid hormones involved in plant growth and development³ and FLS2 is a receptor for bacterial flagellin or flg22 (a 22-amino-acid peptide derived from flagellin) involved in plant immunity⁴. Both BRI1 and FLS2 interact with a subgroup of RLKs, the SERKs, which consist of five members in *Arabidopsis*. Except for SERK5, which is likely to be a non-functional kinase⁵, SERK1, SERK2, SERK3 and SERK4 are involved in a wide range of physiological responses⁶. SERK1 and SERK2 play a crucial and redundant role in male gametophyte development^{7,8}. SERK3, also known as BAK1, and SERK4 function in plant immunity by association with FLS2 and other immune receptors^{9–12}. SERK1, BAK1 and SERK4 function in brassinosteroid signalling by association with BRI1^{5,13,14}. SERK1, SERK2, BAK1 and SERK4 regulate stomatal patterning by means of EPF peptide ligand-induced association with ERECTA family RLKs¹⁵. In addition, SERK1, SERK2 and BAK1 regulate PSK peptide hormone-mediated root growth by association with its receptor PSKR^{16,17}.

In addition, BAK1 and SERK4 negatively regulate the plant cell death process^{18,19}. The *bak1-4/serk4-1* null mutant is post-embryonic seedling lethal associated with spontaneous cell death and constitutive H₂O₂ production¹⁸. In contrast to the well-defined signalling framework of BAK1/SERK-mediated plant growth and immunity, the mechanisms underlying BAK1/SERK4-regulated cell death control are poorly understood. Notably, BIR1, a BAK1-interacting RLK, also negatively regulates cell death and the *bir1* mutant exhibits post-embryonic seedling lethality, which depends on another RLK SUPPRESSOR OF BIR1,1 (SOBIR1)²⁰. It remains unknown whether the same or distinct mechanisms operate *bir1* and *bak1/serk4* cell death. As no viable seeds are produced by the *bak1-4/serk4-1* null mutant plants, the conventional forward genetic screen of *bak1/serk4* cell death suppressors is not feasible. Here, we have developed an *Agrobacterium-mediated* tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) system²¹ with the TRV-RNA2 vector harbouring fragments of both *BAK1* and *SERK4*. Mutants that suppress *bak1/serk4* cell death were identified using VIGS screening of *Arabidopsis* transfer (T)-DNA insertion lines. We report that the protein glycosylation pathway and specific ERQC components are essential for *bak1/serk4* cell death. Transcriptomic analysis revealed that the plasma membrane-associated genes, including members of cysteine-rich receptor-like kinase (*CRK*) genes, were highly enriched among upregulated genes in *bak1-4/serk4-1*. Further biochemical and genetic investigations have suggested that *CRK4* is one of the client proteins of protein glycosylation involved in the BAK1/SERK4-regulated cell death process.

Results

Arabidopsis wild-type (WT) Col-0 plants silenced with *BAK1/SERK4* showed severe growth defects with chlorotic leaves and dwarfism two weeks after *Agrobacterium*-inoculation, which resembled *bak1-4* plants silenced with *SERK4* (Fig. 1a). Analysis using polymerase chain reaction with reverse transcription (RT-PCR) revealed the reduced transcripts of *BAK1* and *SERK4*, but not *SERK1* or *SERK2* in *BAK1/SERK4*-silenced plants (Fig. 1b). Trypan blue and 3,3'-diaminobenzidine (DAB) staining indicated that WT plants following silencing of *BAK1/SERK4* or the *bak1-4* mutant silenced with *SERK4* displayed spontaneous cell death and the elevated H₂O₂ accumulation (Fig. 1c). In addition, these plants showed increased levels of *PR1* and *PR2* gene expression (Fig. 1d). The *bak1-4/serk4-1* cell death is partially dependent on the plant defence hormone salicylic acid¹⁸. Consistently, the transgenic plants carrying the bacterial salicylate hydroxylase gene *NahG* and the *sid2* mutant that is deficient in salicylic acid biosynthesis partially suppressed the cell death and H₂O₂ production triggered by VIGS of *BAK1/SERK4* (Fig. 1e and Supplementary 1a). Taken together, these results demonstrate that silencing of *BAK1/SERK4* by means of VIGS phenocopies cell death observed in the *bak1-4/serk4-1* null mutant.

We tested whether *bak1/serk4* cell death depends on SOBIR1, which is required for *bir1* cell death. Similar to the *bir1* null mutant, cell death could be observed in WT plants silenced with *BIR1* by means of VIGS (Fig. 1f). The dwarfism and leaf chlorosis associated with silencing of *BIR1* were almost completely suppressed in *sobir1-12* (Fig. 1f). Interestingly, the *sobir1-12* mutant did not affect cell death triggered by VIGS of *BAK1/SERK4* (Fig. 1f). We further generated the *bak1-4/serk4-1/sobir1-12* triple mutant, which showed the same level of seedling lethality, elevated cell death and H₂O₂ accumulation with *bak1-4/serk4-1* (Fig. 1g). Activation of resistance (R) protein-mediated defence is a common mechanism of plant cell death. However, the *pad4* and *ndr1* mutants, which had impaired R protein pathways, did not significantly suppress the cell death caused by VIGS of *BAK1/SERK4* (Fig. 1h), suggesting that activation of R protein-mediated defences may not play a major role in *bak1/serk4* cell death. In contrast, the *pad4* mutant largely alleviated cell death caused by silencing of *BIR1* (Supplementary Fig. 1b). Consistently, *BAK1/SERK4*-regulated cell death does not require R proteins RPS2 and RPM1 (Supplementary Fig. 1c). Together, the data indicate distinct mechanisms underlying *BAK1/SERK4*- and *BIR1*-regulated cell death.

To identify components involved in *BAK1/SERK4*-regulated cell death, we carried out a VIGS-based genetic screen of a sequence-indexed library of *Arabidopsis* T-DNA insertion lines. After screening ~6,000 homozygous lines, a series of mutants were isolated based on the suppression of cell death by silencing of *BAK1/SERK4*. One mutant (line CS800052 with an annotated T-DNA insertion at *STAUROSPORIN AND TEMPERATURE SENSITIVE3 (STT3a)*, *stt3a-2* (Supplementary Fig. 2a)) largely suppressed dwarfism and leaf chlorosis triggered by VIGS of *BAK1/SERK4* (Fig. 2a). MEKK1, a MAP kinase (MAPK) kinase kinase downstream of *BAK1/SERK4* in flagellin signalling, is also involved in cell death regulation²². Silencing of *MEKK1* by VIGS in WT plants resulted in severe dwarfism and cell death (Fig. 2a). However, the *stt3a-2* mutant did not suppress MEKK1-

regulated cell death (Fig. 2a), suggesting different mechanisms underlying BAK1/SERK4- and MEKK1-regulated cell death. The data also suggest that *stt3a-2* did not affect the gene silencing machinery. RT-PCR analysis demonstrated a similar silencing efficiency of *BAK1/SERK4* by VIGS in WT and *stt3a-2* mutant (Supplementary Fig. 2b). Furthermore, the cell death and elevated H₂O₂ accumulation caused by VIGS of *BAK1/SERK4* were almost completely abolished in *stt3a-2* (Fig. 2b). Compared with WT, *stt3a-2* showed much reduced accumulation of *PR1* and *PR2* genes by VIGS of *BAK1/SERK4* (Fig. 2c). Another T-DNA insertion mutant *stt3a-1*, which is in the ecotype C24 background, also suppressed cell death by silencing of *BAK1/SERK4* (Fig. 2d). Furthermore, transformation of a genomic fragment containing the *STT3a* gene into *stt3a-2* restored BAK1/SERK4-regulated cell death (Fig. 2e and Supplementary Fig. 2c). To investigate if *stt3a-2* could genetically suppress *bak1-4/serk4-1* seedling lethality and defence activation, we generated the *bak1-4/serk4-1/stt3a-2* triple mutant. The *bak1-4/serk4-1/stt3a-2* triple mutant overcame seedling lethality of *bak1-4/serk4-1* and resembled WT plants at the two-week-old stage when grown on ½ Murashige and Skoog medium (½MS) medium plates (Fig. 2f and Supplementary Fig. 2d). In addition, cell death, H₂O₂ accumulation, and *PR1* and *PR2* expression were significantly ameliorated in *bak1-4/serk4-1/stt3a-2* compared with those in *bak1-4/serk4-1* (Fig. 2g,h). The *stt3a-2* mutant also partially suppressed VIGS of *BIR1*-induced cell death (Supplementary Fig. 2e,f). The elevated expression of *PR1* and *PR2* was also significantly reduced in *stt3a-2* silenced with *BIR1* (Supplementary Fig. 2g). This is consistent with *STT3a* being required for the activation of defence responses in the *bir1* genetic mutant²³.

Plant cell death and defence activation are often modulated by temperature and other environmental factors²⁴. When grown on ½MS medium plates, seedling lethality of *bak1-4/serk4-1* was largely ameliorated at 30 °C when compared with that at 23 °C (Supplementary Fig. 3a,b). The elevated expression of *PR1* and *PR2* in *bak1-4/serk4-1* was also reduced when grown at 30 °C (Supplementary Fig. 3c). The alleviation of seedling lethality and growth defects of *bak1-4/serk4-1* by the elevated temperature was a relatively subtle effect for plants grown on soil (Supplementary Fig. 3d). However, the *bak1-4/serk4-1/stt3a-2* triple mutant grew significantly better on soil at 30 °C than that at 23 °C (Supplementary Fig. 3d). The *bak1-4/serk4-1/stt3a-2* mutant could ultimately develop to maturity and occasionally produce some viable seeds when grown on soil at 30 °C.

STT3 is the catalytic subunit of the oligosaccharyltransferase (OST) complex that is involved in protein *N*-glycosylation modifications (Fig. 3a)²⁵. There are two family members of STT3, STT3a and STT3b, in *Arabidopsis*. The mutations in *STT3b* (*stt3b-2* and *stt3b-3*) did not affect cell death by silencing of *BAK1/SERK4*, suggesting a specific function of *STT3a* in this process (Fig. 3b). This is consistent with the observation that STT3a, but not STT3b, is involved in *Arabidopsis* salt and osmotic stresses²⁵. In addition to the catalytic STT3 subunit, the OST complex also consists of several non-catalytic subunits that regulate substrate specificity, stability or complex formation²⁶. The highly conserved subunit OST3/6 regulates overall protein glycosylation and is involved in plant immunity by controlling biogenesis of the EF-Tu receptor EFR (ref. 26). The *ost3/6* mutant also suppressed BAK1/SERK4-regulated cell death (Fig. 3b).

The OST complex transfers the lipid (dolichol)-linked *N*-glycan precursor Glc₃Man₉GlcNAc₂ (Glc, glucose; Man, mannose; GlcNAc, *N*-acetylglucosamine) to the acceptor proteins in the ER (Fig. 3a)^{27,28}. The *N*-glycan precursor is preassembled by a series of glucosyltransferases encoded by asparagine-linked glycosylation (ALG) proteins (Fig. 3a). Loss-of-function of some ALG proteins prevented assembly of the *N*-glycan precursor and reduced protein *N*-glycosylation efficiency in *Arabidopsis*^{29,30}. The *alg10*, but not *alg3* or *alg12* mutant, suppressed *BAK1/SERK4* silencing-mediated cell death (Fig. 3c), suggesting that proper assembly of the *N*-glycan precursor is essential in *bak1/serk4* cell death. ALG10 catalyses the last step of *N*-glycan precursor assembly by transferring the terminal glucose residue to the precursor, an essential step for OST complex recognition. The *Arabidopsis alg10* mutant had a severe defect in protein *N*-glycosylation and increased sensitivity to salt stress²⁹.

After the *N*-glycan precursor was transferred to the acceptor proteins by the OST complex, the two terminal glucose residues of the oligosaccharides were subsequently removed by glucosidase I and glucosidase II (RSW3 in *Arabidopsis*), and then recognized by the ER chaperone-like lectins calnexin (CNX) and calreticulin (CRT3) for proper protein folding and secretion (Fig. 3a)^{27,28}. The incompletely folded or misfolded proteins will be recognized by UDP-glucose:glycoprotein glucosyltransferase (UGGT) for additional CNX/CRT cycles and another round of folding (Fig. 3a)^{27,28,31}. ER-localized HSP70 proteins, BiPs and their associated factors ERdj3 and SDF2 also play important roles to prevent export of misfolded proteins (Fig. 3a)³¹. Some specific ERQC components, such as CRT3, UGGT, ERdj3b and SDF2, were genetically implicated in the protein folding and degradation of mutated bri1 receptor and immune receptor EFR and in BIR1-mediated cell death^{23,32–40}. We observed that *erdj3b-1* and *sdf2-2*, but not *crt3-1* or *rsw3-1*, partially suppressed *BAK1/SERK4* silencing-mediated cell death (Fig. 3d). RT-PCR analysis indicated a similar silencing efficiency of *BAK1/SERK4* by VIGS in WT and different mutants (Supplementary Fig. 4a). As reported for genetic mutants³², *crt3-1*, *erdj3b-1* and *sdf2-2* suppressed *BIR1* silencing-mediated cell death (Fig. 3d). We further generated genetic mutants of *bak1-4/serk4-1/crt3-1*, *bak1-4/serk4-1/erdj3b-1* and *bak1-4/serk4-1/sdf2-2*. Similar to VIGS assays, *bak1-4/serk4-1/erdj3b-1* and *bak1-4/serk4-1/sdf2-2*, but not *bak1-4/serk4-1/crt3-1*, alleviated *bak1-4/serk4-1* seedling lethality and H₂O₂ production (Supplementary Fig. 4b). The data further support that distinct mechanisms, but with certain overlapping features, control *BAK1/SERK4*- and *BIR1*-regulated cell death.

Proteins with native conformation after ERQC will be exported to the Golgi apparatus for further modifications, such as formation of complex and hybrid *N*-glycans catalysed by stepwise enzymatic reactions, including β1,2-*N*-acetylglucosaminyltransferase I (CGL1/GNTI), α-mannosidase II (HGL1/MANII), GNTII, β1,2-xylosyltransferase (XYLT), α1,3-fucosyltransferase (FUCTa and FUCTb), β1,3-galactosyltransferase (GALT) and α1,4-fucosyltransferase (FUCTc) (Fig. 3a)⁴¹. To investigate if *N*-glycan maturation in the Golgi apparatus also plays a role in *bak1/serk4* cell death, we silenced *BAK1/SERK4* in the *cgl1-3* and *hgl1-1* single mutants and *fucTa/fucTb/xyIT* triple mutant (Fig. 3e). All these mutants showed similar levels of cell death and growth retardation as WT plants after silencing of *BAK1/SERK4* by VIGS (Fig. 3e). Apparently, these mutants also did not affect the *BIR1* silencing-mediated cell death (Fig. 3e). These data suggest that protein

glycosylation modification for proper folding in ER, but not *N*-glycan modification in the Golgi apparatus, is essential for the initiation of *bak1/serk4* and *bir1* cell death. *N*-glycan modification in the Golgi apparatus is also not required for EFR maturation in plant immunity³⁷.

Preventing protein *N*-glycosylation often leads to protein misfolding, a major contributor to ER stress, thereby resulting in unfolded protein response (UPR)³¹. Defects in protein glycosylation and ERQC in the *stt3a*, *erdj3b* and *sdf2* mutants is likely to induce ER stress and UPR. The *Arabidopsis* UPR signalling pathway is composed of two arms: one involving the bifunctional protein kinase/ RNA ribonuclease IRE1 and its target RNA bZIP60, and another involving ER membrane-associated transcription factors, such as bZIP28 (ref. 31). To test if UPR may contribute to *bak1/serk4* cell death, we silenced *BAK1/SERK4* in the *ire1a/ire1b* and *bzip28/bzip60* double mutants, both of which are deficient in UPR induction⁴². Neither *ire1a/ire1b* nor *bzip28/bzip60* affected cell death by VIGS of *BAK1/SERK4* (Supplementary Fig. 4c), suggesting that UPR may not directly link to BAK1/SERK4-regulated cell death.

In contrast to the negative regulation in cell death, BAK1 and SERK4 positively regulate plant immunity and brassinosteroid signalling^{5,9-14}. We tested whether the mutation in *stt3a* also suppressed *bak1* or *bak1/serk4* deficiency in flagellin and brassinosteroid signalling. Flg22 triggers rapid phosphorylation of MAPKs and receptor-like cytoplasmic kinase BIK1 (ref. 43). The *bak1-4* and *bak1-4/serk4-1* mutants displayed compromised flg22-induced MAPK activation (Fig. 4a,b) and BIK1 phosphorylation (Fig. 4c). The *stt3a* mutant did not affect flg22 signalling as previously reported (Fig. 4a)³⁷. The compromised flg22-induced MAPK activation and BIK1 phosphorylation remained the same in *bak1-4/stt3a-2* and *bak1-4/serk4-1/stt3a-2* as those in *bak1-4* or *bak1-4/serk4-1* (Fig. 4a-c). Similar to *bak1-4/serk4-1*, *bak1-4/serk4-1/stt3a-2* showed compromised expression of flg22-induced genes, *WRKY30*, *MYB15* and *LOX4*, compared with WT or *stt3a-2* (Fig. 4d). Taken together, these results suggest that *stt3a-2* did not suppress *bak1-4* or *bak1-4/serk4-1* deficiency in flagellin-mediated immune signalling. When grown in the dark, the hypocotyls of the *bak1-4/serk4-1* mutant elongated slightly, but were significantly shorter than those of WT plants (Fig. 4e,f). In the presence of brassinazole (BRZ), an inhibitor of brassinosteroid biosynthesis, *bak1-4/serk4-1* displayed shorter hypocotyl elongation than WT. The *stt3a-2* mutant exhibited hypocotyl elongation similar to WT plants. The hypocotyl elongation of *bak1-4/serk4-1/stt3a-2* was similar to that of *bak1-4/serk4-1* in the absence or presence of BRZ (Fig. 4e,f), suggesting that *stt3a-2* did not interfere with the responsiveness of *bak1-4/serk4-1* to BRZ.

As *PR* genes are highly upregulated in the *bak1/serk4* cell death process, we determined the genome-wide transcriptional changes in *bak1-4/serk4-1* by RNA-sequencing (RNA-seq) analysis. Among 24,572 detectable transcripts (Fig. 5a and Supplementary Table 1), we identified 3,637 differentially expressed genes (fold change ≥ 2 and false discovery rate (FDR) < 0.1), including 1,920 induced genes (Supplementary Table 2) and 1,717 reduced genes (Supplementary Table 3) in *bak1-4/serk4-1* compared with WT plants (Fig. 5b). Interestingly, gene ontology enrichment analysis indicated that genes encoding proteins associated with membrane, especially plasma membrane, were highly enriched among the

upregulated genes in *bak1-4/serk4-1* (Fig. 5c). Remarkably, among 44 *CRK* genes, the expression of 22 *CRKs* was upregulated in *bak1-4/serk4-1* (Fig. 5d and Supplementary Table 4). The induction of *CRK4*, *CRK5*, *CRK7*, *CRK8*, *CRK19* and *CRK20* in *bak1-4/serk4-1* was confirmed by quantitative RT-PCR (qRT-PCR) (Fig. 5e).

It has been reported that overexpression of certain *CRKs*, such as *CRK4*, *CRK5*, *CRK13*, *CRK19* and *CRK20*, was able to induce cell death in *Arabidopsis* transgenic plants^{44–46}. When transiently expressed in *Nicotiana benthamiana*, *CRK4* and *CRK5* elicited water-soaking and subsequent cell death (Fig. 6a and Supplementary Fig. 5a). We tested whether *CRK* proteins were glycosylated and whether STT3a-mediated protein glycosylation was required for *CRK*-mediated cell death. Glycosylated proteins often display slower migration than non-glycosylated proteins in an immunoblot. When treated with tunicamycin, an inhibitor of protein *N*-glycosylation, both *CRK4* and *CRK5* proteins exhibited faster migration than proteins without treatment, suggesting that *CRK4* and *CRK5* proteins are likely to be *N*-glycosylated in *Arabidopsis* cells (Fig. 6b). Consistently, Endoglycosidase H treatment released *CRK4* proteins with reduced molecular weight (Supplementary Fig. 5b). *CRK4* proteins when expressed in *stt3a-2* migrated faster than that in WT (Fig. 6c), indicating that STT3a is required for *CRK4* glycosylation. Notably, we have consistently observed that the accumulation of *CRK4* proteins after tunicamycin treatment or in *stt3a* appeared to be reduced when compared with proteins without treatment or in WT (Fig. 6b,c), suggesting that *N*-glycosylation may regulate *CRK4* protein stability.

We further introduced *CRK4* under the control of an oestrogen-inducible promoter into WT and *stt3a-2*. The cell death caused by ectopic expression of *CRK4* in WT plants was largely alleviated in *stt3a-2* when oestradiol was applied (Fig. 6d). Similar to the above transient assays, the *CRK4* protein level was lower in *stt3a-2* than that in WT in multiple transgenic lines (Fig. 6e). We aligned the extracellular domain of *CRK4* with its closest homologue *CRK5*. Out of five potential *N*-glycosylation sites (Asn in Asn-X-Ser/Thr, where X is any amino acid except Pro), we identified two sites that are conserved between *CRK4* and *CRK5* (Supplementary Fig. 5c). We mutated them in *CRK4* to Gln (*CRK4*^{N181Q} and *CRK4*^{N286Q}) and found that *CRK4*^{N181Q} showed faster migration than WT *CRK4* when expressed in *Arabidopsis* protoplasts (Fig. 6f) or *N. benthamiana* (Supplementary Fig. 5d), resembling the *CRK4* expression in *stt3a* (Fig. 6c). The protein level of *CRK4*^{N181Q} was similar to that of WT *CRK4*, suggesting that mutation of one glycosylation site may not affect *CRK4* protein stability. Significantly, the *CRK4*^{N181Q} mutant reduced cell death intensity and H₂O₂ accumulation compared with WT *CRK4* when transiently expressed in *N. benthamiana* (Fig. 6g). Since *CRK4*^{N181} is a conserved site between *CRK4* and *CRK5*, it is likely to be that the corresponding site in *CRK5* is required for its cell death-inducing ability. The results suggest that *N*-glycosylation is essential for *CRK4*-mediated cell death, and *CRK4* is one of the substrates of STT3a in *bak1/serk4* cell death. To test whether loss of *CRK4* suppresses *bak1/serk4* cell death, we silenced *BAK1/SERK4* in the *crk4* mutant. However, the *crk4* mutant did not affect cell death by VIGS of *BAK1/SERK4* (Supplementary Fig. 5e,f), indicating that mutation of *CRK4* is not sufficient to suppress *bak1/serk4* cell death.

Discussion

The mechanisms of cell death control are poorly understood in plants. To uncover the pathways and mechanisms regulating *bak1/serk4* cell death, we have developed an unbiased and highly efficient genetic screen which combines the features of both forward and reverse genetics. We have identified *stt3a* as a suppressor of *bak1/serk4* cell death. Systematic investigation of components in protein *N*-glycosylation pathways and ERQC indicates that *N*-glycan production in ER and specific ERQC components involved in protein folding are essential in the activation of *bak1/serk4* cell death. By combining RNA-seq, genetic and biochemical analyses, we provide evidence that CRK4 serves as one of the client proteins for STT3a-mediated protein *N*-glycosylation and ERQC in *bak1/serk4* cell death.

Although BAK1 and BIR1 are in a complex, the mechanisms regulating their functions in cell death control are distinct. The *bir1*-mediated cell death depends on SOBIR1 and the R protein-mediated signalling²⁰. However, *sobir1* and R protein signalling mutants did not affect *bak1/serk4* cell death (Fig. 1f–h). Both *bak1/serk4* and *bir1* cell death require STT3a (Fig. 3), although the SOBIR1 protein level was not affected in *stt3a* (ref. 23). It has been shown that SOBIR1 protein accumulation was reduced in *erdj3b*, suggesting that ERQC is important for biogenesis of SOBIR1 (ref. 32). It is likely that activation of SOBIR1 also requires *N*-glycosylation modification in ER. Thus, protein glycosylation and ERQC are common features in BAK1/SERK4- and BIR1-regulated cell death, but different client proteins are likely to be deployed for *N*-glycosylation modification.

BAK1 family RLKs serve as a shared signalling node that modulates the interconnected architecture of the complex cellular signalling networks yet disseminates different biological outcomes^{6,47}. Mounting evidence suggests that BAK1 family RLKs function independently in different signalling pathways. For instance, the involvement of BAK1 and SERK4 in cell death control can be separated from their involvement in brassinosteroid signalling^{18,19}. Similarly, their function in stomatal patterning is uncoupled from function in brassinosteroid signalling¹⁵. The *bak1-5* mutant had severe defects in plant immunity, but did not affect brassinosteroid signalling and cell death control⁴⁸. These results are consistent with our observation that *stt3a* did not suppress *bak1* or *bak1/serk4* deficiency in *flg22* and brassinosteroid signalling, reinforcing that the signalling pathway of BAK1/SERK4-regulated cell death is uncoupled from their functions in *flg22*-triggered immunity and brassinosteroid-mediated development.

Many *CRK* genes are induced by defence hormone salicylic acid treatment and bacterial pathogen infections^{44,45}. Constitutive expression of *CRK4* or *CRK5* at a modest level enhanced plant resistance to virulent bacteria *Pseudomonas syringae*^{45,49}. In addition, chemical-induced expression of *CRK4* or *CRK5* triggered cell death in *Arabidopsis*^{44,45}. These observations point to the potential role of CRKs in plant defence responses. We observed an enrichment of *CRK* genes among upregulated genes in *bak1/serk4* (Fig. 5d,e). In addition, CRK4-induced cell death depends on STT3a-mediated protein *N*-glycosylation (Fig. 6d). The CRK4 *N*-glycosylation mutant reduced the ability to trigger cell death (Fig. 6g). It is likely to be that the non-glycosylated or under-glycosylated CRK4 protein is misfolded in ER and finally removed by ER-associated degradation, thereby resulting in the

reduced protein level in *stt3a* (Fig. 6e). However, *crk4* did not suppress *bak1/serk4* cell death (Supplementary Fig. 5e,f). This may be due to the redundant function of several *CRKs*, which are able to induce cell death. We also could not exclude the possible contribution of other genes. Notably, *SOBIR1* is also moderately induced in *bak1/serk4*. Thus, *CRK4* is one of the client proteins of *N*-glycosylation and ERQC involved in *BAK1/SERK4*-regulated cell death.

Methods

Plant materials and growth conditions

Arabidopsis accessions Col-0 and C24 (WT), various mutants and transgenic plants used in this study were grown in soil (Metro Mix 366) in a growth room at 23 °C, 60% relative humidity, 70 $\mu\text{E m}^{-2} \text{s}^{-1}$ light with a 12-h light/12-h dark photoperiod for 2 weeks before VIGS assay or 30 days for protoplast isolation. The *bak1-4*, *serk4-1*, *sobir1-12*, *stt3a-1*, *hgl1-1*, *rsw3-1*, *fucTa/fucTb/xyIT*, *erdjb3b-1*, *sdf2-2* and *crt3-1* mutants and *NahG* transgenic plants have been reported previously^{15,25,32,41}. The *ire1a/ire1b* and *bzip60/bzip28* double mutants were obtained from Stephen H. Howell⁴². The sets of confirmed *Arabidopsis* T-DNA insertion lines (CS27941, CS27943 and 0827944), *stt3a-2*, *ost3/6* (SALK_067271C), *stt3b-2* (SALK_078498C), *stt3b-3* (Salk_134449C), *alg3* (sALK_040296C), *alg10-1* (SAIL_515_F10), *alg12* (SALK_200867C) and *crk4* (CS859967) were obtained from the *Arabidopsis* Biological Resource Center (ABRC) and confirmed by PCR using primers listed in Supplementary Table 5. Seedlings were grown on agar plates containing 1/2 Murashige and Skoog medium (1/2MS) with 0.5% sucrose, 0.8% agar and 2.5 mM MES at pH 5.7, in a growth chamber at 23 °C or 30 °C, 70 $\mu\text{E m}^{-2} \text{s}^{-1}$ light with a 12-h light/12-h dark photoperiod.

Plasmid construction and generation of transgenic plants

To generate the VIGS *BAK1/SERK4* construct, fragments of *BAK1* (319 bp) and *SERK4* (310 bp) were PCR amplified from *Arabidopsis* Col-0 cDNA with primers listed in Supplementary Table 5, digested with EcoRI and NcoI for the *SERK4* fragment, and NcoI and KpnI for the *BAK1* fragment, and ligated with the *pYL156 (pTRV-RNA2)* vector pre-cut with EcoRI and KpnI²¹. To generate VIGS constructs for individual genes, fragments of *SERK4* (310 bp), *CLAI* (541 bp), *MEKK1* (520 bp) and *BIR1* (491 bp) were PCR amplified from *Arabidopsis* Col-0 cDNA using primers listed in Supplementary Table 5, digested with EcoRI and KpnI, and ligated into the *pYL156 (pTRV-RNA2)* vector pre-cut with EcoRI and KpnI. All the clones were confirmed by sequencing.

The *CRK4* and *CRK5* genes were amplified from Col-0 cDNA with primers containing BamHI or NcoI at the amino (N) terminus and StuI at the carboxy (C) terminus (Supplementary Table 5), and ligated into a plant protoplast expression vector *pHBT* with a *CaMV 35S* promoter at the N terminus and haemagglutinin (HA) epitope tag at the C-terminus. The point mutations of *CRK4*^{N181Q} and *CRK4*^{N286Q} were generated by site-directed mutagenesis with primers listed in Supplementary Table 5. To construct the *pCB302* binary vector containing *CRKs* for *Agrobacterium-mediated* transient expression assay in *N. benthamiana*, the *CRKs* fragment was released from the *pHBT* vector digested with BamHI

or NcoI and StuI and ligated into the *pCB302* binary vector. The *Est::CRK4* binary vector construct was generated by inserting the PCR products of the *CRK4* open reading frame with an HA epitope tag at its C terminus from the *pHBT-CRK4-HA* vector into the *pER8* vector using the XhoI and SpeI sites¹⁵. All the clones were confirmed by sequencing.

STT3a complementation transgenic plants in the *stt3a-2* background have been reported previously²⁵. *Arabidopsis* transgenic plants were generated using *Agrobacterium-mediated* transformation by the floral-dip method. For oestradiol induction of *CRK4* expression, the detached leaves of *Est::CRK4* T1 transgenic plants were treated with 10 μ M oestradiol for 24 h and transgene expression was detected using immunoblotting with α -HA antibody.

***Arabidopsis* protoplast and *Nicotiana benthamiana* transient assays**

For *Arabidopsis* protoplast transient expression, protoplasts from WT or *stt3a* mutant were transfected with HA-tagged *CRKs* in the *pHBT* vector and incubated for 12 h. Proteins were isolated with 2 \times SDS loading buffer and subjected to immunoblot analysis with anti-HA antibody.

For *N. benthamiana* transient expression, *Agrobacterium tumefaciens* strain GV3101 containing *pCB302* vector was cultured overnight in LB medium at 28 °C. Bacteria were harvested by centrifugation and resuspended with buffer (10 mM MES, pH 5.7, 10 mM MgCl₂, 200 μ M acetosyringone) at $A_{600} = 0.75$. Leaves of 4-week-old soil-grown *N. benthamiana* were hand-infiltrated using a needleless syringe with *Agrobacterium* cultures. Leaf samples were collected 36 h after infiltration for protein isolation and immunoblot analysis. The cell death phenotype was observed and leaf pictures were taken 4 days after infiltration under UV light with a ChemiDoc system.

Trypan blue and DAB staining

Trypan blue staining and 3,3'-diaminobenzidine (DAB) staining were performed according to procedures described previously with modifications⁵⁰. Briefly, the excised plant tissues were immersed in trypan blue staining solution (2.5 mg ml⁻¹ trypan blue in lactophenol (lactic acid, glycerol, liquid phenol and H₂O in a ratio of 1:1:1:1)) or DAB solution (1 mg ml⁻¹ DAB in 10 mM Na₂HPO₄ and 0.05% Tween 20). Samples were vacuum-infiltrated for 30 min and then incubated for 8 h at 25 °C with gentle shaking at 75 rpm. Subsequently, samples were transferred to trypan blue destaining solution (ethanol and lactophenol in a ratio of 2:1) or DAB destaining solution (ethanol, acetic acid and glycerol in a ratio of 3:1:1) and incubated at 65 °C for 30 min. The samples were then incubated in fresh destaining solution at room temperature until complete destaining. Pictures were taken under a dissecting microscope with samples in 10% glycerol.

***Agrobacterium-mediated* virus-induced gene silencing assay**

Plasmids containing binary TRV vectors *pTRV-RNA1* and *pTRV-RNA2* derivatives, *pYL156-BAK1/SERK4*, *pYL156-SERK4*, *pYL156-MEKK1*, *pYL156-BIR1*, *pYL156-GFP* (the vector control) or *pYL156-CLA1* were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. *Agrobacterium* cultures were first grown overnight in LB medium containing 50 pg ml⁻¹ kanamycin and 25 pg ml⁻¹ gentamicin and then subcultured

in fresh LB medium containing 50 µg ml⁻¹ kanamycin and 25 µg ml⁻¹ gentamicin supplemented with 10 mM MES and 20 µM acetosyringone overnight at 28 °C in a roller drum. Cells were pelleted by 4,200 r.p.m. centrifugation, resuspended in a solution containing 10 mM MgCl₂, 10 mM MES and 200 µM acetosyringone, adjusted to A₆₀₀ of 1.5 and incubated at 25 °C for at least 3 h. *Agrobacterium* cultures containing *pTRV-RNA1* and *pTRV-RNA2* derivatives were mixed in a 1:1 ratio and inoculated into the first pair of true leaves of 2-week-old soil-grown plants using a needleless syringe. The *pYL156-CLA1* construct, which leads to plant albino phenotype 2 weeks after *Agrobacterium* infiltration, was included as a visual marker for VIGS efficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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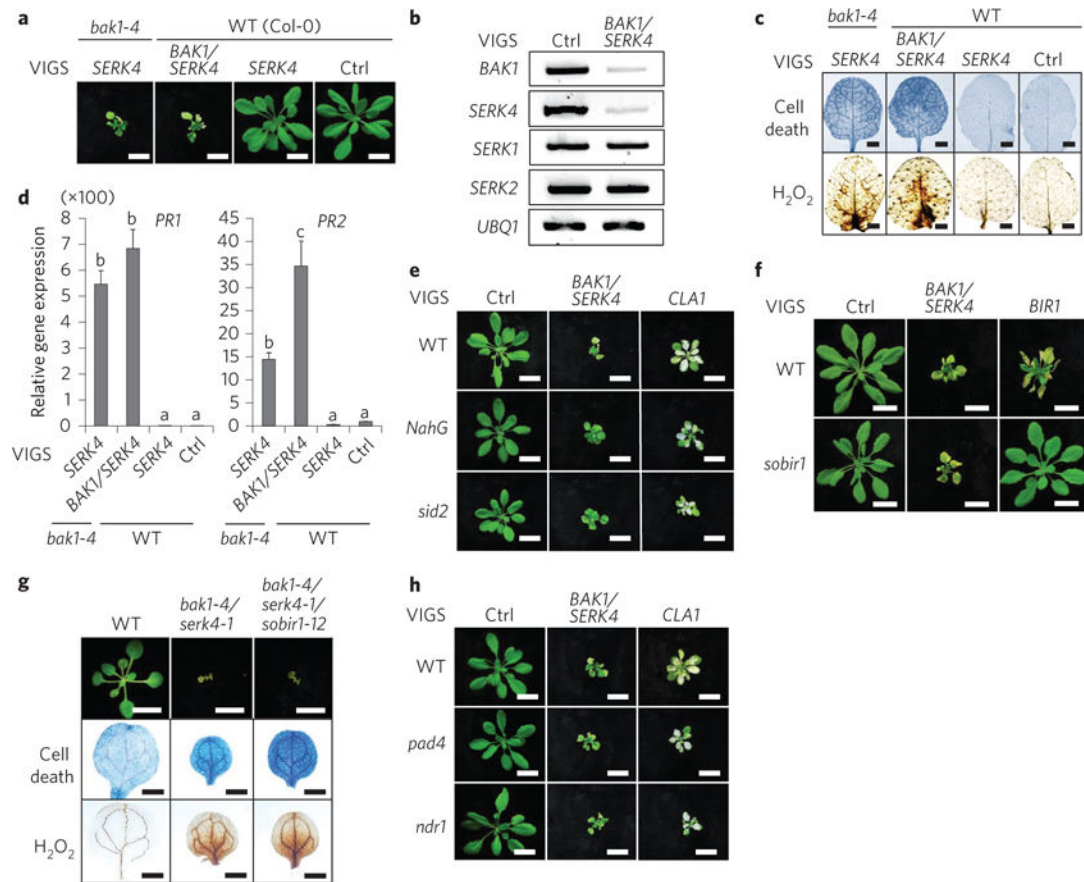


Figure 1. The *sobir1* mutant did not suppress *bak1/serk4* cell death

a, Silencing of *BAK1/SERK4* by VIGS causes plant dwarfism and leaf chlorosis. Plants were photographed 2 weeks after inoculation of *Agrobacterium* carrying the indicated VIGS vectors in *bak1-4* or WT plants. Scale bar, 1 cm. **b**, RT-PCR analysis of *BAK1/SERK4*-silenced plants after VIGS. *BAK1*, *SERK4*, *SERK1* and *SERK2* expression was analysed by RT-PCR from WT plants 2 weeks after VIGS. *UBQ1* was used as an internal control. **c**, Silencing of *BAK1/SERK4* by VIGS triggers cell death and H₂O₂ production. Trypan blue staining for cell death (upper panel) and DAB staining for H₂O₂ production (lower panel) are shown for true leaves of VIGS plants. Scale bar, 2 mm. **d**, Silencing of *BAK1/SERK4* by VIGS induces *PR1* and *PR2* expression. The data are shown as mean ± s.e.m. from three independent repeats. The different letters denote a statistically significant difference according to one-way analysis of variance (ANOVA) followed by the Tukey test ($P < 0.05$). **e**, *BAK1/SERK4*-regulated cell death is partially dependent on salicylic acid. Plants silenced with *CLA1* (*Chloroplastos alterados 1*) showed the albino phenotype as a visual marker of VIGS efficiency. *NahG* and *sid2* plants after VIGS of *BAK1/SERK4* remained greener with reduced leaf chlorosis than WT. Scale bar, 1 cm. **f**, *BAK1/SERK4*-regulated cell death is independent of *SOBIR1*. Scale bar, 1 cm. **g**, The *sobir1-12* mutant did not suppress *bak1-4/serk4-1* cell death in the *bak1-4/serk4-1/sobir1-12* mutant. Seedlings were grown on a ½MS plate and photographed at 16 days after germination (top; scale bar, 1 cm). Cotyledons were stained with trypan blue for cell death (middle) and DAB for H₂O₂ accumulation (bottom). Scale bar, 1 mm in middle and bottom panels. **h**, *BAK1/SERK4*-regulated cell death is

independent of PAD4 and NDR1. Scale bar, 1 cm. The above experiments were repeated at least three times with similar results.

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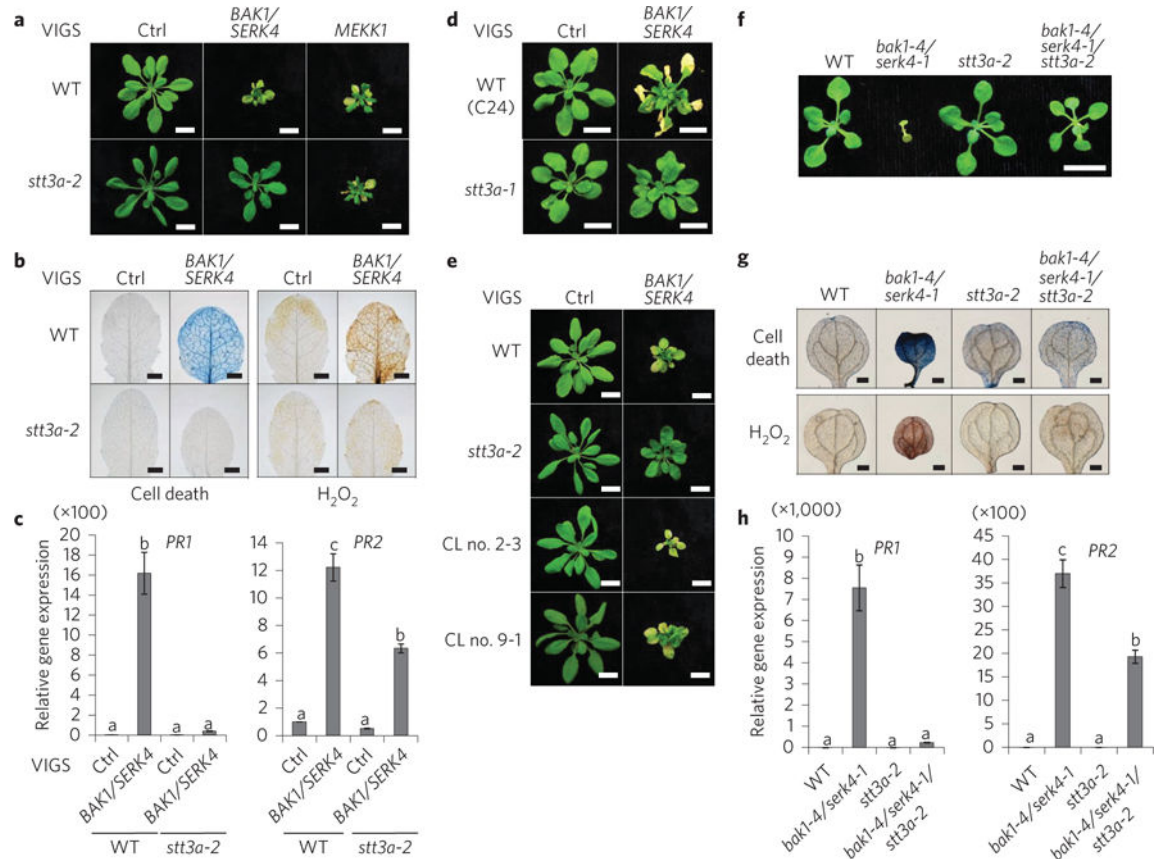


Figure 2. The *stt3a* mutants suppress BAK1/SERK4-regulated cell death

a, The *stt3a-2* mutant suppresses growth defects triggered by VIGS of *BAK1/SERK4* but not *MEKK1*. Scale bar, 1 cm. **b**, The *stt3a-2* mutant suppresses cell death (left panel) and H₂O₂ production (right panel) by VIGS of *BAK1/SERK4*. Scale bar, 2 mm. **c**, The *stt3a-2* mutant suppresses *PR1* and *PR2* expression by VIGS of *BAK1/SERK4*. **d**, The *stt3a-1* mutant suppresses growth defects by VIGS of *BAK1/SERK4*. Plant phenotypes are shown from WT (C24) and *stt3a-1* 2 weeks after VIGS. Scale bar, 1 cm. **e**, Complementation of the *stt3a-2* mutant with *STT3a* restores growth defects by VIGS of *BAK1/SERK4*. CL no. 2-3 and CL no. 9-1 are two homozygous complementation lines. Scale bar, 1 cm. **f**, The *stt3a-2* mutant rescues the seedling lethality of the *bak1-4/serk4-1* mutant. Seedlings were grown on ½MS plate and photographed at 16 days after germination. Scale bar, 1 cm. **g**, The alleviated cell death and H₂O₂ accumulation in *bak1-4/serk4-1/stt3a-2*. Scale bar, 2 mm. **h**, The reduced *PR1* and *PR2* expression in *bak1-4/serk4-1/stt3a-2*. The data in **c** and **h** are shown as mean ± s.e.m. from three independent repeats. The different letters denote a statistically significant difference according to one-way ANOVA followed by the Tukey test ($P < 0.05$). The above experiments were repeated three times with similar results.

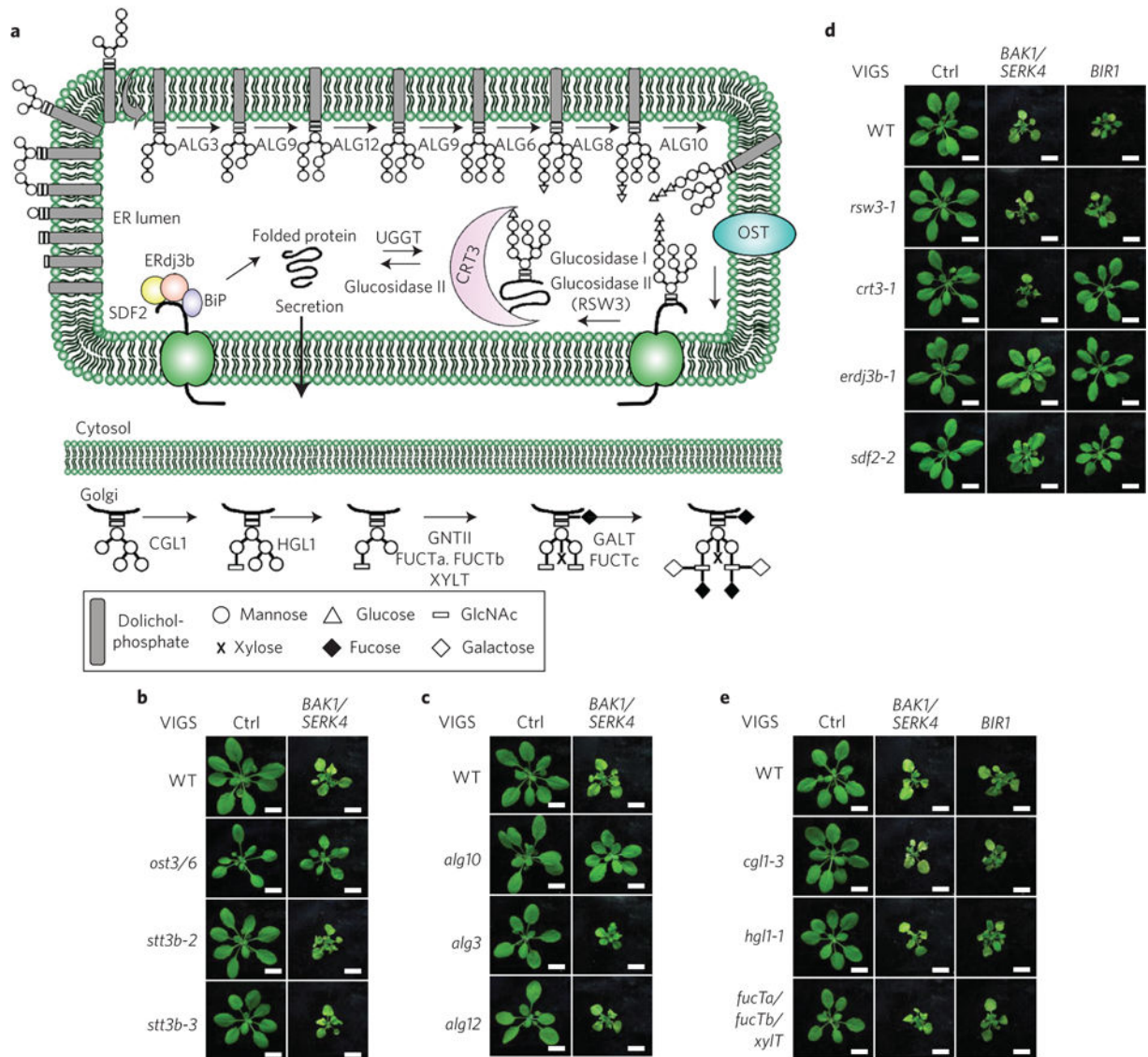


Figure 3. Control of BAK1/SERK4-regulated cell death by protein *N*-glycosylation and specific components of ERQC

a, A schematic overview of protein *N*-glycan assembly in the ER and modification in the Golgi apparatus and ERQC system. The OST complex transfers the dolichol-linked *N*-glycan precursor preassembled by different ALG glycosyltransferases to the acceptor proteins in the ER. The *N*-glycan precursor is then modified by ER glucosidase, and recognized by CNX and CRT3 for proper protein folding. The misfolded proteins undergo additional CNX/CRT cycles by UGGT and glucosidase for another round of folding. The BiP-ERdj3-SDF2 complex also controls proper protein folding. The correctly folded proteins will be exported to the Golgi apparatus to form complex *N*-glycans catalysed by different enzymes. **b**, The *ost3/6*, but not *stt3b*, mutant suppresses cell death by VIGS of *BAK1/SERK4*. Phenotypes of different plants are shown 2 weeks after VIGS. Scale bar, 1 cm. **c**, The *alg10*, but not the *alg3* or *alg12*, mutant suppresses cell death by VIGS of *BAK1/SERK4*. Scale bar, 1 cm. **d**, Differential ERQC components are required for *BAK1/SERK4*-

and BIR1-regulated cell death. The *erdj3b-1* and *sdf2-2* mutants partially suppressed BAK1/SERK4- and BIR1-regulated cell death by VIGS. However, the *crt3-1* mutant only suppressed BIR1- but not BAK1/SERK4-regulated cell death by VIGS. Scale bar, 1 cm. **e**, The *N*-glycan modification in the Golgi apparatus may not be required for BAK1/SERK4-regulated cell death. Various mutants impaired in *N*-glycan modification in the Golgi apparatus did not suppress cell death by VIGS of *BAK1/SERK4* or *BIR1*. Scale bar, 1 cm. The above experiments were repeated three times with similar results.

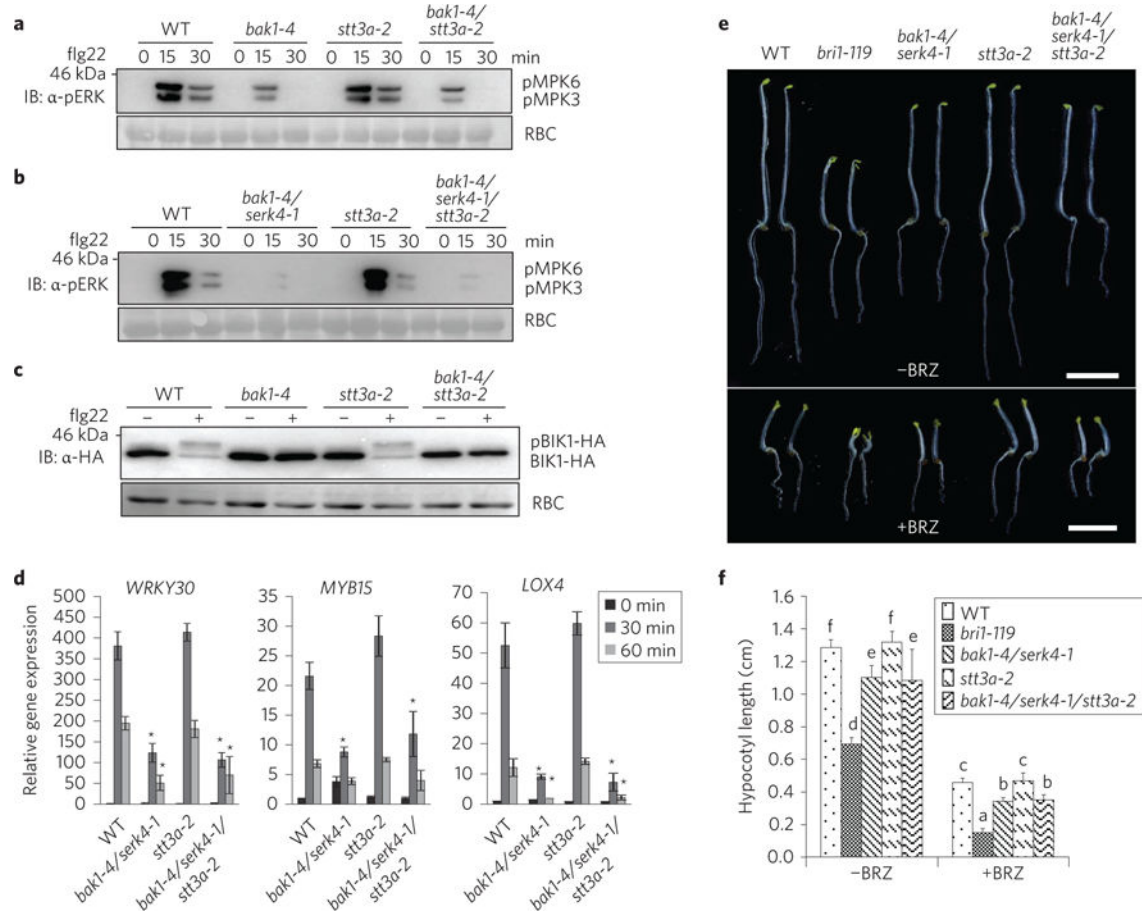


Figure 4. Uncoupled BAK1 functions in cell death control, immunity and brassinosteroid signalling

a, The *stt3a-2* mutation did not interfere with the compromised flg22-induced MAPK activation in the *bak1-4* mutant. Ten-day-old seedlings were treated without or with 100 nM flg22 for 15 and 30 min. MAPK activation was analysed by immunoblotting with α -pERK antibody (top panel); protein loading is shown by Ponceau S staining for Rubisco (RBC) (bottom panel). **b**, The *stt3a-2* mutation did not interfere with the compromised flg22-induced MAPK activation in the *bak1-4/serk4-1* mutant. **c**, The *stt3a-2* mutation did not affect the compromised flg22-induced BIK1 phosphorylation in the *bak1-4* mutant. Protoplasts from different plants were transfected with haemagglutinin (HA)-tagged BIK1 and treated with 100 nM flg22 for 10 min. BIK1-HA proteins were detected by immunoblotting using the α -HA antibody (top panel); protein loading is shown by Ponceau S staining for RBC (bottom panel). **d**, The *stt3a-2* mutation did not affect the compromised flg22-induced marker gene expression in the *bak1-4/serk4-1* mutant. Ten-day-old seedlings were treated without or with 100 nM flg22 for 30 or 60 min for qRT-PCR analysis. The data are shown as mean \pm s.e.m. from three independent repeats. Asterisks indicate statistically significant differences from the WT within the same time point according to two-way ANOVA followed by the Tukey test ($P < 0.05$). **e,f**, The *bak1-4/serk4-1* sensitivity to BRZ still occurs in the *bak1-4/serk4-1/stt3a-2* mutant. The seedlings of indicated genotypes were grown in the dark for 5 days on $\frac{1}{2}$ MS plates with or without 2 μ M BRZ (**e**), and hypocotyl

lengths were quantified (**f**). The data are shown as mean \pm s.e.m. from 20 seedlings. Scale bar, 0.5 cm. The different letters denote a statistically significant difference according to two-way ANOVA followed by the Tukey test ($P < 0.05$). The experiments in **a–d** were repeated three times and in **e,f** the experiments were repeated twice with similar results.

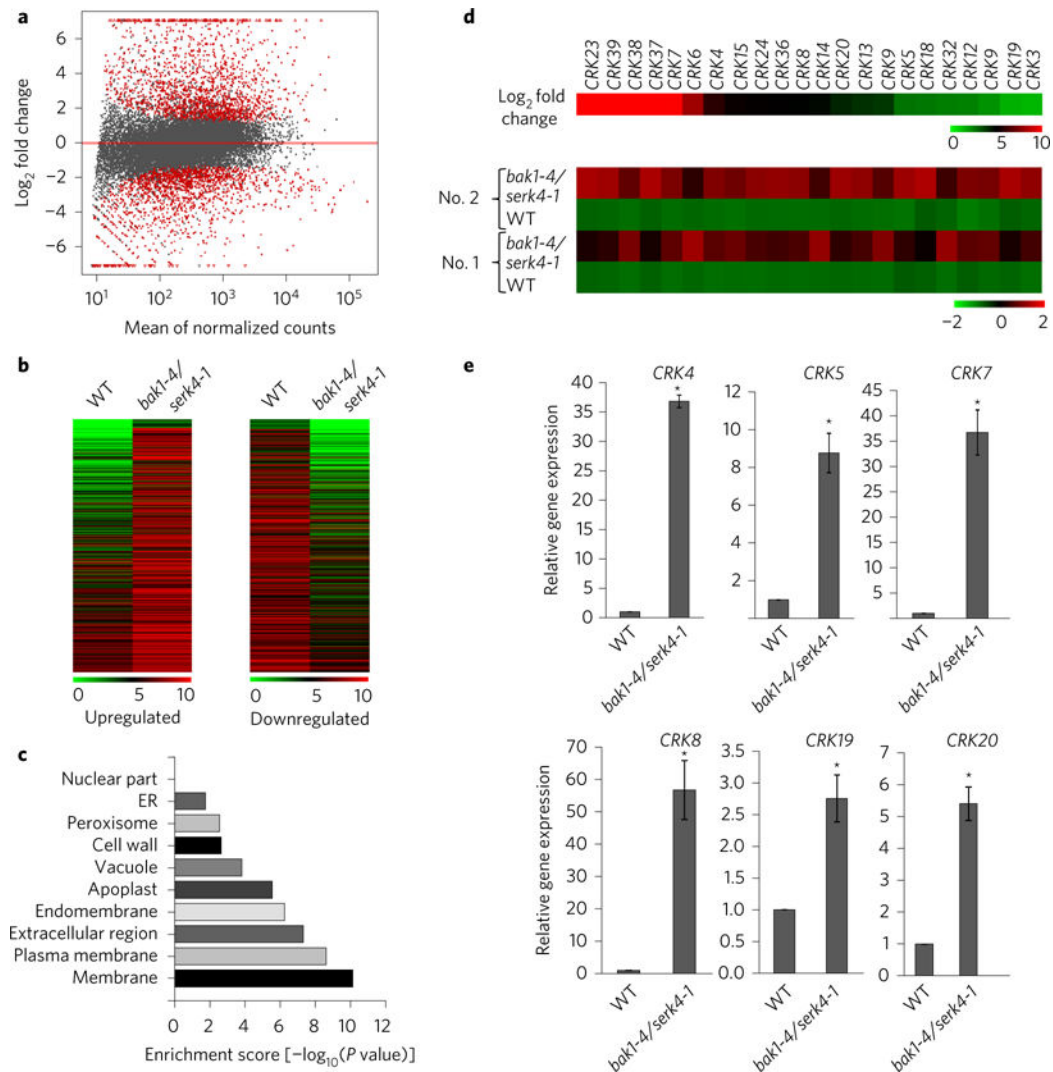


Figure 5. Members of *CRK* genes are upregulated in the *bak1/serk4* mutant

a, DESeq's *plotMA* displays differential expression (\log_2 fold changes between *bak1-4/serk4-1* and WT on the y axis) versus expression strength (the mean of normalized counts on the x axis). Genes with significantly differential expression with $FDR < 0.10$ and fold change ≥ 2 are in red. **b**, Heatmaps of upregulated and downregulated genes in the *bak1-4/serk4-1* mutant compared with WT. The original means of read counts were subjected to data adjustment by \log_2 transformation using MeV4.0 for the heatmaps. The genes are plotted in the order of fold changes. **c**, Gene ontology (GO) enrichment analysis based on the cellular component. The x axis indicates the enrichment scores [$-\log_{10}(P \text{ value})$] (the P value indicates the possibility of significant enrichment) for each cellular component GO item on the y axis. **d**, Members of *CRKs* are induced in *bak1/serk4*. The upper panel indicates \log_2 fold change of different *CRK* genes (*bak1-4/serk4-1* vs. WT) and the bottom panel shows normalized expression level from two independent repeats (No. 1 and No. 2). **e**, Expression of some *CRKs* by qRT-PCR analysis. Ten-day-old seedlings grown on $\frac{1}{2}$ MS plates were subjected to qRT-PCR analysis. The data are shown as means \pm s.e.m. from three

biological replicates. * $P < 0.05$ significant difference compared with WT using Student's t -test.

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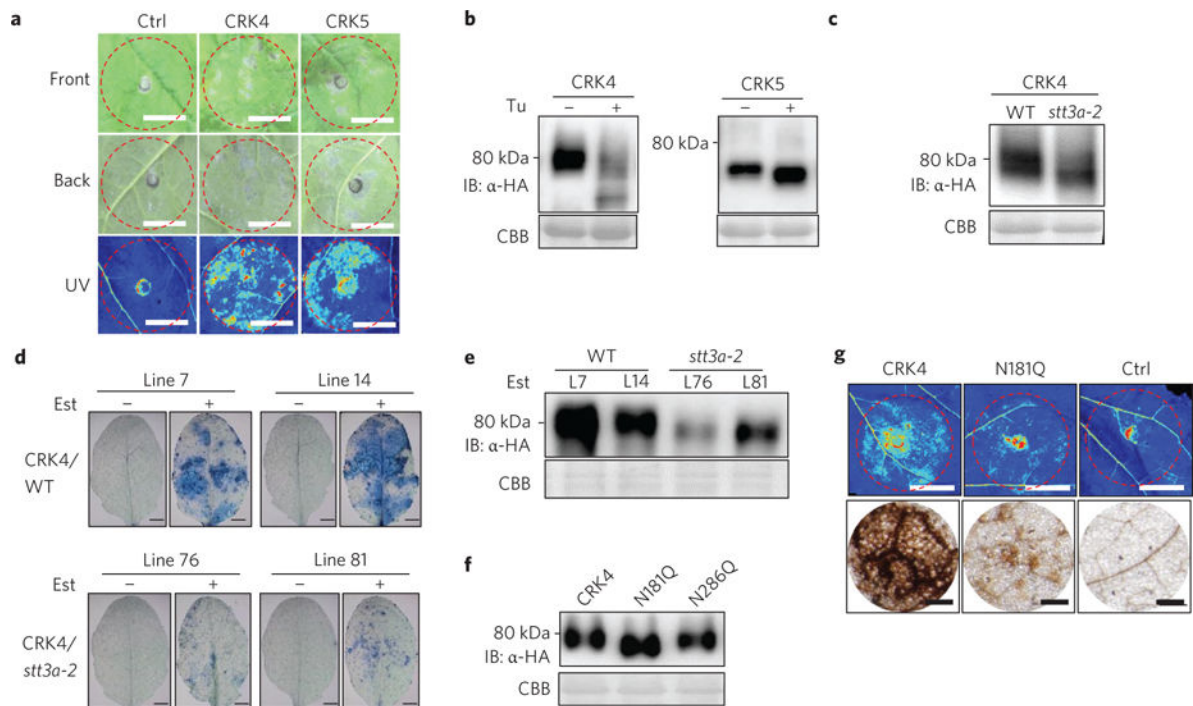


Figure 6. CRK4-induced cell death requires STT3a-mediated N-glycosylation

a, Expression of *CRK4* or *CRK5* in *N. benthamiana* induces cell death. Cell death was visualized on the front (top panel) or back (middle panel) side of different leaves or under UV light with the ChemiDoc Imaging System (bottom panel) 4 days after infiltration. The infiltrated areas are labelled with red dashed circles. Scale bar, 1 cm. **b**, Tunicamycin treatment modifies protein migration of *CRK4* and *CRK5*. Protoplasts from Col-0 WT were transfected with *CRK4-HA* or *CRK5-HA* and treated without or with tunicamycin (1 μ M). The proteins were detected by immunoblotting with α -HA antibody (upper panel). Coomassie Brilliant Blue (CBB) staining is a loading control (lower panel). **c**, *CRK4* exhibits elevated protein migration rate in *stt3a-2*. Protoplasts from Col-0 WT or the *stt3a-2* mutant were transfected with *CRK4-HA*. **d**, STT3a is required for *CRK4*-induced cell death in *Arabidopsis*. Leaves of four-week-old transgenic plants of oestradiol-inducible *CRK4-HA* in WT and *stt3a* background were infiltrated with oestradiol (10 μ M) or mock. Cell death staining was performed 4 days after infiltration. Two independent lines for each background are shown. Scale bar, 2 mm. **e**, *CRK4* protein expression in transgenic plants. Detached leaves from 4-week-old transgenic plants were soaked in oestradiol solution (10 μ M) for 24 h. The HA-tagged *CRK4* protein was detected by immunoblotting with α -HA antibody. **f**, Expression of *CRK4* and its putative N-glycosylation mutants in *Arabidopsis* protoplasts. **g**, *CRK4*-induced cell death is partially blocked by N181Q mutation in *N. benthamiana*. Cell death was documented 4 days after inoculation of *Agrobacterium* carrying different constructs in *N. benthamiana*. Upper panel, cell death was visualized as autofluorescence under UV light with the ChemiDoc Imaging System. Scale bar, 1 cm. Bottom panel, H₂O₂ accumulation by DAB staining. Scale bar, 100 μ m.