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. We found previously that acquired osmotolerance after salt stress is widespread among Arabidopsis thaliana accessions. 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. 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. 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. Plants have evolved the ability to acclimatize to various stresses after initial exposure to a related stress cue. A large-scale analysis of 350 A. thaliana accessions revealed extensive variation in acquired osmotolerance upon mild salt exposure. When 7-day-old seedlings were pre-exposed to 100 mM NaCl for 7 d (acclimation period), the A. thaliana accessions Bu-5 and Bur-0, but not Col-0 or Wl-0, acquired osmotolerance to 750 mM sorbitol (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 BC3F3
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 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 \( \text{NLR}^{\text{Bu-5}} \) 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, \( \text{NLR}^{\text{Bu-5}} \) did not confer acquired osmotolerance in the Col-0
background (Supplementary Fig. 4). By contrast, Col-0 \( \text{NLR}^4 \), but not \( \text{NLR}^3 \),
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 [5-(3,4-dichlorophenyl)furan-2-yl]-piperidine-1-ylmethanethione (DFPM) in Col-0\textsuperscript{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)\textsuperscript{3,6}. In plants and animals, NLR proteins are typically immune sensors for pathogen molecules or pathogen-induced modifications of host cell components\textsuperscript{7}. There are 104 annotated TIR-NLRs in the genome of A. thaliana Col-0. The closest homolog 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 Figs. 7). In
addition, F₁ 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 homologs, including those from Arabidopsis lyrata as an outgroup (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 homolog (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 NLRGroup 4, whereas NLR3 of Groups 1–3 and ACQOS belong to the same clade as NLRGroup 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 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 NLRGroup 5, suggesting that heterologous recombination due to unequal crossing over or gene conversion between NLRGroup 5 and ACQOS may have contributed to the high level of variation in the ACQOS gene (Supplementary Figs. 11, 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 PRI 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 due to the 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\(^\text{14}\) and NLR genes have been reported to influence plant development, growth and cold tolerance in \textit{Arabidopsis thaliana}\(^\text{16,17}\). Under our conditions, plant growth was largely indistinguishable between Col-0, Bu-5, NIL-Bu-5 and \textit{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 \textit{RAB18, RS6} and \textit{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: \textit{aba2-1}\(^\text{18}\) (\textit{aba2-1}_\text{NIL-Bu-5}) and \textit{nced3-2}\(^\text{19}\) (\textit{nced3-2}_\text{NIL-Bu-5}) which are defective in ABA biosynthesis, or \textit{abi1-1}\(^\text{20,21}\) (\textit{abi1-1}_\text{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 \textit{ACQOS} is independent of ABA.

The observed species-wide variation in acquired osmotolerance, in particular retention of the \textit{ACQOS} allele that disables this trait, might be explained if \textit{ACQOS} has fitness benefits under certain conditions. As a trade-off often occurs between biotic and abiotic stress adaptation\(^\text{22}\), we tested whether Group 1 \textit{ACQOS} influences plant immunity. In \textit{A. thaliana}, acquired osmotolerance and pathogen resistance are not
necessarily correlated at the level of accessions\textsuperscript{23}, likely reflecting 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 \textit{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\textsuperscript{24}, with a degree of species-wide variation in \textit{A. thaliana}\textsuperscript{25}. We tested flg22-triggered induction of the defence markers \textit{PROPEP3} and \textit{NHL10} in Col-0 and NIL-Bu-5 plants, and in \textit{efr fls2} plants that lack the flg22 receptor FLS2 and are insensitive to flg22\textsuperscript{26}. Induction of these two markers in response to flg22 was lower in NIL-Bu-5 plants compared to Col-0 plants, suggesting that flg22-triggered defences are lowered in the absence of \textit{ACQOS} (Fig. 3e). As accumulation of FLS2 and its coreceptor BAK1\textsuperscript{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 \textit{ACQOS} influences bacterial resistance. NIL-Bu-5 and Col-0 plants were indistinguishable in basal resistance (without flg22 pretreatment) to virulent \textit{Pseudomonas syringae pv. tomato} strain DC3000 (\textit{Pst DC3000}) (Fig. 3f). Following flg22 pretreatment, however, NIL-Bu-5 plants exhibited lower suppression of bacterial growth compared to Col-0 plants which strongly reduced bacterial growth, as described previously\textsuperscript{28} (Fig. 3f). These data suggest that Group 1 \textit{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 \textit{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.
Figure 1

**Identification of the ACQOS locus.**

a, Acquired osmotolerance of *A. thaliana* accessions. Upper panel: A flow chart of the acquired osmotolerance assay. Middle panel: Salt tolerance when grown on soil. Three-week-old plants grown in pots were exposed to 500 mM NaCl in water for 49 d. Lower panel: Acquired osmotolerance. Salt-acclimated 2-week-old seedlings were mesh-transferred to MS agar plates containing 750 mM sorbitol for 21 d.

b, High-resolution mapping of the ACQOS locus using NILs. Upper panel: Acquired osmotolerance of Col-0, Bu-5, NIL-Col-0, and NIL-Bu-5. Lower panel: Graphical genotypes of NILs. Chromosomal segments of Col-0, off-white; Bu-5, green. Numbers above the genes are the last 3 digits of their Arabidopsis Genome Initiative (AGI) numbers (*At5g46XXX*).

c, Genome-wide association study for acquired osmotolerance. Upper panel: Manhattan plot of GWAS results for acquired osmotolerance. Middle panel: Close-up of the major GWAS peak in the vicinity of the ACQOS locus on chromosome 5. The position of the ACQOS gene is indicated by a red line. Lower panel: Linkage disequilibrium patterns within ± 500 kb upstream and downstream of the ACQOS locus.

d, Complementation test performed by transforming NIL-Bu-5 with *NLR4 (ACQOS)*. T3 homozygous plants transformed with native promoter: *NLR4 (ACQOS)* derived from Col-0 were used.

e, Acquired osmotolerance of *nlr2*, *nlr3*-*1*, and *nlr4*-*1 (acqos-1)* mutants.
Expression of ACQOS in Col-0 plants under normal, salt acclimated, and subsequent osmotic stress conditions; gene expression was determined by qRT-PCR (mean ± se, n = 3).

Histochemical analysis of the expression pattern of 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 analyzed by Student’s t-test. (mean ± se, 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) d. Similar results were obtained in three independent experiments; representative data are shown.

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 nonsynonymous substitution compared to 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 d.

d, Nucleotide diversity at all sites across the ACQOS locus (Groups 1 and 2). A dotted horizontal line indicates average genome-wide nucleotide diversity of A. thaliana."
Complementation test for acquired osmotolerance using Group 1 ACQOS (upper part) and Group 2 ACQOS (lower part). Salt-acclimated seedlings were grown in the presence of 750 mM sorbitol for 15 d. Arrowheads indicate T$_2$ seedlings with introduced Group 1 ACQOS.

**Figure 3**

**Contribution of ACQOS to immune responses and pathogen resistance after MAMP treatment.**

**a,** Salicylic acid (SA) contents in Col-0 and NIL-Bu-5 plants under normal, salt stress, and subsequent osmotic stress conditions (mean ± se, n = 3).

**b,** Expression of PR1, PR2, and EDS1 in Col-0 and NIL-Bu-5 plants under normal, salt stress, and subsequent osmotic stress conditions determined by qRT-PCR (mean ± se, n = 3). Differences between Col-0 and NIL-Bu-5 were analyzed by Student’s t-test. *P <0.05; ***P <0.001.

**c,** Acquired osmotolerance of the immune signaling mutants eds1-2, pad4-1, and npr1-1$^{20}$, R protein accumulation and hence function mutants rar1-21 and sgt1b$^{15}$, an SA-depleted 35S:nahG transgenic plant$^{30}$, and the SA-deficient mutants eds5-1$^{21}$ (mutation in an SA transporter) and sid2-2$^{32}$ (mutation in isochorismate synthase). All the mutants were in the Col-0 background.
Similar results were obtained in three times independent experiments; representative data are shown.

**d**, Chlorophyll content of immune deficient mutants as described in **e**.

Within each lines, bars with different letters are significantly different (P < 0.01, one-way ANOVA with post-hoc Tukey HSD test, mean ± se, n=6).

**e**, Expression of *NHL10* and *PROPEP3* in Col-0, NIL-Bu-5 and *efr fls2* plants exposed to 1 µM flg22 for 8h determined by qRT-PCR (mean ± se, 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 ± se, n = 5). **e** and **f**, Differences between pretreatment with Mock and flg22 were analyzed by Student’s t-test. *P <0.05; **P <0.01; ***P <0.001.
Supplementary Figure 1

Fine mapping of acquired osmotolerance.

Fine mapping was performed using 1993 osmo-sensitive F$_2$ (Col-0 × Bu-5) plants. The \textit{ACQOS} locus exhibited strong linkage within approximately 100 kb between At5g_126 and At5g_136. The scores indicate recombination frequencies (%). The number under the markers shows the number of recombinants. \textit{NLR} genes in the \textit{ACQOS} locus are shown as colored arrows. Other genes are shown as black arrows.

Supplementary Figure 2

Chlorophyll contents for the acquired osmotolerant assays.

a. Chlorophyll contents of Col-0, NIL-Col-0, NIL-Bu-5 and Bu-5 as described in Fig. 1b.

b. Chlorophyll contents of accessions as described in Fig. 2c.

c. Chlorophyll contents of \textit{acqos} complementation lines as described in Fig. 2e.

Within each lines, bars with different letters are significantly different (P < 0.05, one-way ANOVA with post-hoc Tukey HSD test, mean ± se, n=5-6).

Supplementary Figure 3

Graphical genotype of a BAC clone derived from the Bu-5 genome.

The BAC clone contained the entire 100-kb region (red line) narrowed down by fine mapping (see Supplementary Figure 1). \textit{NLR} genes in the \textit{ACQOS} locus are shown as colored arrows. Other genes are shown as gray arrows.

Supplementary Figure 4
Phenotypes of T2 plants transformed with native promoter: NLR\textsuperscript{Bu-5} in Col-0 or native promoter: NLR\textsuperscript{Col-0} in NIL-Bu-5.

\textbf{a,} Salt-acclimated 2-week-old seedlings were grown in the presence of 750 mM sorbitol for 12 days. None of the transgenic plants showed osmotolerance, indicating that NLR\textsuperscript{Bu-5} did not confer acquired osmotolerance. Experiments were repeated three times.

\textbf{b,} Chlorophyll contents of Col-0, NIL-Bu-5 and transgenic lines as described in \textbf{a} (mean ± se, n=6).

\textbf{c.} Expression levels of NLR3 in NLR3_NIL-Bu-5 lines and NIR\textsuperscript{Bu-5} in NIR\textsuperscript{Bu-5}_Col-0 lines (mean ± se, n=3).

\textbf{Supplementary Figure 5}

\textbf{Acquired osmotolerance of NLR T-DNA insertion mutants.}

\textbf{a,} Schematic representation of the ACQOS locus and the sites of T-DNA insertions.

\textbf{b,} Salt-acclimated 2-week-old seedlings were grown in the presence of 750 mM sorbitol for 17 days. Only acqos knockout mutants showed the acquired osmotolerance. Experiments were repeated three times.

\textbf{c,} Chlorophyll content of each plants as described in \textbf{b.}

Within each lines, bars with different letters are significantly different (P < 0.01, one-way ANOVA with post-hoc Tukey HSD test, mean ± se, n=6).

\textbf{d,} Expression of the neighboring NLRs in the acqos mutatns. Expression levels were normalized to that of β-actin (mean ± se, n = 3).

\textbf{Supplementary Figure 6}
Phylogenetic tree of 104 Arabidopsis (Col-0) TIR-NLRs.

Phylogenetic tree was drawn using amino acid sequence of 104 TIR-NB-LRRs from Col-0. Red frame shows a magnified branch with tandem NLR genes (graphical genotypes) in the ACQOS locus.

Supplementary Figure 7

Acquired osmotolerance of plants overexpressing osmotic stress–inducible ACQOS<sup>Col-0</sup>.

a and b, Relative ACQOS expression in T<sub>3</sub> plants under (a) normal growth conditions and (b) osmotic stress for 3 days. Expression levels were normalized to that of β-actin (mean ± se n = 3). Differences between Col-0 and the T<sub>3</sub> lines were analyzed by Student’s t-test. **P<0.01, ***P<0.001.

c, Phenotypes of T<sub>3</sub> homozygous plants with introduced ACQOS<sup>Col-0</sup>. Salt-acclimated 2-week-old seedlings were grown in the presence of 750 mM sorbitol for 7 (upper panel) or 14 days (lower panel). Experiments were repeated three times.

d, Chlorophyll contents of ACQOS<sup>Col-0</sup> introduced lines as described in c. Within each lines, bars with different letters are significantly different (P < 0.01, one-way ANOVA with post-hoc Tukey HSD test, mean ± se, n=6).

Supplementary Figure 8

Phenotypes of F<sub>1</sub> progeny derived from a cross between Col-0 and NIL-Bu-5.

a, Osmotolerance of F<sub>1</sub> seedlings was intermediate between those of Col-0 and NIL-Bu-5, indicating that ACQOS reduces acquired osmotolerance. Experiments were repeated three times.
b, Chlorophyll contents of the F1 plants after 14 days of osmotic stress (mean ± se, n = 4). Differences were analyzed by Student’s t-test. **P < 0.01, ***P < 0.001.

**Supplementary Figure 9**

Alignments of five A. thaliana ACQOS haplogroupes (Group 1-5) and A. lyrata ACQOS locus using Progressive MAUVE.

a. Alignments of five A. thaliana ACQOS haplogroupes (Group 1-5)

Regions of significant synteny between the species or groups are shown as colored blocks in the mauve alignment. Regions of sequence not shared between genotypes are seen as white gaps within the blocks or spaces between the blocks. Black bars and colored arrows show genes. Red bars show TIR-NB-LRRs used in Fig. 2f.

b. Alignments of the region around ACQOS locus between A. thaliana (Col-0) and A. lyrata.

**Supplementary Figure 10**

Polymorphism and divergence levels between Group 1 and 2 ACQOS genes.

Sliding window analysis of \( \pi_a/\pi_s \) in the coding region of Group 1 and 2 ACQOS genes.

TIR, Toll/interleukin 1 receptor domain; NB, nucleotide-binding domain; Leucine-rich repeat domain.

**Supplementary Figure 11**

Phylogenetic trees of Group 1 ACQOS, Group 2 ACQOS, and Group 5 NLR.

To reveal phylogenetic relationships between the ACQOS genes of Groups 1 (blue) and 2 (orange) and the NLR genes of Group 5 (yellow), three maximum-likelihood
phylogenetic trees were drawn using different regions of the genes (shaded in gray in
the upper panel). The graph showing nucleotide diversity of Group 1 and 2 ACQOS
genes is identical to Fig 2d. The values on the branches indicate the percentage of 1,000
bootstrap replicates.

Supplementary Figure 12
Polymorphisms between the Group 5 NLR gene and Group 1 and 2 ACQOS genes.
The region of Group 5 NLR corresponding to the high-diversity region of ACQOS
harbors two clearly distinct haplotypes, and Group 1 and 2 ACQOS are even closer to
each haplotype of Group 5 NLR. See also Supplementary Figure 11 Arrowheads
indicate polymorphisms between the Group 5 NLR gene in some Group 5 accessions
and Group 2 ACQOS gene.

Supplementary Figure 13
Group 3 NLR3 is derived from Group 1 NLR3 and ACQOS via gene deletion.
To reveal phylogenetic relationships between NLR3 and ACQOS genes from Groups 1
(blue), 2 (orange), and 3 (green), maximum-likelihood phylogenetic trees were drawn
using the indicated 5’ and 3’ regions of the genes. The values on the branches indicate
the percentage of 1,000 bootstrap replicates.

Supplementary Figure 14
Effect of temperature on acquired osmotolerance in Col-0 and NIL-Bu-5.
Salt-acclimated seedlings grown under normal conditions (22 ºC) were transferred to
plates containing 750 mM sorbitol and grown at 22 ºC (control) or 28 ºC for 8 days.
Experiments were repeated three times.

Supplementary Figure 15

**ABA is dispensable for acquired osmotolerance in absence of ACQOS.**

a, ABA contents in Col-0 and NIL-Bu-5 (mean ± se, n = 3).

b, Expression profiles of the ABA responsible genes *RAB18* and *Raffinose synthase 6* (*RS6*), and the ABA synthesis gene *NCED3* in Col-0 and NIL-Bu-5 (mean ± se, n = 3).

c, Acquired osmotolerance of Col-0 and NIL-Bu-5 carrying mutations in the ABA signaling component gene *ABI1* (*abi1-1*_Col-0 and *abi1-1*_NIL-Bu-5) or the ABA biosynthesis genes *ABA2* (*aba2-1*_Col-0 and *aba2-1*_NIL-Bu-5), and *NCED3* (*ncde3-2*_Col-0 and *ncde3-2*_NIL-Bu-5). After salt acclimation, seedlings were grown in the presence of 750 mM sorbitol for 21 days. Experiments were repeated three times.

Supplementary Figure 16

**Immunoblot analysis for FLS2 and BAK1.**

Ten-day-old seedlings of Col-0, NIL-Bu-5, *acqos-1*, *acqos-2*, *bak1-4* and *efr fls2* under normal growth conditions were subjected to immunoblot analysis with the indicated antibodies. Equal loading of protein lysates was verified by Ponceau S staining of the protein blots. There were no significant alterations in FLS2 and BAK1 accumulation among Col-0, NIL-Bu-5 and *acqos* mutants.

Supplementary Figure 17

**Osmotolerance after acclimation with mild osmotic stress.**
a. Plants acclimated with 150 mM sorbitol were grown in the presence of 750 mM sorbitol for 14 days. As well as salt-acclimation, Bu-5, NIL-Bu-5 and acqos-1 showed osmotolerance, whereas Col-0 plants did not. Experiments were repeated three times.

b. Chlorophyll content of each plants as described in a. Within each line, bars with different letters are significantly different (P < 0.05, one-way ANOVA with post-hoc Tukey HSD test, mean ± se, n=6).
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 ºC 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 ºC) 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), abil-1 (CS22), pad4-1 (CS3806), sid2-2 (CS16438), eds5-1 (CS3735), and npr1-l (CS3726). The eds1-2 mutant\(^{24}\) 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 mutations and ACQOS locus, the F\(_2\) seedlings were genotyped by sequencing or SSLP markers (Supplementary Table 2). The F3 progeny was used in this study. To generate abil-1_Col-0 and abil-1 NIL-Bu-5, abil-1 (Ler background) was backcrossed three times to Col-0 or NIL-Bu-5.

Stress treatment for acquired osmotolerance assay
7-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 d. The 14-day-old seedlings were then
mesh transferred to a plate supplemented with 750mM sorbitol for 14 d. Mild osmotic stress (e.g., 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$_3$F$_2$ 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$_3$F$_2$ 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 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 analyzed in this study, 250k SNP dataset is available only for 173 accessions. We excluded some accessions whose phenotype is not penetrated (e.g., 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 250k SNP data as a genotype set$^{35}$. To deal with the confounding effect of population structure, we employed a mixed model incorporating a genome-wide kinship matrix as a
random effect\textsuperscript{36}. We used the GWAPP platform\textsuperscript{37} 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 Ascl linker primers and cloned into the Ascl sites introduced into the binary vectors pGreen0029 and pGreen0129. The ACQOS promoter: 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\textsuperscript{38}. 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\(^{-1}\) claraor 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. \(\beta\)-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-MUG fluorometric assay\(^{39}\). 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 200
mM Na$_2$CO$_3$ and 4-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 $\pi_a/\pi_s^{40}$. 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$^{41}$.

**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$^{42}$. 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, Waters)$^{42}$.

**Bacterial inoculation assays**

Bacterial inoculation assays were performed as described previously$^{43}$ with the following modifications. Following 1 µM flg22 or water (mock) pretreatment for 24 h, *Pst* DC3000 suspension at 1 x 10$^5$ cfu/mL was syringe-infiltrated into 3 leaves of 5 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 (5 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\(^4\). 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).
References


Supplementary information is available in the online version of the paper.

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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.

Author information

The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to T.Taji (t3teruak@nodai.ac.jp).
Figure 1
Identification of the ACQOS locus.

a, Acquired osmotolerance of *A. thaliana* accessions. Upper panel: A flow chart of the acquired osmotolerance assay. Middle panel: Salt tolerance when grown on soil. Three-week-old plants grown in pots were exposed to 500 mM NaCl in water for 49 d. Lower panel: Acquired osmotolerance. Salt-acclimated 2-week-old seedlings were mesh-transferred to MS agar plates containing 750 mM sorbitol for 21 d.
b, High-resolution mapping of the ACQOS locus using NILs. Upper panel: Acquired osmotolerance of Col-0, Bu-5, NIL-Col-0, and NIL-Bu-5. Lower panel: Graphical genotypes of NILs. Chromosomal segments of Col-0, off-white; Bu-5, green. Numbers above the genes are the last 3 digits of their Arabidopsis Genome Initiative (AGI) numbers (At5g40XXX).
c, Genome-wide association study for acquired osmotolerance. Upper panel: Manhattan plot of GWAS results for acquired osmotolerance. Middle panel: Close-up of the major GWAS peak in the vicinity of the ACQOS locus on chromosome 5. The position of the ACQOS gene is indicated by a red line. Lower panel: Linkage disequilibrium patterns within ±500 kb upstream and downstream of the ACQOS locus.
d, Complementation test performed by transforming NIL-Bu-5 with *NLR4 (ACQOS)*. T₁ homozygous plants transformed with native promoter: *NLR4 (ACQOS)* derived from Col-0 were 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 ± se, n = 3).
g, Histochemical analysis of the expression pattern of 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 analyzed by Student’s t-test. (mean ± se, 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) d. Similar results were obtained in three independent experiments; representative data are shown.
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 nonsynonymous substitution compared to 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 d. 
d, Nucleotide diversity at all sites across the ACQOS locus (Groups 1 and 2). A dotted horizontal line indicates average genome-wide nucleotide diversity of A. thaliana (Nordborg et al. 2005).
e, Complementation test for acquired osmotolerance using Group 1 ACQOS (upper part) and Group 2 ACQOS (lower part). Salt-acclimated seedlings were grown in the presence of 750 mM sorbitol for 15 d. Arrowheads indicate T2 seedlings with introduced Group 1 ACQOS.
f, Maximum-likelihood based phylogenetic tree of NLR genes in the ACQOS locus with three homologs from Arabidopsis lyrata as an outgroup. The values on the branches indicate the percentage of 1,000 bootstrap replicates. Similar results of Fig. 2c and 2e were obtained in at least three times independent experiments; representative data are shown.
Figure 3
Contribution of ACQOS to immune responses and pathogen-resistance after MAMP treatment.

a, Salicylic acid (SA) contents in Col-0 and NIL-Bu-5 plants under normal, salt stress, and subsequent osmotic stress conditions.
b, Expression of PR1, PR2, and EDS1 in Col-0 and NIL-Bu-5 plants under normal, salt stress, and subsequent osmotic stress conditions determined by qRT-PCR (mean ± se, n=3). Differences between Col-0 and NIL-Bu-5 were analyzed by Student’s t-test. *P <0.05; ***P <0.001.
c, Acquired osmotolerance of the immune signaling mutants eds1-2, pad4-1, and npr1-1. R protein accumulation and hence function mutants rar1-21 and sgt1b14, an SA-depleted 35S:NahG transgenic plant29, and the SA-deficient mutants eds5-130 (mutation in an SA transporter) and sid2-231 (mutation in isochorismate synthase). All the mutants were in the Col-0 background. Similar results were obtained in three times independent experiments; representative data are shown.
d, Chlorophyll content of immune deficient mutants as described in c. Within each lines, bars with different letters are significantly different (P < 0.01, one-way ANOVA with post-hoc Tukey HSD test, mean ± se, n=6).
e, Expression of NHL10 and PROPEP3 in Col-0, NIL-Bu-5 and efr fls2 plants exposed to water (Mock) or 1 mM flg22 for 8h determined by qRT-PCR (mean ± se, 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 mM flg22 for 24 h. (mean ± se, n=5). e and f, Differences between samples were analyzed by Student’s t-test. *P <0.05; **P <0.01.