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NLR locus-mediated trade-off between abiotic and biotic stress adaptation in *Arabidopsis*

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Osmotic stress caused by drought, salt or cold decreases plant fitness. Acquired stress tolerance defines the ability of plants to withstand stress following an initial exposure[1]. We found previously that acquired osmotolerance after salt stress is widespread among *Arabidopsis thaliana* accessions[2]. Here, we identify *ACQOS* as the locus responsible for ACQUIRED OSMOTOLERANCE. Of its five haplotypes, only plants carrying group 1 *ACQOS* are impaired in acquired osmotolerance. *ACQOS* is identical to *VICTR*, encoding a nucleotide-binding leucine-rich repeat (NLR) protein[3]. In the absence of osmotic stress, group 1 *ACQOS* contributes to bacterial resistance. In its presence, *ACQOS* causes detrimental autoimmunity, thereby reducing osmotolerance. Analysis of natural variation at the *ACQOS* locus suggests that functional and non-functional *ACQOS* alleles are being maintained due to a trade-off between biotic and abiotic stress adaptation. Thus, polymorphism in certain plant *NLR* genes might be influenced by competing environmental stresses.

Natural genetic variation has facilitated the identification of genes underlying complex traits such as growth, flowering and stress tolerance while creating opportunities for adaptation to changing environmental conditions[4]. Studies of several hundred *A. thaliana* accessions have provided new insights into genome evolution, differentiation among geographic populations and selective mechanisms that shape complex trait variation in nature[5]. Plants have evolved the ability to acclimatize to various stresses after initial exposure to a related stress cue[1]. A large-scale analysis of 350 *A. thaliana* accessions revealed extensive variation in acquired osmotolerance on mild salt exposure[2]. When 7-day-old seedlings were pre-exposed to 100 mM NaCl for 7 days (acclimation period), the *A. thaliana* accessions Bu-5 and Bur-0, but not Col-0 or Wl-0, acquired osmotolerance to 750 mM sorbitol[2] (Fig. 1a). Using the progeny of a Bu-5 × Col-0 cross, we mapped a single locus on chromosome 5, which we named acquired osmotolerance (*ACQOS*).

Here, we resolved the *ACQOS* locus to a 100 kilobase (kb) region on chromosome 5 containing 24 annotated genes (Supplementary Fig. 1). We then developed two BC_5F_3 near-isogenic lines, NIL-Col-0 and NIL-Bu-5, which carried different sized small chromosomal segments from Bu-5 containing the *ACQOS* region in the genetic background of Col-0. Retention of acquired osmotolerance in NIL-Bu-5 but not NIL-Col-0 narrowed down the *ACQOS* locus to a 67 kb region (Fig. 1b and Supplementary Fig. 2a). To investigate whether the *ACQOS* locus accounts for species-wide variation in acquired osmotolerance, we performed a genome-wide association study (GWAS) using 179 accessions (Supplementary Table 1). This revealed a significant ~200-kb-wide peak on chromosome 5 that coincided with large linkage disequilibrium patterns within ±500 kb of the *ACQOS* locus, consistent with the fine mapping data (Fig. 1c). To identify polymorphisms in the region, we constructed a bacterial artificial chromosome (BAC) library derived from Bu-5 genomic DNA and sequenced a BAC clone containing the region. Sequencing revealed a 17 kb deletion in Bu-5. In the corresponding region, Col-0 has a tandem repeat of four *Toll and interleukin1 receptor–nucleotide binding leucine-rich repeat* (*TIR–NLR*) genes (*NLR1–NLR4*; *NLR2* encodes a truncated, apparently non-functional protein), whereas Bu-5 has one *TIR–NLR* gene (Fig. 1b and Supplementary Fig. 3). We tested whether this single $NLR^{Bu\text{-}5}$ confers osmotolerance in Bu-5 or one or more of the four Col-0 *NLRs* impairs acquired osmotolerance, by introducing different *NLRs* into Col-0 and NIL-Bu-5. In these complementation assays, $NLR^{Bu\text{-}5}$ did not confer acquired osmotolerance in the Col-0 background (Supplementary Fig. 4). By contrast, Col-0 *NLR4*, but not *NLR3*, abolished osmotolerance in the NIL-Bu-5 background (Fig. 1d and Supplementary Fig. 4). Also, disruption of *NLR4* but not *NLR2* or *NLR3* in Col-0 by T-DNA insertion conferred acquired osmotolerance equivalent to that of NIL-Bu-5 (Fig. 1e and Supplementary Fig. 5). Therefore, Col-0 *NLR4* suppresses the acquired osmotolerance of Bu-5. These results suggest that *NLR4* is the *ACQOS* locus underlying variation in acquired osmotolerance.

Col-0 *ACQOS* was described previously as *VICTR* (*VARIATION IN COMPOUND TRIGGERED ROOT* growth response), which mediates root growth arrest induced by the small molecule

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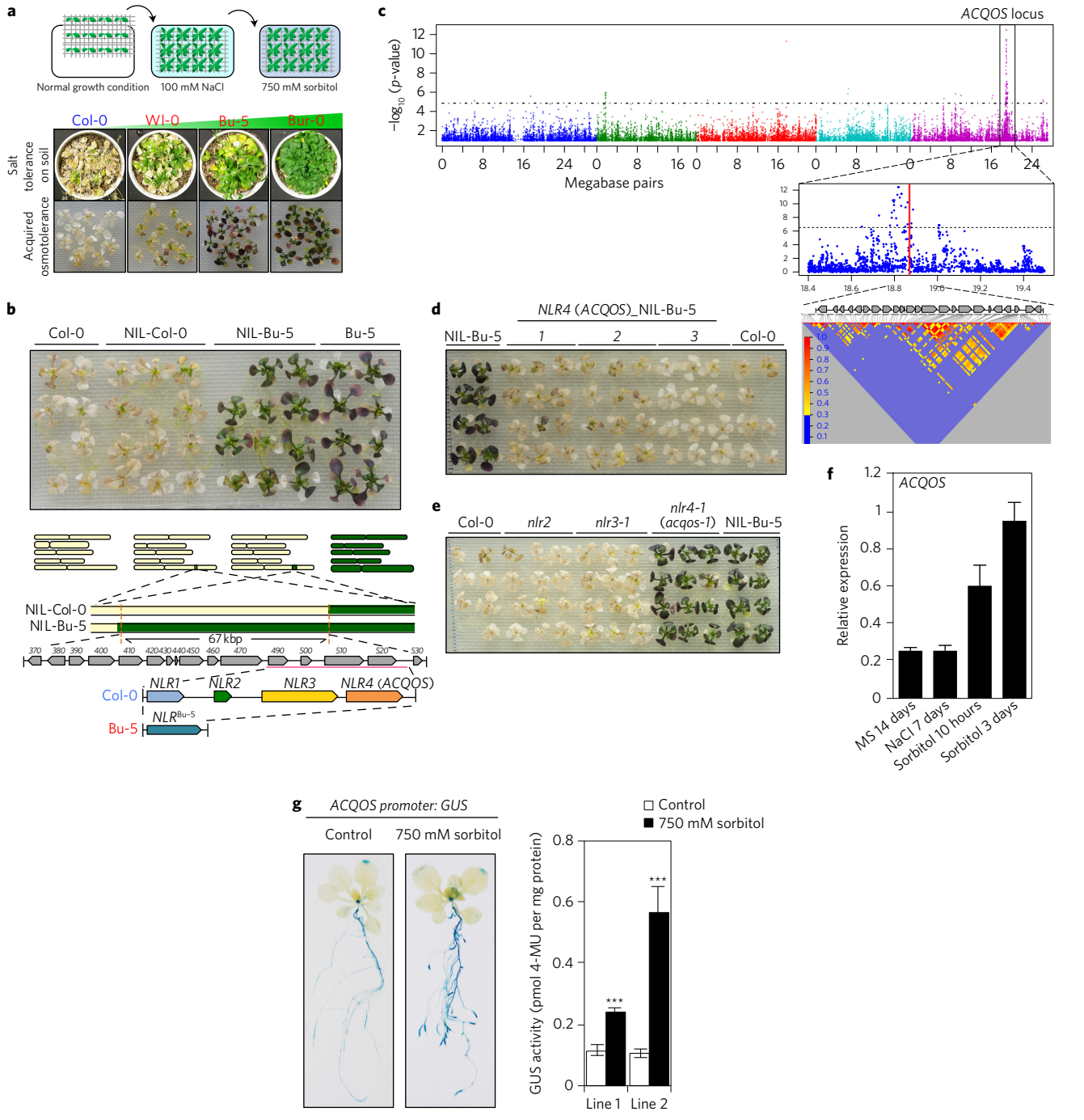

Figure 1 | Identification of the *ACQOS* locus. a, Acquired osmotolerance of *A. thaliana* accessions. A flow chart of the acquired osmotolerance assay (top). Salt tolerance when grown on soil (middle). Three-week-old plants grown in pots were exposed to 500 mM NaCl in water for 49 days. Acquired osmotolerance (bottom). Salt-acclimated 2-week-old seedlings were mesh-transferred to MS agar plates containing 750 mM sorbitol for 21 days. **b**, High-resolution mapping of the *ACQOS* locus using NILs. Acquired osmotolerance of Col-0, Bu-5, NIL-Col-0 and NIL-Bu-5 (top). Graphical genotypes of NILs (bottom). Chromosomal segments of Col-0, off-white; Bu-5, green. Numbers above the genes are the last three digits of their *Arabidopsis* Genome Initiative (AGI) numbers (*At5g46XXX*). **c**, Genome-wide association study for acquired osmotolerance. Manhattan plot of GWAS results for acquired osmotolerance (top; colours distinguish chromosomes 1–5). Close-up of the major GWAS peak in the vicinity of the *ACQOS* locus on chromosome 5 (middle). The position of the *ACQOS* gene is indicated by a red line. Linkage disequilibrium patterns within ±500 kb upstream and downstream of the *ACQOS* locus (bottom). **d**, Complementation test performed by transforming NIL-Bu-5 with *NLR4 (ACQOS)*. T_3 homozygous plants transformed with *native promoter*: *NLR4* (*ACQOS*) derived from Col-0 was used. **e**, Acquired osmotolerance of *nlr2*, *nlr3-1* and *nlr4-1* (*acqos-1*) mutants. **f**, Expression of *ACQOS* in Col-0 plants under normal, salt acclimated and subsequent osmotic stress conditions; gene expression was determined by qRT–PCR (mean ± s.e.m., $n = 3$). **g**, Histochemical analysis of the expression pattern of the *ACQOS* promoter: *GUS* in Col-0 seedlings grown under normal or osmotic stress conditions. GUS activities in two independent transgenic lines were measured using 4-MUG fluorometric assay. Differences between normal (white bars) and osmotic stress (black bars) conditions were analysed using Student's *t*-test. (mean ± s.e.m., $n = 7$, $^{***}P < 0.001$) After salt acclimation, seedlings were grown in the presence of 750 mM sorbitol for 21 (**b**), 15 (**d**) or 20 (**e**) days. Similar results were obtained in three independent experiments; representative data are shown. All error bars are S.E.M.

[5-(3,4-dichlorophenyl)furan-2-yl]-piperidine-1-ylmethanethione (DFPM) in Col-0[3]. ACQOS/VICTR protein associated with and required the TIR–NLR immunity regulators Enhanced Disease Susceptibility1 (EDS1) and Phytoalexin-Deficient4 (PAD4) for DFPM-induced immunity and antagonism of certain osmotic stress responses mediated by the hormone abscisic acid (ABA)[3,6]. In plants and animals, NLR proteins are typically immune sensors for pathogen molecules or pathogen-induced modifications of host cell components[7]. There are 104 annotated *TIR–NLR*s in the genome of *A. thaliana* Col-0. The closest homologue of *ACQOS* in Col-0 is *NLR3*, which is also missing in Bu-5 (Supplementary Fig. 6). In Col-0, *ACQOS* gene expression was induced predominantly in roots in response to osmotic stress (Fig. 1f,g). To investigate whether *ACQOS* expression levels influence the extent of acquired osmotolerance, we exploited an osmotic stress-inducible *ACQOS* overexpression line identified among the *ACQOS* transgenic lines in the NIL-Bu-5 background (see Fig. 1d). Osmotic stress-inducible overexpression of *ACQOS* (line 3), without a significant increase in basal expression, rendered the seedlings more sensitive to osmotic stress than other less strongly inducible lines or Col-0 plants (Supplementary Fig. 7). In addition, F_1 progeny of Col-0 × NIL-Bu-5 showed a partial breakdown of acquired osmotolerance (Supplementary Fig. 8). These results show that *ACQOS* suppresses the acquisition of osmotolerance in a dose-dependent manner.

To explore nucleotide variation at the *ACQOS* locus, we performed PCR-based cloning and Sanger sequencing of a ~23 kb genomic region encompassing the *ACQOS* gene in 79 *A. thaliana* accessions. We chose Sanger sequencing because standard Illumina short-read sequencing is often unreliable if there are large deletions, insertions or tandem repeats, as found in the *ACQOS* region. Based on the pattern of indels and tandem repeats, we classified the tested accessions into five *ACQOS* haplogroups (groups 1–5) (Fig. 2a and Supplementary Fig. 9a). Group 1, which includes Col-0, was rare (10%), whereas group 4 including Bu-5 and group 5 were most frequent (72%; Fig. 2b). As expected from the prominent GWAS peak around the *ACQOS* locus (Fig. 1c), we found a strong correlation between the haplogroup and acquired osmotolerance: groups 2–5 displayed osmotolerance, whereas group 1 did not (Fig. 2c and Supplementary Fig. 2b). Notably, group 2 carrying polymorphisms in the *ACQOS* gene (Fig. 2d) had acquired osmotolerance (Fig. 2c and Supplementary Fig. 2b). This suggests that nucleotide substitutions between the group 1 and 2 *ACQOS* genes explain the presence or absence of acquired osmotolerance. To test this possibility, we introduced the corresponding *ACQOS* genes from Col-0 (group 1) or Rou-0 (group 2) into *ACQOS* knockout mutants. In these complementation experiments, group 1 but not group 2 *ACQOS* strongly reduced acquired osmotolerance (Fig. 2e and Supplementary Fig. 2c), indicating that the nucleotide substitutions render group 2 *ACQOS* non-functional in osmotolerance suppression.

To explore haplotype and allelic diversity at the *ACQOS* locus, we conducted a phylogenetic analysis of the tandemly duplicated *ACQOS* homologues, including those from *Arabidopsis lyrata* as an outgroup[8] (Fig. 2f). The corresponding region of *A. lyrata* contains three *TIR–NLR* genes which differ from *A. thaliana*, suggesting that this locus has evolved independently after species divergence (Supplementary Fig. 9b). The phylogeny revealed that *NLR* genes within the *ACQOS* locus fall into two major clades, one containing group 4 *NLR* and an *A. lyrata* homologue (named haplogroup A) and the other containing group 5 *NLR* (named haplogroup B) (Fig. 2f). *NLR1* in groups 1–3 appears to be closest to the $NLR^{group\ 4}$, whereas *NLR3* of groups 1–3 and *ACQOS* belong to the same clade as $NLR^{group\ 5}$. These results suggest that two divergent single-copy *NLR* haplogroups (A and B) evolved initially, and that *NLR3* and *ACQOS* originated through tandem duplication in the haplogroup A. Nucleotide diversity at *ACQOS*, especially in the LRR domain, is higher than the genome-wide average[9] and that of *NLR1-NLR3* in the *ACQOS* locus, and is associated with an excess of non-synonymous over synonymous substitutions between group 1 and group 2 *ACQOS* genes, suggesting diversifying selection (Fig. 2d; Supplementary Fig. 10). In one of three *ACQOS* high-diversity regions, polymorphisms were shared between *ACQOS* and $NLR^{group\ 5}$, suggesting that heterologous recombination because of to unequal crossing over or gene conversion between $NLR^{group\ 5}$ and *ACQOS* may have contributed to the high level of variation in the *ACQOS* gene (Supplementary Figs 11 and 12). Also, because *A. thaliana* group 3 accessions showed acquired osmotolerance, we reasoned that this trait is due to a non-functional *ACQOS* gene. The 3′ portion of group 3 *NLR3* is more closely related to that of group 1 *ACQOS* than to group 1 or 2 *NLR3*. It seems that deleting the majority of *ACQOS* 5′ region by gene fusion with *NLR3* suppressed *ACQOS* function in group 3 (Supplementary Fig. 13). Our data suggest that acquired osmotolerance was impaired when *ACQOS* originated, and was then restored in *A. thaliana* after repeated rearrangements, recombination and/or mutations at the *ACQOS* locus, giving rise to the haplotype groups 2, 3 and 5.

In pathogen-triggered TIR–NLR immunity and autoimmunity, EDS1/PAD4 nuclear complexes transcriptionally reprogram cells for pathogen resistance via salicylic acid (SA) and SA-independent pathways[10,11]. When exposed to osmotic stress, SA accumulation and the defence marker genes *PR1* and *EDS1* (SA dependent) and *PR2* (SA independent[12]) were strongly induced in Col-0 but not in NIL-Bu-5 plants (Fig. 3a,b). These results suggest that immune responses are de-repressed under osmotic stress in the presence of *ACQOS*. Given that SA antagonizes ABA signalling in *A. thaliana*[13], we tested for roles of *EDS1*, *PAD4* and SA in the impaired Col-0 acquired osmotolerance. Notably, Col-0 plants displayed acquired osmotolerance when *EDS1* or *PAD4* were mutated (Fig. 3c,d). Consistent with this, group 1 *ACQOS* failed to suppress acquired osmotolerance at 28 °C, at which TIR–NLR and EDS1/PAD4 immune responses are compromised in several *A. thaliana* accessions[14] (Supplementary Fig. 14). By contrast, acquired osmotolerance remained suppressed in mutants of *EDS5*, *SID2* or *NPR1*, encoding an SA transporter, an SA biosynthetic enzyme (Isochorismate Synthase 1) and a SA signalling regulator, respectively (Fig. 3c,d), pointing to SA independence of ACQOS suppression of osmotolerance. We further tested whether ACQOS relies on RAR1 and SGT1, which facilitate stable NLR accumulation and function[15]. Acquired osmotolerance was observed in *rar1* and *sgt1b* plants, albeit to a lesser extent in the latter compared with *rar1*, *eds1* and *pad4* plants, possibly because of retention of *SGT1a* (Fig. 3c,d). None of these four genes was associated with acquired osmotolerance in our GWAS (Fig. 1c). Our findings suggest that under osmotic stress, de-repression of TIR–NLR ACQOS-mediated defences via EDS1/PAD4 leads to a loss of acquired osmotolerance. Misactivated immunity often results in stunted growth and necrotic lesioning[14] and *NLR* genes have been reported to influence plant development, growth and cold tolerance in *A. thaliana*[16,17]. Under our conditions, plant growth was largely indistinguishable between Col-0, Bu-5, NIL-Bu-5 and *acqos* knockout plants when transferred to 4 °C after 100 mM NaCl treatment. These results suggest that ACQOS de-repression connects to auto-immunity specifically under osmotic stress conditions. Osmotic tolerance often depends on ABA, which increases with osmotic stress. Induced ABA accumulation and expression of the ABA-responsive genes *RAB18, RS6* and *NCED3* was higher in NIL-Bu-5 than Col-0 when plants were exposed to high osmotic stress, although their induction was not detectable during initial salt stress (Supplementary Fig. 15a,b). To assess the role of ABA in acquired osmotolerance, we introduced mutations into the NIL-Bu-5 background, *aba2-1* (ref. 18) (*aba2-*

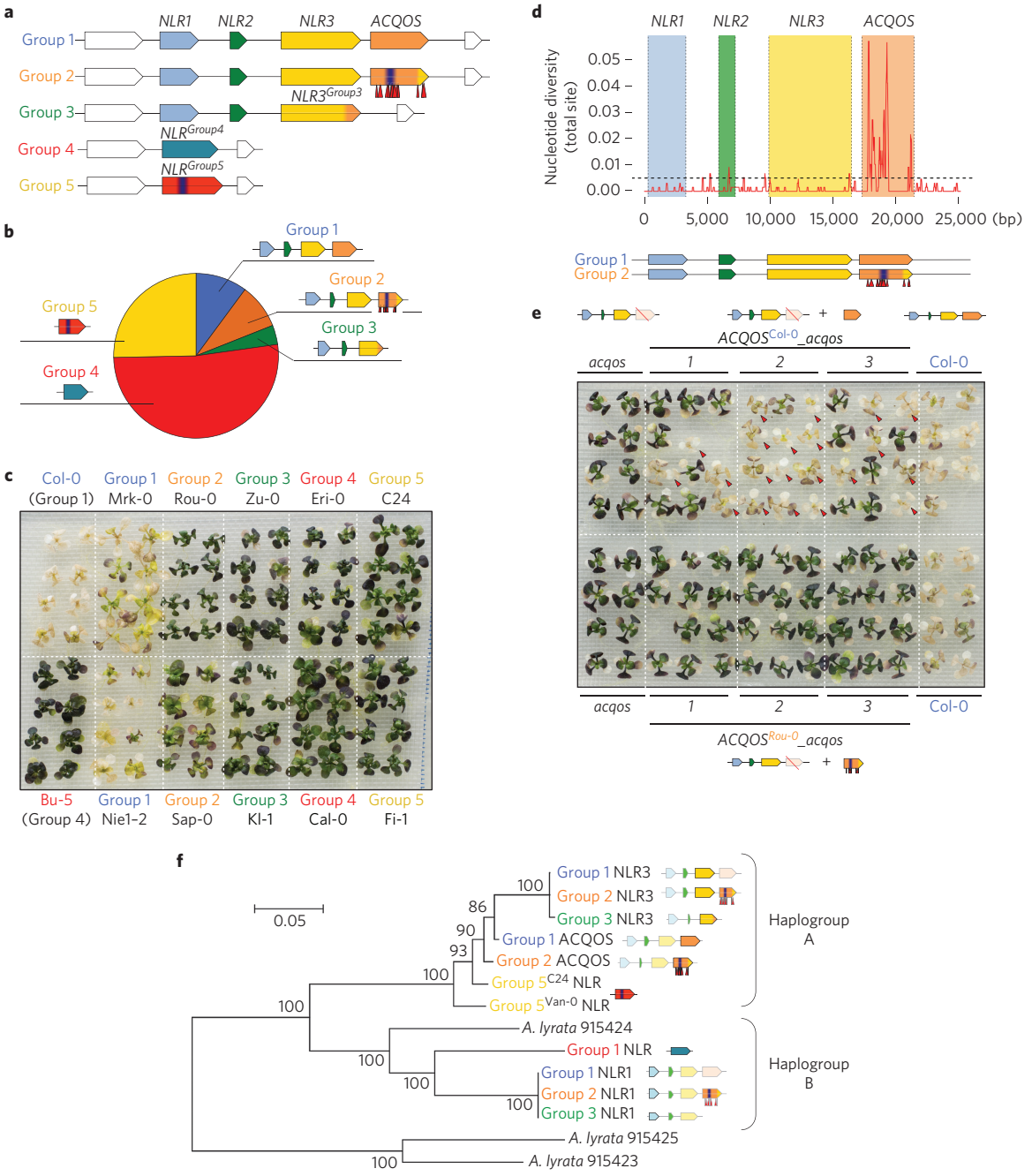

Figure 2 | Haplotype diversity and functional evolution of the *ACQOS* locus. a, Schematic representation of five haplogroups at the *ACQOS* locus, which differ by *NLR* tandem copy numbers and by nucleotide substitutions. Arrowheads below group 2 *ACQOS* show non-synonymous substitution compared with group 1 *ACQOS*. **b**, Relative frequencies of the five haplogroups among the 79 surveyed natural accessions. **c**, Acquired osmotolerance of the five haplogroups. Salt-acclimated seedlings were grown in the presence of 750 mM sorbitol for 21 days. **d**, Nucleotide diversity at all sites across the *ACQOS* locus (groups 1 and 2). A dotted horizontal line indicates the average genome-wide nucleotide diversity of *A. thaliana*[9]. **e**, Complementation test for acquired osmotolerance using group 1 *ACQOS* (top) and group 2 *ACQOS* (bottom). Salt-acclimated seedlings were grown in the presence of 750 mM sorbitol for 15 days. Arrowheads indicate T_2 seedlings with introduced group 1 *ACQOS*. **f**, Maximum-likelihood based phylogenetic tree of *NLR* genes in the *ACQOS* locus with three homologues from *Arabidopsis lyrata* as an outgroup. The values on the branches indicate the percentage of 1,000 bootstrap replicates. Similar results for **c** and **e** were obtained in at least three independent experiments; representative data are shown.

*1*_NIL-Bu-5) and *nced3-2* (ref. 19) (*nced3-2*_NIL-Bu-5), which are defective in ABA biosynthesis, or *abi1-1* (refs 20,21) (*abi1-1*_NIL-Bu-5), which is ABA-insensitive. Unexpectedly, acquired osmotolerance in NIL-Bu-5 was unaffected by these mutations (Supplementary Fig. 15c), indicating that the osmotolerance suppressed by *ACQOS* is independent of ABA.

The observed species-wide variation in acquired osmotolerance, in particular retention of the *ACQOS* allele that disables this trait, might be explained if *ACQOS* has fitness benefits under certain conditions. As a trade-off often occurs between biotic and abiotic stress adaptation[22], we tested whether group 1 *ACQOS* influences plant immunity. In *A. thaliana*, acquired osmotolerance and pathogen resistance are not necessarily correlated at the level of accessions[23], and are likely to reflect complex genetic interactions in the control and/or coordination of the two traits. We therefore compared Col-0 and NIL-Bu-5 plants to assess directly a role for group 1

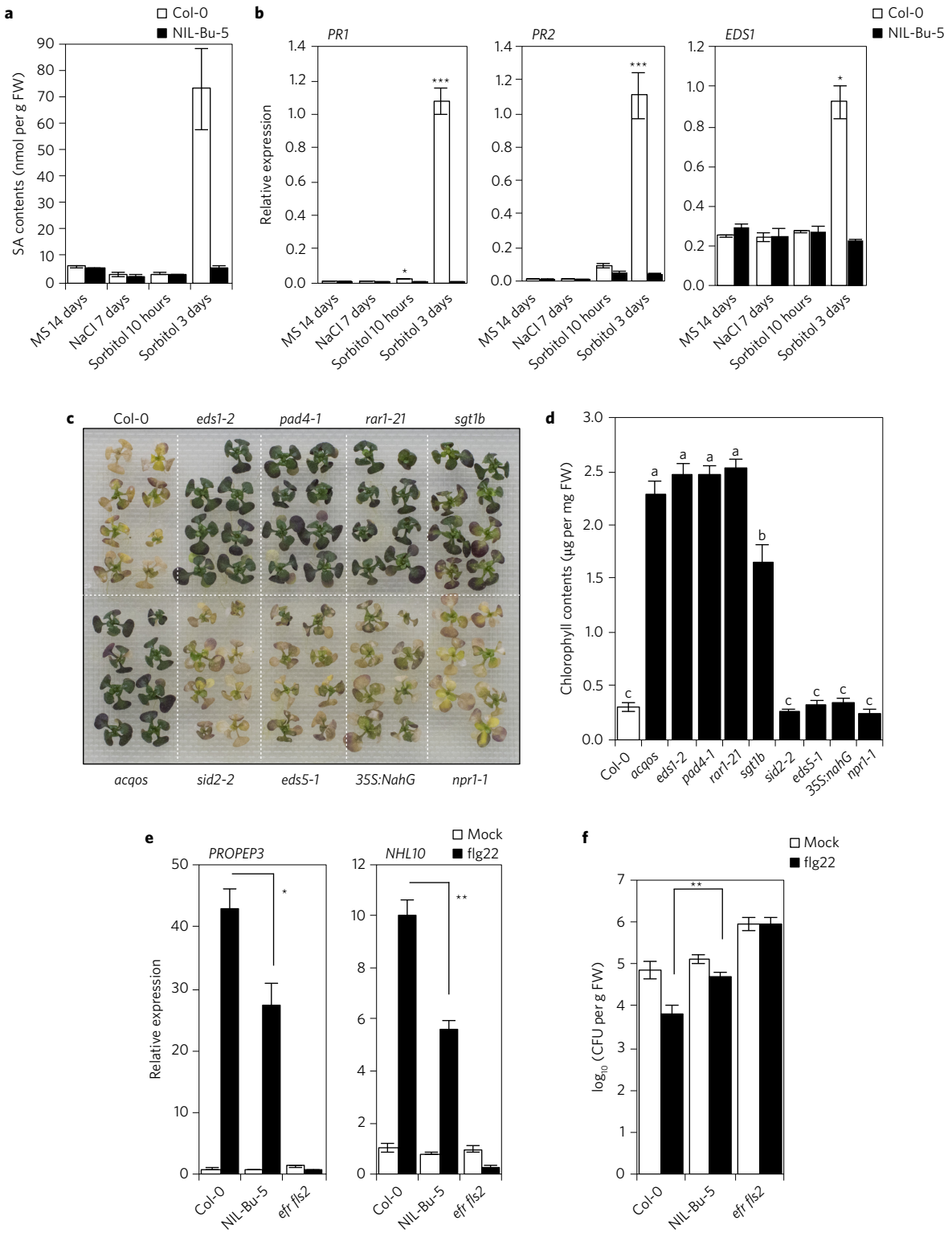

Figure 3 | Contribution of *ACQOS* to immune responses and pathogen resistance after MAMP treatment. a,**b**, Salicylic acid (SA) contents (**a**) and expression of *PR1*, *PR2* and *EDS1* (**b**) in Col-0 and NIL-Bu-5 plants under normal, salt stress and subsequent osmotic stress conditions (mean ± s.e.m., $n = 3$). Differences between Col-0 and NIL-Bu-5 were analysed using Student's *t*-test. $^{*}P < 0.05$; $^{***}P < 0.001$. **c**, Acquired osmotolerance of the immune signalling mutants *eds1-2*, *pad4-1* and *npr1-1* (ref. 29), R protein accumulation and hence function mutants *rar1-21* and *sgt1b* (ref. 15), an SA-depleted *35S:nahG* transgenic plant30 and the SA-deficient mutants *eds5-1* (ref. 31) (mutation in an SA transporter) and *sid2-2* (ref. 32) (mutation in isochorismate synthase). All the mutants were in the Col-0 background. Similar results were obtained in three independent experiments; representative data are shown. **d**, Chlorophyll content of immune deficient mutants as described in **c**. Within each line, bars with different letters are significantly different ($P < 0.01$, one-way ANOVA with post hoc Tukey HSD test, mean ± s.e.m., $n = 6$). **e**, Expression of *NHL10* and *PROPEP3* in Col-0, NIL-Bu-5 and *efr fls2* plants exposed to 1 µM flg22 for 8 h determined by qRT–PCR (mean ± s.e.m., $n = 3$). **f**, Growth of syringe-infiltrated *Pst* DC3000 in rosette leaves of 4-week-old Col-0, NIL-Bu-5 and *efr fls2* plants pretreated with water (mock) or 1 µM flg22 for 24 h (mean ± s.e.m., $n = 5$). **e**,**f**, Differences between pretreatment with Mock and flg22 were analysed using Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

ACQOS in defence responses. Recognition of bacterial flagellin (flg22 epitope), a pathogen-associated molecular pattern (PAMP), and subsequent defence activation is critical in bacterial resistance and largely conserved in higher plants[24], with a degree of species-wide variation in *A. thaliana*[25]. We tested flg22-triggered induction of the defence markers *PROPEP3* and *NHL10* in Col-0 and NIL-Bu-5 plants, and in *efr fls2* plants that lack the flg22 receptor FLAGELLIN-SENSITIVE 2 (FLS2) and are insensitive to flg22 (ref. 26). Induction of these two markers in response to flg22 was lower in NIL-Bu-5 plants than in Col-0 plants, suggesting that flg22-triggered defences are lowered in the absence of *ACQOS* (Fig. 3e). As accumulation of FLS2 and its coreceptor BAK1 (ref. 27) was intact in NIL-Bu-5 plants (Supplementary Fig. 16), this implies a role for ACQOS in defence signalling downstream of PAMP perception. To assess the biological significance of this finding, we tested whether loss of *ACQOS* influences bacterial resistance. NIL-Bu-5 and Col-0 plants were indistinguishable in basal resistance (without flg22 pretreatment) to virulent *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000) (Fig. 3f). Following flg22 pretreatment, however, NIL-Bu-5 plants exhibited lower suppression of bacterial growth than Col-0 plants, which strongly reduced bacterial growth, as described previously[28] (Fig. 3f). These data suggest that group 1 *ACQOS* is required for full activation of FLS2-mediated bacterial resistance, and that a contribution to this key branch of PAMP-triggered immunity might present an advantage for retaining functional *ACQOS*.

Polymorphism associated with rearrangements and mutations in the single *ACQOS* locus implies that acquired osmotolerance has evolved independently several times by *ACQOS* disruption, despite its potential for compromising immunity effectiveness. This might reflect a need to manage *ACQOS*-mediated autoimmunity, which becomes significant under severe osmotic stress and dominates in stress acclimation conferred by pre-exposure to mild salinity. Our findings suggest that the genetic variability of certain *NLR* genes in *A. thaliana* populations is not only shaped by coevolution between plants and pathogens but also the need to balance responsiveness to biotic and abiotic stresses in the environment.

Methods

Plant material and growth conditions. *Arabidopsis* seeds were sown on agar (0.8%, w/v) plates containing full-strength Murashige and Skoog (MS) salts with a vitamin mixture (10 mg l^{-1} myoinositol, 200 µg l^{-1} glycine, 50 µg l^{-1} nicotinic acid, 50 µg l^{-1} pyridoxine hydrochloride, 10 µg l^{-1} thiamine hydrochloride, pH 5.7) and 1% sucrose. Plates were sealed with surgical tape; the seeds were stratified at 4 °C for 4–7 days and then transferred to a growth chamber (80 µmol photons m^2 s^{-1}; 16 h/8 h light/dark cycle; 22 °C) for germination and growth.

Seeds of the following *Arabidopsis* mutants were obtained from the *Arabidopsis* Biological Resource Center (Ohio State University): *acqos* (SALK_122941, SALK_072727), *nlr2* (SALK_147652C), *nlr3* (SALK_145278, SALK_097845), *aba2-1* (CS156), *abi1-1* (CS22), *pad4-1* (CS3806), *sid2-2* (CS16438), *eds5-1* (CS3735) and *npr1-1* (CS3726). The *eds1-2* mutant[29] and *35S:NahG* transgenic line[30] were described previously. The *nced3-2* mutant[19] was kindly provided by Dr. Kaoru Urano. To generate *aba2-1*_NIL-Bu-5 and *nced3-2*_NIL-Bu-5, *aba2-1* and *nced3-2* mutants were crossed with NIL-Bu-5 (see below), respectively. To identify the homozygous of each mutation and *ACQOS* locus, the F_2 seedlings were genotyped by sequencing or simple sequence length polymorphysism (SSLP) markers (Supplementary Table 2). The F_3 progeny was used in this study. To generate *abi1-1*_Col-0 and *abi1-1*_NIL-Bu-5, *abi1-1* (Ler background) was backcrossed three times to Col-0 or NIL-Bu-5.

Stress treatment for acquired osmotolerance assay. Seven-day-old seedlings grown on nylon mesh on an MS agar plate were mesh-transferred to a plate supplemented with 100 mM NaCl for 7 days. The 14-day-old seedlings were then mesh transferred to a plate supplemented with 750 mM sorbitol for 14 days. Mild osmotic stress (for example, 150 mM sorbitol) is able to induce the acquired osmotolerance as well as the mild NaCl stress does (Supplementary Fig. 17).

High-resolution mapping of *ACQOS*. BC_5F_2 plants were generated by backcrossing F_2 plants (derived from a cross between Bu-5 and Col-0 and showing acquired salt tolerance) to Col-0 plants five times. We screened the BC_5F_2 plants for recombination events within the mapped 100 kb region containing *ACQOS*. We also developed two near-isogenic lines, named NIL-Col-0 and NIL-Bu-5, which carried a small chromosomal segment from Bu-5 containing the *ACQOS* region in the genetic background of Col-0. Genotyping was performed with SSLP markers and using single nucleotide polymorphism (SNP) detection by sequencing (Supplementary Table 1).

Genome-wide association study. A GWAS was performed to find loci associated with the absence or presence of acquired osmotolerance in 179 worldwide natural accessions (Supplementary Table 1). Of 350 accessions analysed in this study, the 250 k SNP dataset is available only for 173 accessions. We excluded some accessions whose phenotype is not penetrated (for example, a within line variation), and added some accessions obtained from ABRC. As for the GWAS, the osmotolerance phenotype was scored in a binary (absent or present) way because this 'all or nothing' difference of the phenotype was so clear. We used the 250 k SNP data as a genotype set[31]. To deal with the confounding effect of population structure, we employed a mixed model incorporating a genome-wide kinship matrix as a random effect[32]. We used the GWAPP platform[33] to perform GWAS and to generate the Manhattan and linkage disequilibrium plots.

Generation of a BAC library from the Bu-5 genome and sequencing of the *ACQOS* locus. A BAC library derived from the Bu-5 genome was generated by Amplicon Express (USA). BAC clones were extracted with a NucleoBond BAC 100 kit (Macherey-Nagel) and sequenced. The *ACQOS* loci of 79 accessions (Supplementary Table 3) were amplified using a haplogroup-specific primer set (Supplementary Table 4), the PCR fragments were cloned into pCR-TOPO (Invitrogen) and sequenced.

Plasmid construction and transformation. For complementation analysis, the genomic region of each *NLR* (2.0 kb upstream of the ATG initiation codon and 1.0 kb downstream region as a terminator in the *ACQOS* locus of Col-0) were amplified by PCR with AscI linker primers and cloned into the AscI sites introduced into the binary vectors pGreen0029 and pGreen0129. The *ACQOS promoter:* β-glucuronidase (*GUS*) plasmid was constructed by amplifying a 2.0 kb DNA fragment upstream of the *ACQOS* initiation codon by PCR and cloning it into the BamHI site of pBI101.

All constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 carrying pSoup, a helper plasmid necessary for pGreen replication[34]. Agrobacteria were then used for plant transformation by the floral dip method. Primers for cloning are listed in Supplementary Table 5. Transgenic plants were selected on MS agar plates containing 200 µg ml^{-1} claforan and 25 µg ml^{-1} kanamycin or 20 µg ml^{-1} hygromycin. Ten-day-old seedlings (T_1 plants) were transferred to the soil pots.

Quantitative RT–PCR. Total RNA (2 µg) was isolated with an RNeasy Plant Mini Kit (QIAGEN), treated with DNase I (Invitrogen) and used as a template to synthesize first-strand cDNA using SuperScript II Reverse Transcriptase (Invitrogen) and an oligo dT primer. qRT–PCR was performed using a LightCycler 96 (Roche Diagnostics) with FastStart Essential DNA Green Master (Roche Diagnostics) in a total volume of 12 µl under the following conditions: 95 °C for 10 min followed by 45–50 cycles of 95 °C for 20 s, 54 °C for 20 s and 72 °C for 20 s. β-*Actin* was used as an internal standard. Primers and their efficiencies are listed in Supplementary Table 6.

GUS staining and quantification

ACQOS *promoter*. *GUS* transgenic seedlings were salt-acclimated under 100 mM NaCl for 7 days and subsequently subjected to 750 mM sorbitol for 7 days. Seedlings were then washed twice with phosphate buffer and incubated in GUS buffer (10 mM phosphate buffer (pH 7), 0.5% Triton X-100, 1 mg ml^{-1} X-Gluc, 2 mM potassium ferricyanide) for 3–5 h at 37 °C. Chlorophyll was subsequently removed by incubation in 100% ethanol. Quantification of GUS activity was performed according to 4-4-methlyumbelliferyl β-D-glucronide (MUG) fluorometric assay[35]. Transgenic seedlings with or without osmotic stress were homogenized with GUS extraction buffer (100 mM sodium phosphate, 10 mM EDTA, 10 mM DTT, 0.1% Triton X-100, 20% methanol and 1 mM 4-MUG) and incubated at 37 °C for 60 min. After incubation, 100 µl of each samples were mixed with 4 ml of 200 mM Na_2CO_3 and 4-4-methylumbelliferone (MU) fluorescence was measured with excitation at 365 nm, emission at 455 nm on a spectrofluorimeter. Fluorescence intensity was calculated using 4-MU standards (0.001~1 mM). Then GUS activity was normalized with protein concentration quantified with Bradford (Bio-Rad).

Population genetic analysis. DnaSP v.5 was used to calculate nucleotide diversity and πa/πs (ratio of nucleotide diversity πa/πs between nonsynonymous (a) and synonymous (s) sites)[36]. In the sliding window analysis, window length was 100 bp and step size was 25 bp. We generated phylogenetic trees using the maximum-likelihood method implemented in the MEGA5 software[37]. Alignments of five *A. thaliana* ACQOS haplogroupes (Group 1-5) and *A. lyrata* ACQOS locus using Progressive MAUVE[38].

Analysis of plant hormone contents. About 100 mg (fresh weight) of tissues were subjected to hormone quantification. The hormone extraction and fractionation

were performed using the method described previously[39]. Hormones were measured with an UPLC-ESI-qMS/MS (AQUITY UPLC System/Xevo-TQS; Waters) with an ODS column (AQUITY UPLC BEH C_{18}, 1.7 μm, 2.1 × 100 mm^2, Waters)[39].

Bacterial inoculation assays. Bacterial inoculation assays were performed as described previously[40] with the following modifications. Following 1 μM flg22 or water (mock) pretreatment for 24 h, *Pst* DC3000 suspension at 1×10^5 cfu ml^{-1} was syringe-infiltrated into three leaves of five plants per genotype per treatment. Three days after inoculation, these leaves were collected and then their fresh weight was determined before the quantification of leaf bacteria using leaf fresh weight (g) for normalization. These experiments (five replicates each) have been repeated three times with the same conclusions.

Immunoblot analysis. Ten-day-old seedlings were subjected to immunoblot analysis with the indicated antibodies, essentially as described previously[41]. Equal loading of protein lysates was verified by Ponceau S staining of the protein blots.

Data availability. DNA sequences that support the findings of this work have been deposited to DNA Data Bank of Japan (DDBJ) with the following accession numbers: ACQOS_Col-0 (LC214887), ACQOS_Rou-0 (LC214888), ACQOS_ Zu-0 (LC214889), ACQOS_ Kl-1 (LC214890), ACQOS_ Van-0 (LC214891), ACQOS_ Bu-5 (LC214892), ACQOS_ C24 (LC214893) and ACQOS_ Bs-1 (LC214894). The data are available from the National Center for Biotechnology Information (NCBI).

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References

1. Sung, D. Y., Kaplan, F., Lee, K. J. & Guy, C. L. Acquired tolerance to temperature extremes. *Trends Plant Sci.* **8,** 179–187 (2003).
2. Katori, T. *et al.* Dissecting the genetic control of natural variation in salt tolerance of *Arabidopsis thaliana* accessions. *J. Exp. Bot.* **61,** 1125–1138 (2010).
3. Kim, T. H. *et al.* Natural variation in small molecule-induced TIR-NB-LRR signaling induces root growth arrest via EDS1- and PAD4-complexed R protein VICTR in *Arabidopsis*. *Plant Cell* **24,** 5177–5192 (2012).
4. Weigel, D. Natural variation in *Arabidopsis*: from molecular genetics to ecological genomics. *Plant Physiol.* **158,** 2–22 (2012).
5. Mitchell-Olds, T. & Schmitt, J. Genetic mechanisms and evolutionary significance of natural variation in *Arabidopsis*. *Nature* **441,** 947–952 (2006).
6. Kim, T. H. *et al.* Chemical genetics reveals negative regulation of abscisic acid signaling by a plant immune response pathway. *Curr. Biol.* **21,** 990–997 (2011).
7. Maekawa, T., Kufer, T. A. & Schulze-Lefert, P. NLR functions in plant and animal immune systems: so far and yet so close. *Nat. Immunol.* **12,** 817–826 (2011).
8. Shao, Z. Q. *et al.* Large-scale analyses of angiosperm nucleotide-binding site-leucine-rich repeat genes reveal three anciently diverged classes with distinct evolutionary patterns. *Plant Physiol.* **170,** 2095–2109 (2016).
9. Nordborg, M. *et al.* The pattern of polymorphism in *Arabidopsis thaliana*. *PLoS Biol.* **3,** e196 (2005).
10. Shirano, Y., Kachroo, P., Shah, J. & Klessig, D. F. A gain-of-function mutation in an *Arabidopsis* Toll Interleukin1 receptor-nucleotide binding site-leucine-rich repeat type R gene triggers defense responses and results in enhanced disease resistance. *Plant Cell* **14,** 3149–3162 (2002).
11. Cui, H. *et al.* A core function of EDS1 with PAD4 is to protect the salicylic acid defense sector in *Arabidopsis* immunity. *New Phytol.* **213,** 1802–1817 (2017).
12. Zhang, Y., Goritschnig, S., Dong, X. & Li, X. A gain-of-function mutation in a plant disease resistance gene leads to constitutive activation of downstream signal transduction pathways in *suppressor of npr1-1, constitutive 1*. *Plant Cell* **15,** 2636–2646 (2003).
13. Yasuda, M. *et al.* Antagonistic interaction between systemic acquired resistance and the abscisic acid-mediated abiotic stress response in *Arabidopsis*. *Plant Cell* **20,** 1678–1692 (2008).
14. Alcázar, R. & Parker, J. E. The impact of temperature on balancing immune responsiveness and growth in *Arabidopsis*. *Trends Plant Sci.* **16,** 666–675 (2011).
15. Shirasu, K. The HSP90-SGT1 chaperone complex for NLR immune sensors. *Annu. Rev. Plant Biol.* **60,** 139–164 (2009).
16. Huang, X., Li, J., Bao, F., Zhang, X. & Yang, S. A gain-of-function mutation in the *Arabidopsis* disease resistance gene *RPP4* confers sensitivity to low temperature. *Plant Physiol.* **154,** 796–809 (2010).
17. Yang, H. *et al.* A mutant CHS3 protein with TIR-NB-LRR-LIM domains modulates growth, cell death and freezing tolerance in a temperature-dependent manner in *Arabidopsis*. *Plant J.* **63,** 283–296 (2010).
18. Léon-Kloosterziel, K. M. *et al.* Isolation and characterization of abscisic acid-deficient *Arabidopsis* mutants at two new loci. *Plant J.* **10,** 655–661 (1996).
19. Urano, K. *et al.* Characterization of the ABA-regulated global responses to dehydration in *Arabidopsis* by metabolomics. *Plant J.* **57,** 1065–1078 (2009).
20. Leung, J. *et al.* *Arabidopsis* ABA response gene ABI1: features of a calcium-modulated protein phosphatase. *Science* **264,** 1448–1452 (1994).
21. Meyer, K., Leube, M. P. & Grill, E. A protein phosphatase 2C involved in ABA signal transduction in *Arabidopsis thaliana*. *Science* **264,** 1452–1455 (1994).
22. Bostock, R. M., Pye, M. F. & Roubtsova, T. V. Predisposition in plant disease: exploiting the nexus in abiotic and biotic stress perception and response. *Annu. Rev. Phytopathol.* **52,** 517–549 (2014).
23. Ahmad, S. *et al.* Genetic dissection of basal defence responsiveness in accessions of *Arabidopsis thaliana*. *Plant Cell Environ.* **34,** 1191–1206 (2011).
24. Boller, T. & Felix, G. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* **60,** 379–406 (2009).
25. Vetter, M. M. *et al.* Flagellin perception varies quantitatively in *Arabidopsis thaliana* and its relatives. *Mol. Biol. Evol.* **29,** 1655–1667 (2012).
26. Jeworutzki, E. *et al.* Early signaling through the *Arabidopsis* pattern recognition receptors FLS2 and EFR involves Ca-associated opening of plasma membrane anion channels. *Plant J.* **62,** 367–378 (2010).
27. Sun, Y. *et al.* Structural basis for flg22-induced activation of the *Arabidopsis* FLS2-BAK1 immune complex. *Science* **342,** 624–628 (2013).
28. Zipfel, C. *et al.* Bacterial disease resistance in *Arabidopsis* through flagellin perception. *Nature* **428,** 764–767 (2004).
29. Bartsch, M. *et al.* Salicylic acid-independent *ENHANCED DISEASE SUSCEPTIBILITY1* signaling in Arabidopsis immunity and cell death is regulated by the monooxygenase *FMO1* and the nudix hydrolase *NUDT7*. *Plant Cell* **18,** 1038–1051 (2006).
30. Reuber, T. L. *et al.* Correlation of defense gene induction defects with powdery mildew susceptibility in *Arabidopsis* enhanced disease susceptibility mutants. *Plant J.* **16,** 473–485 (1988).
31. Horton, M. W. *et al.* Genome-wide patterns of genetic variation in worldwide *Arabidopsis thaliana* accessions from the RegMap panel. *Nat. Genet.* **44,** 212–216 (2012).
32. Atwell, S. *et al.* Genome-wide association study of 107 phenotypes in *Arabidopsis thaliana* inbred lines. *Nature* **465,** 627–631 (2010).
33. Seren, U. *et al.* GWAPP: A Web application for genome-wide association mapping in *Arabidopsis*. *Plant Cell* **24,** 4793–4805 (2012).
34. Hellens, R. P., Edwards, E. A., Leyland, N. R., Bean, S. & Mullineaux, P. M. Pgreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Mol. Biol.* **42,** 819–832 (2000).
35. Jefferson, R. A., Kavanagh, T. A. & Bevan, M. W. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6,** 3901–3907 (1987).
36. Librado, P. & Rozas, J. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25,** 1451–1452 (2009).
37. Tamura, K. *et al.* MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28,** 2731–2739 (2011).
38. Darling, A. E., Mau, B. & Perna, N. T. Progressivemauve: multiple genome alignment with gene gain, loss and rearrangement. *PLoS ONE* **5,** e11147 (2010).
39. Kojima, M. & Sakakibara, H. Highly sensitive high-throughput profiling of six phytohormones using MS-probe modification and liquid chromatography-tandem mass spectrometry. *Methods Mol. Biol.* **918,** 151–164 (2012).
40. Lu, X. *et al.* Uncoupling of sustained MAMP receptor signaling from early outputs in an *Arabidopsis* endoplasmic reticulum glucosidase II allele. *Proc. Natl Acad. Sci. USA* **106,** 22522–7 (2009).
41. Yamada, K., Saijo, Y., Nakagami, H. & Takano, Y. Regulation of sugar transporter activity for antibacterial defense in *Arabidopsis*. *Science* **354,** 1427–1430 (2016).
42. Cao, H., Bowling, S. A., Gordon, A. S. & Dong, X. Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell* **6,** 1583–1592 (1994).
43. Wildermuth, M. C., Dewdney, J., Wu, G. & Ausubel, F. M. Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* **414,** 562–565 (2001).
44. Rogers, E. E. & Ausubel, F. M. *Arabidopsis* enhanced disease susceptibility mutants exhibit enhanced susceptibility to several bacterial pathogens and alterations in PR-1 gene expression. *Plant Cell* **9,** 305–316 (1997).

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Author contributions

H.A. and T. Taji initiated, conceived and coordinated the project; H.A., identified *ACQOS* locus and characterized plants altered with the *ACQOS* locus; T.K., generated NIL plants; T. Tsuchimatsu performed population genetic analyses; T. Tsuchimatsu, O.H., A.E.L.,

Y. Kobayashi and M.A.G. performed GWAS; T. Hirase, Y.T. and Y. Saijo designed and performed defence-related assays; H.S. and M.K. determined SA and ABA contents; S.I. and M.K. provided *A. thaliana* accession seeds and their markers; J.E.P., R.A., M.K., K.S., T. Hayashi, Y. Sakata and Y. Saijo supervised the project; T. Taji and Y. Saijo wrote the manuscript with assistance from T. Tsuchimatsu, J.E.P., R.A., M.K., K.S. and Y. Sakata.

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Competing interests

The authors declare no competing financial interests.