Oxicam-type nonsteroidal anti-inflammatory drugs inhibit NPR1-mediated salicylic acid pathway

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Abstract

Salicylic acid (SA) and its structural analogs are nonsteroidal anti-inflammatory drugs (NSAIDs) that target mammalian cyclooxygenases. In plants, SA acts as a defense hormone that regulates NON-EXPRESSOR OF PATHOGENESIS RELATED GENES 1 (NPR1), the master transcriptional regulator of immunity-related genes. We identified a number of NSAIDs that enhance bacterial effector-induced cell death. Among them, the oxicam-type NSAIDs tenoxicam (TNX), meloxicam, and piroxicam, but not other types of NSAIDs, exhibit an inhibitory effect on immunity to bacteria and SA-dependent immune responses in plants. TNX treatment reduces NPR1 levels, independently from the proposed SA receptors NPR3 and NPR4. Instead, TNX induces oxidation of cytosolic redox status, which is also affected by SA and regulates NPR1 homeostasis. Surprisingly, however, cysteine modification associated with NPR1 oligomerization *via*

intermolecular disulfide bonds is not affected by either SA or TNX. Therefore, oxycam-type NSAIDs highlight importance of SA effects on the cytosolic redox status, but not on cysteine modification or oligomerization of NPR1.

Introduction

Salicylic acid (SA), originally isolated from the bark of white willow (*Salix alba*), has been used for easing pain and reducing fevers for more than two millennia^{1,2}. SA derivatives, such as aspirin, acetylsalicylic acid, and other structural analogs, belong to a class of drugs known as nonsteroidal anti-inflammatory drugs (NSAIDs) that are commonly used to treat pain and inflammation. The primary target of NSAIDs are cyclooxygenase (COX) enzymes that convert arachidonic acid into prostaglandins in inflammatory processes in human^{3,4}. SA is produced by many plants and functions as a key phytohormone that is required for immunity against pathogens. SA accumulates at high levels in plants upon pathogen infection. Ectopic expression of an SA-degrading enzyme increases the plant's susceptibility to infection, whereas exogenous application of SA potentiates plant defense responses⁵⁻⁷. *Arabidopsis* NON-EXPRESSOR OF PATHOGENESIS RELATED GENES 1 (NPR1) is a transcriptional cofactor that regulates the SA-dependent signaling pathway⁸, and more than 95% of genes responsive to the SA-analog benzothiadiazole are NPR1-dependent⁹. Recently, NPR1 was also shown to function as an adaptor of the Cullin 3 E3 ubiquitin ligase¹⁰.

Recent studies showed that NPR1 and its paralogs, NPR3 and NPR4, have affinity for SA and are involved in the SA perception¹¹. Because SA promotes the NPR1-NPR3 interaction but disrupts the NPR1-NPR4 interaction, NPR3 and NPR4 are proposed to function as adaptors of the Cullin 3 E3 ubiquitin ligases for the turnover of NPR1 in response to SA¹². Structural analysis of NPR4-SA complex showed that SA is completely buried in the hydrophobic core of NPR4 thus causing its drastic conformational change, which presumably disrupts the NPR1-NPR4 interaction¹³. Amino acid residues forming the interaction surface of NPR4 with SA are highly conserved in NPR1 paralogs, corroborating that NPR1 also binds to SA¹³⁻¹⁶. In contrast, Ding et al.¹⁶ demonstrated that NPR3 and NPR4 are transcriptional co-repressors of SA-responsive genes, but that repression is lost upon SA binding. Further, the same study reported that SA binding to NPR1 promotes its transcriptional activation¹⁶.

Arabidopsis NPR1 is a cysteine-rich protein whose activity is regulated by posttranslational modifications at cysteine residues¹⁷. In particular, Mou et al.¹⁸ reported that, under normal conditions, NPR1 exists predominantly in cytoplasm as an oligomer that is formed *via* redox-sensitive intermolecular disulfide bonds between cysteine residues. Later, Tada et al.¹⁹ found that an NO donor, S-nitrosoglutathione, promotes S-nitrosylation of Cys¹⁵⁶, which in turn facilitates NPR1 oligomerization. SA accumulation results in a shift in cellular redox balance toward the reductive state, which then leads to the release of active NPR1 monomers by thioredoxin-mediated reduction of the disulfide bonds^{18,19}. NPR1 was also reported to serve as an intrinsic link between daily redox rhythms and the circadian clock²⁰. Thus, NPR1 has been proposed to be a redox sensor in plants.

Previously, we established a high-throughput screening method by employing an *Arabidopsis* suspension cell-*Pseudomonas syringae* pv. *tomato* DC3000 (*Pto*) *avrRpm1* system to identify plant immune-priming agents^{21–26}. In this system, the suspension cells exhibit immunity-associated programmed cell death triggered by recognition of the effector protein AvrRpm1²⁷. Plant immune-priming compounds, such as SA and tiadinil, potentiate this type of effector-triggered cell death in suspension cells at 10 μ M concentration²⁶. By screening a set of chemical libraries, we isolated several plant immune-priming compounds that enhance effector-triggered cell death in this assay^{21–24}. Here, we report that oxicams, a class of NSAIDs, potentiate effector-triggered cell death but, unlike SA, inhibit immunity against bacteria and down-regulate SA-dependent immune responses in plants. Among them, tenoxicam (TNX) disturbs the SA-induced cellular redox shift, which is important for NPR1 homeostasis, and broadly suppresses SA-responsive genes and reduces NPR1 levels independently of NPR3 and NPR4. Our results demonstrate that oxicam-type NSAIDs can be used to dissect the molecular components of effector-triggered cell death and immunity, and are useful new tools to understand plant immunity governed by the SA signaling pathway.

Results

NSAIDs potentiate effector-triggered cell death in *Arabidopsis* suspension cultures.

In the previous screening assays using *Pto avrRpm1*-induced cell death in *Arabidopsis* suspension cell cultures²⁴, we identified 19 NSAID compounds as cell death

potentiators. Eight NSAID compounds were from the MicroSource library and 13 compounds were from the NPDepo library²⁸, including ibuprofen (IBF) and ketoprofen (KPF), which were included in both libraries (Supplementary Fig. 1 and Supplementary Table 1). We selected eight representative compounds in terms of chemical structure for further studies. Similar to aspirin, *Pto avrRpm1*-induced cell death was significantly enhanced in a dose-dependent manner by the application of propionic acid derivatives (IBF and naproxen (NPX)), acetic acid derivatives (indomethacin (IDM) and sulindac (SLD)), anthranilic acid derivatives (mefenamic acid (MFA)), and oxycam derivatives (TNX, meloxicam (MLX) and piroxicam (PRX)), although some were toxic at higher concentrations in mock-treated controls (Fig. 1a).

Oxicams suppress immunity to *Pto* in *Arabidopsis*.

SA has been shown to enhance plant immunity⁵⁻⁷, therefore we next tested whether NSAIDs could also affect disease resistance in *Arabidopsis*. Soil-grown *Arabidopsis* plants were inoculated with the virulent strain *Pto* with 100 μ M NSAID compounds and bacterial growth was measured. Surprisingly, immunity against *Pto* was significantly suppressed by application of the oxycams TNX, MLX, and PRX, while the non-oxycams IBF, IDM, and MFA did not show the suppressing effect (Fig. 1b). Oxycam derivatives did not promote bacterial growth *in vitro* without host plants at the concentration of 100 μ M (Supplementary Fig. 2), suggesting that these compounds do not directly promote the growth *per se*. Importantly, at 100 μ M, oxycam derivatives alone did not cause visible cell death in *Arabidopsis* leaf tissues, but potentiated *Pto avrRpm1*-induced cell death (Fig. 1c). We conclude that the oxycam-type NSAIDs potentiate effector-triggered cell death but also suppress host immunity in *Arabidopsis* plants.

TNX suppresses SA-responsive genes.

Given that the oxycam-type NSAIDs are structurally similar to SA (Fig. 2a), but unlike SA are able to suppress immunity against *Pto*, we focused on TNX, which is the most potent compound among oxycam derivatives tested in the cell death assay (Fig. 1a), to elucidate their mode or modes of action. First, we tested the effect of TNX on representative marker genes, including *PR1* for SA-dependent immune signaling, and *PDF1.2* for jasmonic acid-dependent immune signaling in leaf tissues in response to *Pto* infection. Application of TNX significantly suppressed *Pto*-induced *PR1* expression at

24 hr after inoculation (Fig. 2b), but increased *Pto*-induced *PDF1.2* expression (Fig. 2c). We also assessed the effect of TNX on SA-induced *PR1* expression in Col-0 wild type (WT) seedlings, and observed its suppression in a dose-dependent manner (Fig. 2d). Consistently, MLX and PRX treatments of transgenic *Arabidopsis* plants carrying *beta-glucuronidase (GUS)* under the regulatory control of the *PR1* promoter²⁹ suppressed SA-induced *PR1* expression similar to TNX treatment (Fig. 2e). Bacterial growth assays in the *sid2-2* and *npr1-1* mutants, which are defective in SA biosynthesis and signaling, respectively^{8,30}, revealed that the application of TNX does not further increase susceptibility to *Pto* (Fig. 2f). These data suggest that the oxicam-type NSAIDs interfere with the SA signaling pathway, resulting in reduced immunity against *Pto*.

TNX represses almost half of SA-induced NPR1-dependent genes.

Transcriptome analysis of fourteen-day-old WT or *npr1-1* seedlings treated with 100 μ M SA and/or 100 μ M TNX for 24 hours (Supplementary Data 1) showed that 2,402 genes were induced and 2,662 were repressed (5,064 total) by more than 2-fold with TNX treatment compared with mock-treated plants (FDR = 0.001, Fig. 3a and Supplementary Data 2 and 3). Gene ontology (GO) analysis revealed that there was significant overrepresentation of environmental stress-related GO terms among TNX-induced genes, including response to abiotic stimulus (GO:0009628), response to inorganic substance (GO:0010035), two oxidative stress-related GO terms, response to oxygen-containing compound (GO:1901700), and response to oxidative stress (GO:0006979) (Fig. 3b). In addition, there were two overrepresented GO terms related to the biosynthesis of flavonoids, which are plant secondary metabolites with antioxidant activity and are responsive to biotic and abiotic stresses³¹ (GO:0009812 and GO:0009813) (Fig. 3b). Response to abiotic stimulus (GO:0009628) was also significantly overrepresented among TNX-repressed genes. These results suggest that TNX broadly stimulates the expression of environmental or oxidative stress-related genes, while negatively affecting the expression of an independent set of stress-response genes (Fig. 3b). Of the SA-inducible genes in this data set, 940 genes were induced at least two-fold upon SA-treatment (FDR = 0.001) (Fig. 3c and Supplementary Data 4). In the *npr1* mutant, 67.1 % (631) of the SA-inducible genes were repressed to less than 0.5-fold, confirming that NPR1 is a master regulator of SA signaling in plants (Fig. 3c and Supplementary Data 5). SA-inducible NPR1-dependent genes were then

categorized into three groups. Group I contained 313 genes whose expression was decreased to less than 0.5-fold by co-treatment with TNX, Group II contained 218 genes whose expression was not significantly suppressed by co-treatment with TNX, nor up-regulated by TNX treatment alone, and Group III consisted of 100 genes whose expression was not suppressed by co-treatment with TNX, but was up-regulated by TNX treatment alone (FDR = 0.001 and greater than 2-fold increase) (Fig. 3d, e and Supplementary Data 6). Nearly half (49.6%) of SA-inducible NPR1-dependent genes (Group I) were repressed following co-treatment with TNX (Fig. 3d, e). Consistently, *PR1* and WRKY transcription factor genes, *WRKY38*, *WRKY53*, and *WRKY62*, which are directly controlled by NPR1^{9,32}, were included in Group I (Supplementary Fig. 3). On the other hand, the other half (50.4 %) of SA-inducible NPR1-dependent genes (Groups II and III) were not affected by co-treatment with TNX (Fig. 3d, e). All 100 of the genes in Group III were up-regulated by TNX treatment alone (Fig. 3d, e). These results show that TNX broadly affects SA-inducible NPR1-dependent genes.

TNX suppresses nuclear accumulation of NPR1 in response to SA.

Because nearly half of NPR1-dependent SA-inducible genes were repressed by TNX, we examined the effect of TNX treatment on subcellular localization and accumulation of NPR1 using a transgenic *Arabidopsis* line expressing *NPR1-YFP* under the control of its native promoter in the *npr1-6* knockout mutant background (*NPR1p:NPR1-YFP*)³³. As had been reported previously³³, 100 μ M SA treatment increased nuclear YFP fluorescence, whereas no fluorescence was detected in either the cytoplasm or nuclei in the absence of SA (Fig. 4a). Under our experimental condition, we did not observe the formation of SA-induced NPR1 condensates previously detected in the *NPR1-GFP* over-expressing seedlings after 5 mM SA treatment¹⁰. Interestingly, SA-inducible nuclear YFP fluorescence was consistently lower when oxycam derivatives were added to SA treatments (Fig. 4a). Immunoblot analysis using anti-NPR1 antibody, which were raised against recombinant NPR1 (Supplementary Fig. 4), revealed that application of the oxycam-type NSAIDs consistently suppressed SA-induced NPR1-YFP accumulation (Fig. 4b). Conversely, non-oxycam-type NSAIDs IBF and IDM did not suppress SA-induced NPR1-YFP accumulation (Supplementary Fig. 5). *NPR1* transcripts were slightly, but not significantly suppressed by TNX in SA-treated plants (Supplementary Fig. 6), so the reduction of NPR1 levels by the oxycam-type NSAIDs is likely due to a

post-transcriptional regulatory mechanism.

As mentioned above, a previous study showed that NPR3 and NPR4 are SA receptors and involved in degrading NPR1 as part of a Cullin 3 E3 ubiquitin ligase¹². To test whether NPR3 and NPR4 are involved in TNX-dependent inhibitory effects on NPR1, we measured NPR1 protein levels in the *npr3npr4* mutant by immunoblot. Endogenous NPR1 levels were increased by SA treatment as had been reported previously^{10,32} and were suppressed by co-treatment with TNX, consistent with the observation in *NPR1p:NPR1-YFP* (Fig. 4b, c). In the *npr3npr4* double mutant, both NPR1 and PR1 accumulated with SA treatment, and TNX suppressed this effect (Fig. 4c), indicating that these phenomena are independent of NPR3 and NPR4. We also tested the effect of TNX treatment on the interactions between NPR1 and NPR3, as well as NPR1 and NPR4, with a yeast two-hybrid assay. Disruption of the NPR1-NPR4 interaction by SA in yeast was confirmed as described previously^{12,34} (Supplementary Fig. 7a, b). Conversely, in contrast to Fu et al.¹², SA application did not promote NPR1-NPR3 interaction (Supplementary Fig. 7a). Instead, our data are consistent with other recent reports by Castello et al.³⁴ and Ding et al.¹⁶ that do not show NPR1-NPR3 interactions in the presence of SA. Importantly, TNX treatment did not result in any apparent effects in any combination of NPR1, NPR3 and NPR4 interactions examined (Supplementary Fig. 7a). These results indicate that neither NPR3 nor NPR4 is involved in the TNX effect on NPR1 protein levels.

TNX inhibits SA-induced cellular redox shift.

Previous studies indicate that a cellular redox shift is important for both activation and nuclear accumulation of NPR1¹⁸. We therefore investigated whether TNX influences the SA-inducible cellular redox shift. For this purpose, we measured total glutathione (GSH) content and the ratio of reduced (GSH) to the oxidized (GSSG) form in SA or SA+TNX-treated *Arabidopsis* seedlings. SA treatment increased both the total GSH amount and the GSH/GSSG ratio, both of which were suppressed by TNX co-treatment (Fig. 5a, b). In addition, TNX alone reduced the GSH/GSSG ratio, indicating that TNX treatment alters the cytoplasmic redox status.

To determine whether TNX alters the redox state of the cysteine residues in NPR1, we employed a thiol-specific labeling strategy using a 2 kDa polyethylene glycol-conjugated maleimide (PEG-Mal) that selectively reacts with free thiol groups

and adds approximately 2 kDa per modified cysteine. Addition of PEG-MAL to a cysteine results in an SDS-PAGE gel mobility shift in proportion to the number of PEG moieties added³⁵. Before labeling with PEG-Mal, proteins were denatured with trichloroacetic acid (TCA)/acetone to prevent further oxidation of cysteine thiols *in vitro*. To monitor the redox state of the cysteine residues in NPR1 in intact plants, we employed *35Sp:NPR1-GFP* plants which accumulate higher amount of NPR1-GFP regardless of SA treatment³⁶. Without reduction, PEG-Mal-labeled PR1 migrated as a 15 kDa polypeptide similar to unlabeled PR1, because PR1 contains 6 cysteines which form three intramolecular disulfide bonds and no free thiols (Fig. 5c)³⁷. Disruption of the disulfide bonds in PR1 by the reducing agent dithiothreitol (DTT) prior to PEG-Mal labeling showed a clear mobility shift of about 12 kDa (Fig. 5c). In the case of NPR1-GFP, which gave a signal around the predicted molecular mass (93 kDa) without PEG-Mal labeling, its mobility was significantly shifted upon PEG-Mal treatment (Fig. 5c). NPR1 and GFP have 17 and 2 cysteine residues, respectively, so NPR1-GFP with all cysteines labeled should yield a protein band at about 131 kDa. However, the actual signal of PEG-labeled NPR1-GFP was above 150 kDa (Fig. 5c). This discordance is likely due to the absence of a charge on PEG, so that PEG-labeling at multiple sites has the potential to affect the charge to mass ratio, and thus its mobility in SDS-PAGE³⁸. SA or SA+TNX treatment did not affect protein band migration patterns, indicating that the redox state of cysteines in NPR1 are not affected by SA or SA+TNX treatment. Reduction of NPR1-GFP with DTT prior to PEG-Mal labeling also did not influence NPR1-GFP migration pattern, indicating that none of the cysteines in NPR1 form disulfide bonds regardless of SA treatment. NPR1-GFP levels were also not affected by either SA or SA+TNX treatment (Fig. 6a), although TNX suppressed PR1 accumulation induced by SA. The absence of any apparent effects on NPR1 levels is likely due to saturation effects in *35S* promoter-driven *NPR1-GFP*-overexpressing plants.

Because TNX does not affect the redox states of cysteines in NPR1 in the PEG-labeling experiment, we next investigate if TNX affects the proposed oligomer-monomer transition of NPR1¹⁸. We used a modification of the method originally developed by Mou et al.¹⁸ to prepare protein extracts from *35Sp:NPR1-GFP* plants using a non-reducing agent buffer, and separated the proteins by SDS-PAGE with or without DTT. Without DTT, NPR1-GFP was detected at around 250 kDa in untreated control plants, and an additional band of around 90 kDa was present in SA treated plants

(Fig. 6a), similar to what had been reported previously¹⁸. The 250 kDa signal was diminished with the addition of DTT, confirming that the 250 kDa and the 90 kDa signals correspond to the NPR1 oligomer and monomer, respectively (Fig. 6a). TNX treatment also induced NPR1 monomer accumulation, and co-treatment with SA resulted in an additive effect (Fig. 6a). This result was contrary to expectations based on the inhibitory effect of TNX on NPR1 levels. To validate these results a TCA/acetone precipitation method was used to avoid potential redox artifacts. To our surprise, even without DTT, no NPR1 oligomer was detected in the TCA/acetone-precipitated samples (Fig. 6b). To check whether NPR1 oligomerization occurs *in vitro*, we prepared protein samples using a buffer containing a thiol-alkylating agent, *N*-ethylmaleimide (NEM). NEM forms covalent bond with the free thiol group, but not with pre-formed disulfide bonds in the protein, thus preventing free thiol groups from further chemical reaction^{39,40}. Samples from WT and *NPR1p:NPR1-YFP* lines were also included to preclude any saturation effects that might have been due to 35S promoter-driven *NPR1-GFP* overexpressing plants. Under non-reducing conditions without NEM (-DTT, -NEM), the protein band corresponding to NPR1 oligomer was detected in SA-treated *NPR1p:NPR1-YFP* and *35Sp:NPR1-GFP* plants, but not in WT plants, possibly due to lower expression of native NPR1 (Fig. 6c). In contrast, under non-reducing conditions but with the inclusion NEM (-DTT, +NEM), the band corresponding to NPR1 oligomer has much weaker signal than in samples without NEM (-DTT, -NEM), whereas the band corresponding to NPR1 monomer has a stronger signal (Fig. 6c). These results suggest that NPR1 oligomerization occurs *in vitro* under non-reducing conditions. Together these observations indicate that NPR1 oligomer-monomer transitions occur *in vitro*, but not *in vivo*. Thus TNX activity is unlikely to be involved in NPR1 oligomer-monomer transitions.

Discussion

All tested NSAIDs potentiate effector-triggered cell death but only oxicams suppress immunity and SA defense. The *Arabidopsis* suspension cell culture-based screening system identified four structurally distinct types of NSAIDs, all of which potentiate cell death induced by *Pto avrRpm1*. These results may not be surprising as those NSAIDs and the plant immune hormone SA, which potentiates cell death upon induction by

avirulent effectors-containing pathogens (Fig. 1A; Shirasu et al.⁶ and Noutoshi et al.²⁴), uniformly target COX enzymes and show anti-inflammatory activity in mammalian cells⁴. Although not yet identified, it is possible that SA and NSAIDs bind to a plant protein (or proteins) to potentiate cell death. There are no close homologs of COX in plants, so NSAIDs must act by some different mechanism in plant than in animals⁴¹.

In contrast, Oxicam-type NSAIDs, including TNX, MLX, and PRX, suppress immunity against *Pto*, while others do not, and they inhibit SA signaling. As a possible clue as to their selective effects on inhibiting immunity and SA signaling, oxicams do not contain a carboxy moiety, compared to other NSAIDs. In humans, the carboxy moiety binds to either the channel entrance or the catalytic site of COX⁴². Instead, oxicams indirectly interact with important residues of both the channel entrance and the catalytic site of COX by bridging with two tightly bound water molecules⁴³. Thus, in plants, the oxicams and other tested NSAIDs may bind to a common target, but potentially in different conformations that result in distinct phenotypes. Alternatively, oxicams may have yet another plant target, one that selectively regulates the SA dependent signaling pathway in plants.

How do oxicam-type NSAIDs suppress immunity while potentiating effector-triggered cell death? Effector-triggered cell death is often associated with immune responses of resistant plants and is potentiated by SA treatment⁶, so it is counterintuitive that cell death augmented by oxicams is accompanied by immune suppression. However, similar observations have been reported. For example, the cell death caused by *avrRpm1* is more enhanced in the *Arabidopsis npr1* mutant, which is immune deficient⁴⁴. Similarly, nuclear NPR1 levels were lower at the infection site of an avirulent pathogen causing cell death by NPR3/4-mediated degradation^{10,12}. In contrast, in cells adjacent to tissues infected with avirulent pathogens, accumulated SA promotes formation of cytoplasmic NPR1 condensates that contain regulators of effector-triggered cell death and facilitates its degradation through NPR1-Cullin 3 complex-mediated ubiquitination, and thus promoting cell survival¹⁰. Thus NPR1 is proposed to function as a negative regulator of effector-triggered cell death¹⁰. In this respect, the reduction of NPR1 levels as a result of TNX treatment (Fig. 4a, b) is likely the reason for potentiation of effector-triggered cell death accompanied by suppression of immunity. In contrast, SA and other NSAIDs, such as IBF and IDM, also potentiate effector-triggered cell death but do not reduce

NPR1 levels (Supplementary Fig. 5). Thus, increased effector-triggered cell death is not always associated with low NPR1 levels.

TNX suppresses SA inducibility in about half of NPR1-dependent genes. Our transcriptome analysis revealed that TNX broadly represses SA-inducible NPR1-dependent genes (Group I in Fig. 3d, e) but almost half of the genes (Groups II and III in Fig. 3d, e) were not affected. TNX at the concentration used only partially reduces NPR1 protein levels, so it is likely that the level of NPR1 activity that remains under our assay conditions to induce genes that require less NPR1 (Group II). In addition, certain genes are redox-change-sensitive, so that both TNX and SA should induce such genes (Group III). These observations suggest that there is a clear difference in TNX sensitivity within the set of SA-inducible genes.

Does TNX target NPR proteins? Because SA has been reported to bind to NPR proteins, we first thought that oxycams directly target NPR proteins to inhibit SA binding. If TNX competitively binds to the SA binding site of NPR proteins, one would assume that TNX prevents the dissociation of NPR1-NPR4 complexes induced by SA^{12,13}. However, TNX did not affect SA-inducible dissociation of the NPR1-NPR4 complex, and did not disrupt NPR1-NPR4 interactions as detected in the yeast two hybrid assay (Supplementary Fig. 7). We did not detect NPR1-NPR3 interactions in this assay in the presence of SA, unlike what had previously been reported¹². Therefore, we cannot eliminate the possibility that our yeast two-hybrid conditions were not sensitive enough to assay for TNX effects, although this assay was used to demonstrate NPR proteins interactions in previous studies¹². Nevertheless, NPR3 and NPR4 are unlikely the targets of TNX, since TNX suppressed SA-induced PR1 accumulation even in *npr3npr4* double mutant plants (Fig. 4c). This is consistent with findings by Ding et al.¹⁶ who showed that NPR3 and NPR4 function independently of NPR1.

TNX shifts the cellular redox state toward oxidation. Although TNX may not directly target NPR proteins, we showed that TNX can affect the cellular redox state, which is proposed to be important for NPR1 homeostasis^{18,19}. TNX increased oxidation of the cellular redox state as indicated by a decrease in the GSH/GSSG ratio (Fig. 5b). The transcriptome analysis indirectly corroborates this result because oxidative stress-

responsive genes were highly expressed in TNX-treated plants (Fig. 3b). In an oxidized cytosolic redox environment modulated by GSH levels, *PR1* expression induced by pathogens or SA is highly reduced⁴⁵⁻⁴⁷. Importantly, in *NPR1-GFP* overexpressing plants, TNX inhibited PR1 accumulation in response to SA, without lowering NPR1-GFP levels (Fig. 6a). Thus, TNX likely interferes with NPR1 activation, in addition to modulating NPR1 levels in response to cellular redox shifts (Fig. 4a, b and 6a). Alternatively, a large amount of newly synthesized NPR1-GFP, which is produced constitutively in *NPR1-GFP* overexpressing plants, may mask the TNX effects on NPR1. Another important point is that interacting partners of NPR1 are also regulated in a redox-dependent manner. For example, a TGA-type transcription factor, TGA1, contains a redox-sensitive intramolecular disulfide bond that affects NPR1 interactions⁴⁸. Reduction of the disulfide bond upon SA accumulation stimulated the interaction between NPR1 and TGA1⁴⁸. Therefore, we speculate that oxidation of the cellular redox state by TNX most likely disturbs the function of NPR1 indirectly through its interacting transcription factors, resulting in attenuated SA responses.

What is the status of NPR1 in different redox environments in vivo? Although an NPR1 oligomer band was clearly observed in protein sample preparations under non-reducing neutral conditions (pH7.0), oligomerization was mostly abolished following the application of the thiol-blocking reagent (NEM) during sample preparation (Fig. 6a, c). This was quite surprising, as it contradicts a previous report¹⁸. Notably, the acidification by TCA also reduced the signal of presumptive NPR1 oligomer (Fig. 6b). In addition, the PEG-labeling experiment indicated that the number of free thiols in NPR1 *in vivo* is not affected by SA application (Fig. 5c). These results indicate that NPR1 contains reactive (low pKa) cysteines and can form oligomers *in vitro*, but oligomerization is unlikely *in vivo*. This is not unreasonable, as the GSH/GSSG pool in the nucleus and cytosol are generally maintained in a highly reducing environment that normally disfavors disulfide-bond formation⁴⁹. Thus, the apparent inconsistency with previous work might be accounted for by a difference in pH of the protein extraction buffer. We employed HEPES buffer (pH 7.0) for protein extraction, while Mou et al.¹⁸ used slightly basic tris buffer (pH 7.5). As the proportion of thiolate anions increases in proportion to the pH of the buffer, the thiols readily form disulfide bonds. *In vitro* NPR1 oligomerization could therefore proceed more quickly under such conditions¹⁸. In

addition, since the hydrolysis of NEM proceeds faster under basic conditions⁴², the instability of NEM in the buffer could be another reason for the discrepancy.

Why does an NPR1 monomer band appear with SA treatment? SA treatment was associated with the appearance of monomeric NPR1 under non-reducing condition (in the absence of DTT) as previously described¹⁸ (Fig. 6a), whereas our data suggest that oligomerization mainly occurs *in vitro* during sample preparation. TNX also induced the appearance of the NPR1 monomer band under non-reducing conditions, and the effect was cumulative in the presence of SA, although PR1 accumulation was suppressed by TNX (Fig. 6a). Thus, the increase in signal of an NPR1 monomer band under non-reducing condition does not always correlate with PR1 levels. The underlying mechanism that could explain the appearance of the SA-induced NPR1 monomer band under non-reducing condition remains to be discovered. SA is known to promote interactions between NPR1 and several members of the TGA transcription factor group, or components of the COP9 signalosome complex^{32,50}. The interacting partners may shield surface-exposed cysteine residues, resulting in the interference of intermolecular disulfide bond formation during sample preparation. Because SA can also promote dephosphorylation of Ser55/Ser59 and sumoylation, as well as subsequent phosphorylation at Ser11/Ser15 of NPR1⁵¹, post-transcriptional modifications of NPR1 may cause conformational changes that influence the reactivity of surface-exposed cysteine residues. SA binding to NPR1 also may induce such changes, as shown in the NPR4-SA interaction¹³. The precise mechanism underlying the appearance of a monomer band under non-reducing conditions needs to be further investigated.

What could be the target of oxicams in plants? TNX up-regulated the expression of oxidative stress-related genes, thus it may be assumed that any potential molecular target(s) are regulators of cellular redox homeostasis or antioxidant enzyme(s). Previously, the cytosolic thioredoxins TRX-h3 and TRX-h5 were identified as regulators of the NPR1 redox state¹⁹. However, susceptibility to *Pseudomonas spp.* bacteria did not increase either in *trx* mutants or in a cytosolic TRX reductase mutant¹⁹, suggesting that the cytosolic thioredoxin system is not a target of TNX. Target identification will be required to delineate how TNX interferes with SA-dependent immune signaling in plants. Nonetheless, our study suggests that TNX and other

NSAIDs are useful chemical tools for investigating the regulatory mechanisms of SA-dependent signal transduction. In addition, these immunity inhibitors could be used for *Agrobacterium*-based transformation where plant immunity should be suppressed for better efficiency. Thus, in future experiments, it will be important to investigate the TNX immunosuppression effect on plant crop species.

Materials and methods

Chemicals

Two chemical libraries, spectrum collection (1,920 chemicals in DMSO (10 mM); MicroSource Discovery Systems Inc.) and NPDepo800 (768 chemicals in DMSO (10 mg/ml); RIKEN Natural Products Depository) were screened for activity. Sodium salicylate (S3007), Tenoxicam (T0909), piroxicam (P5654), meloxicam (M3935), naproxen (N8280), indomethacin (I7378), sulindac (S8139), and mefenamic acid (M4267) were purchased from Sigma-Aldrich. Ibuprofen (098-02641) was purchased from Wako Chemicals.

Plant materials and growth conditions

Suspension cultures of *Arabidopsis thaliana* MM1 (Landsberg *erecta* accession) were maintained as described⁵². In brief, *Arabidopsis* MM1 cells were grown in 300 mL conical flasks containing 100 mL Murashige and Skoog (MS) medium supplemented with Gamborg's B5 vitamin, 3% (w/v) sucrose, 0.5 mg/L MES, 0.5 mg/L naphthaleneacetic acid, and 0.05 mg/L 6-benzylaminopurine (pH adjusted to 5.7 with KOH) at 22°C on a shaker under an 16/8-hour light/dark cycle. For experiments using seedlings, *Arabidopsis* seeds were sown on 1/2 MS medium, 0.8% (w/v) agar and 1% (w/v) sucrose and incubated at 22°C under an 16/8-hour light/dark cycle for 7 to 10 days. Germinated seedlings were transferred to 1/2 MS liquid medium with 1% (w/v) sucrose and grown for 3 more days before performing experiments. 5- to 6-week-old soil-grown *Arabidopsis* plants (22°C, under an 8/16-hour light/dark cycle) were used for disease resistance assays.

Cell death assays in *Arabidopsis* suspension cell culture

Pto avrRpm1-induced cell death in *Arabidopsis* suspension culture was monitored as

previously described²⁶. Briefly, 58.5 µL of *Arabidopsis* suspension cells were dispensed into 96-deep-well plates (260252; Thermo Fisher Scientific), and 0.5 µL of compound was applied to each of two duplicate wells. DMSO and sodium salicylate (20 mM in DMSO) were used as negative and positive controls, respectively. After 1 hour incubation, 41 µL of *Pto avrRpm1* suspension (final concentration; OD = 0.2) was applied into one of the duplicate wells. A mock solution without effector was added to the other, control well. After 21 hour, cells were stained with 1% Evans blue dye and washed 4 x with water. The dye was extracted from cells with 400 µL of 50% methanol, 1% SDS. Absorbance at 595 nm was measured with a microplate reader (iMark Microplate Absorbance Reader; Bio-Rad) to quantify cell death.

Cell death staining in leaves

Pseudomonas syringae pv. *tomato* DC3000 (*Pto*) *avrRpm1* was grown in King's medium B liquid supplemented with 100 µg/mL rifampicin at 28°C. *Arabidopsis* seeds were sown as described above and seedlings were grown for a week, then transferred onto rockwool and hydroponically cultivated for 4 weeks under an 8/16-hour light/dark cycle. Three days before infection, plants were transferred into small pots containing 100 µM chemicals from the two chemical libraries. A bacterial suspension (1×10^7 cfu/mL in 10 mM MgCl₂) was injected with a needleless syringe into the abaxial side of leaves, and infected plants were incubated for 6 hours. Leaves were stained in lactophenol-trypan blue solution (10 mL of lactic acid, 10 mL of glycerol, 40 mL ethanol, 10 g of phenol and 8 mg of trypan blue, dissolved in 10 mL of water) and cleared in chloral hydrate (2.5 g chloral hydrate in 1 mL water). Stained cells were observed using an optical microscope (Olympus; BX51).

Pathogen growth assay

Pseudomonas syringae pv. *tomato* DC3000 was grown on King's medium B liquid containing 100 µg/mL rifampicin at 28°C. Five to six-week-old soil-grown plants grown at 22°C under an 8/16-hour light/dark cycle were syringe-infiltrated with a bacterial suspension of 1×10^5 cfu/mL in 10 mM MgCl₂ and 1% DMSO containing 100 µM oxamic compounds. Bacterial growth in plants was monitored at 3 or 4 days post inoculation.

RNA extraction and quantitative RT-PCR

Total RNA samples were extracted using Qiagen RNeasy Plant Mini kit (Qiagen) with DNase I digestion. RNA concentration was quantified with a NanoDrop (Thermo Fisher Scientific). Reverse-transcription was performed with ReverTra Ace qPCR RT master mix (Toyobo). Quantitative PCR was performed using a Thunderbird SYBR qPCR Mix (Toyobo) and the analysis was carried out with a real-time thermal cycler (Stratagene Mx3000p; Agilent). Primers for quantitative RT-PCR are as follows: for *PRI*, 5'-TTCTTCCCTCGAAAGCTCAA -3' and 5'-AAGGCCCACCAGAGTGTATG -3'; for *PDF1.2*, 5'-TTTGCTGCTTTCGACGCAC -3' and 5'-CGCAAACCCCTGACCATG -3'; *ACT2*, 5'-GATGGCATGAGGAAGAGAGAAAC -3' and 5'-AGTGGTCGTACAACCGGTATTGT -3'.

GUS activity

Quantitative measurement of GUS activity was performed according to the protocol described by Jefferson et al.⁵³. Plant samples were ground with liquid nitrogen and then homogenized in extraction buffer (50 mM NaHPO₄, pH 7.0, 10 mM beta-mercaptoethanol, 10 mM EDTA, 0.1% Sodium Lauryl Sarcosine, 0.1% Triton X-100). The homogenate was centrifuged at 20,000 g for 10 min at 4°C. The supernatant was collected and assayed by fluorometric quantitation of 4-methylumbelliferone produced from the glucuronide precursor using a multiplate reader (365-nm excitation filter and 455-nm emission filter; Mithras LB940; Berthold Technologies).

RNA-sequencing

Fourteen-day-old seedlings grown on 1/2 MS medium with 0.8% (w/v) agar and 1% (w/v) sucrose were transferred to 1/2 MS liquid medium with 1% (w/v) sucrose and grown for one more day. The following day, SA or SA+TNX were added to the liquid medium (100 μM final concentrations) and the seedlings were incubated for 1 day. RNA sequencing was performed as described previously⁵⁴. Purified double stranded cDNAs were subjected to Covaris shearing (parameters: intensity, 5; duty cycle, 20%; cycles/burst, 200; duration, 60 sec). The libraries were sequenced on an Illumina HiSeq 2500. The sequence data have been deposited in the NCBI Gene Expression Omnibus (GEO) database and are accessible through GEO Series accession number GSE154593. Sequence reads to gene associations were carried out using the considerations described

previously⁵⁴. Quality-filtered libraries were aligned to *Arabidopsis* TAIR10 genome using Bowtie version 0.12.8⁵⁵. Unaligned reads from the previous step were aligned to transcript sequences of *Arabidopsis* Col-0 (ftp://ftp.Arabidopsis.org/home/tair/Sequences/blast_datasets/TAIR10_blastsets/TAIR10_cdna_20101214_updated) using Bowtie version 0.12.8⁵⁵. The uniquely aligned reads were used for downstream analysis. Differential expression analysis was performed using the R statistical language version 2.11.1 with the Bioconductor⁵⁶ package, edgeR version 1.6.15⁵⁷ with the exact negative binomial test using tagwise dispersions. Gene ontology analysis was performed using DAVID Bioinformatics Resources 6.8 (<http://david.abcc.ncifcrf.gov/home.jsp>)⁵⁸.

Microscopy

Subcellular localization of NPR1-YFP was observed in cotyledon epidermal cells using an inverted laser scanning confocal microscope (LSM700; ZEISS). NPR1-YFP was excited at 488 nm, and the emission was collected using a BP 490 to 555 nm filter. Autofluorescence from chlorophyll was excited at 555 nm and the emission was collected using a LP 640 nm filter. Obtained images were processed using ZEN 2.1 SP1 software (ZEISS) and Photoshop CC 2019 (Adobe).

Generation of anti-NPR1 antibody

Antigen was prepared with reference to the method described by Mou et al.¹⁸ A truncated cDNA clone of *NPR1* (*tNPR1*, 1-1395 bp) was inserted into the *Nde I/Not I* sites of the pET-26 vector (Novagen) in-frame with C-terminal His tag. The pET-26-*tNPR1*-His plasmid was then transformed into Rosetta (DE3) pLysS (Novagen). The overnight culture at 37°C was transferred to fresh LB medium containing 50 µg/mL kanamycin and was incubated to OD₆₀₀=1.0 at 37°C. Protein synthesis was induced with isopropyl β-D-thiogalactopyranoside at a final concentration of 0.2 mM. The induced cells were incubated overnight at 16°C. The *E.coli*-expressed *tNPR1*-His protein was extracted and purified using TALON Metal Affinity Resin (Clontech). For rabbit immunization, the *tNPR1*-His proteins were further purified by separation on SDS-PAGE. Anti-NPR1 antibodies were purified from antisera by affinity chromatography using a *tNPR1*-His-immobilized column (Hitrap NHS-activated HP; GE Healthcare). To prepare for the *tNPR1*-His-immobilized column, *tNPR1*-His proteins were further

purified by HiLoad 16/600 Superdex 200 (GE Healthcare) with 200 mM NaHCO₃, pH 8.3, 500 mM NaCl, 2 mM DTT, 0.1% CHAPS as a mobile phase.

Protein extraction and immunoblotting

Plant samples were ground with liquid nitrogen and then homogenized in extraction buffer (20 mM HEPES-NaOH, pH 7.0, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 2 mM DTT, 100 µM MG115, and 1 x cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche)). For protein extraction under non-reducing conditions, DTT was omitted from this extraction buffer. For protein extraction under conditions where the maintenance of the thiol group redox state was required, 20 mM *N*-ethylmaleimide (E0136; Tokyo Chemical Industry Co.) was added to the extraction buffer instead of DTT. The homogenate was centrifuged at 20,000 g for 10 min at 4°C, and the supernatant was collected and subjected to SDS-PAGE. Proteins were then electroblotted onto a PVDF membrane using a semidry blotter (trans-blot turbo transfer system; Bio-Rad). Membranes were blocked overnight at 4°C in TBS-T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) with 5% skim milk. Membranes were then incubated with either anti-UGPase (1:5,000; AS05 086; Agrisera), anti-PR1 (1:5,000; AS10 687; Agrisera), anti-HA (1:5,000; 12013819001; Roche), anti-Myc (1:1,000; sc-789; Santa Cruz Biotechnology), or anti-NPR1 antibodies (1:2,000) diluted with TBS-T with 5% skim milk at room temperature for 1 hour. After washing with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:10,000; NA934; GE healthcare) diluted with TBS-T for 1 hour at room temperature. Bound antibodies were visualized using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) and bands were imaged using an image analyzer (ImageQuant LAS 4000 imager; GE healthcare). Experiments were repeated three times with similar results.

Yeast two-hybrid assay

Yeast strain AH109 transformed with pGADT7-NPR3/4 and strain Y187 transformed with pGBKT7-NPR1 were kindly provided by Dr. Xinnian Dong (Duke University). The transformed yeast strains were streaked on synthetic dropout (SD) media lacking Leu and Trp, respectively. For yeast mating, single colonies were grown overnight in YPDA liquid media. The cultures were then streaked on SD media lacking Leu and Trp

(-LT). Single colonies were grown in SD (-LT) liquid media for 1 day and spotted (1:100 dilution) on SD lacking Leu, Trp and His (-LTH) supplemented with 3 mM 3-aminotriazole, with or without 100 μ M SA and/or 100 μ M TNX and incubated at 30°C to monitor the interaction between bait and prey.

TCA/acetone precipitation

Plant samples were ground with liquid nitrogen and then homogenized in 10% TCA in acetone. The homogenate was incubated at -20°C for 1 hour and centrifuged at 20,000 g for 10 min at 4°C. The supernatant was discarded and the pellet was washed 2 times with ice-cold acetone. The pellet was dried and homogenized in buffer (50 mM HEPES pH 7.0, and 2% SDS). The homogenate was centrifuged at 20,000 g for 10 min at RT, and the supernatant was subjected to immunoblot analysis.

Protein labeling with 2k PEG-maleimide

Protein pellets obtained by TCA/acetone precipitation as described above were homogenized in buffer containing either 7.5 mM 2k PEG-maleimide (PEG-Mal; SUNBRIGHT ME-020MA; NOF) or 20 mM DTT. The homogenate was incubated at room temperature for 1 hour and centrifuged at 20,000 g for 10 min. The supernatant was collected and subjected to immunoblot analysis. Half of the DTT-treated sample was precipitated with chloroform/methanol⁵⁹. The precipitated protein was washed 2 times with ice-cold acetone and labeled with PEG-Mal as described above.

GSH measurement

GSH measurements were performed as described previously⁶⁰. Plant samples were ground with liquid nitrogen and then homogenized in 6% HClO₄. The homogenate was centrifuged at 20,000 g for 10 min at 4°C. The supernatant was collected and neutralized with 1.25 M K₂CO₃, then centrifuged at 20,000 g for 10 min at 4°C. The supernatant was divided in two to measure total GSH (reduced GSH + 2 x GSSG) and GSSG. For total GSH measurements, the sample was incubated in assay buffer consisting of 100 mM potassium phosphate buffer, pH 7.5, 5 mM EDTA, 0.6 mM 5,5'-dithiobis(2-nitrobenzoic acid), 0.2 mM NADPH, and 0.5 U/mL glutathione reductase from yeast (46541005; Oriental yeast). The reaction rate was monitored by a microplate reader (Infinite F200; Tecan) equipped with a 405 nm (bandwidth 10 nm) filter. For

GSSG measurements, the sample was immediately mixed with thiol-masking reagent 2-vinylpyridine (20 mM final concentration) after the sample separation and incubated for 1 hour at 25°C, and then measured by the same method as total GSH. The concentrations of total GSH and GSSG were calculated using GSH or GSSG standard curves simultaneously in the assay.

Data availability

RNA-seq data from this study have been deposited in the NCBI Gene Expression Omnibus (GEO) and are accessible through GEO series accession number GSE154593.

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Author Contributions

N.I. and K.S. conceived the project. N.I., S.C., Y.N., I.S., S.A., and K.T. conducted experiments and analyzed results. N.I., S.C., Y.N., I.S., and K.S. conceived and designed experiments. H.O. provided the RIKEN Natural Product Depository compounds. S.Y.H provided *NPR1p:NPR1-GFP* plants prior to publication. N.I., S.C., Y.N., S.A., S.Y.H., and K.S. wrote the paper. All authors reviewed the manuscript.

Fig. 1 Effect of NSAIDs on *Pto avrRpm1*-induced cell death and growth of *Pto* in plants. **a** *Pto avrRpm1* suspension (final OD₅₉₅ = 0.2) was added to *Arabidopsis* suspension cells after adding compounds (concentrations indicated in the right panel). After 20 hours incubation, dead cells were stained with Evans blue, and cell death was determined. Relative values were calculated based on means from controls (DMSO + *Pto*) for each experimental group. Data are shown as means $\pm$ SE ($n = 7$). Asterisks indicate significant differences from DMSO ‘mock’ control (Dunnett test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0001$). **b** Col-0 WT plants were syringe-infiltrated with 1×10^5 cfu/mL of *Pto* with indicated compounds at 100 μM , or with 0.5% DMSO as negative control. Bacterial growth was determined 4 days-post-inoculation. Data are shown as mean $\pm$ SE ($n = 3$). Asterisks indicate significant differences from DMSO (Dunnett test, $p < 0.05$). Experiments were performed three times with similar results. **c** Col-0 WT plants were treated with 100 μM chemicals 3 days before infection. *Pto avrRpm1* suspension (1×10^7 cfu/mL in 10 mM MgCl₂) was infiltrated into the abaxial side of leaves and the plants were incubated for 6 hours. Dead cells were stained with Trypan blue and observed under a microscope. Bar = 1 μm .

Fig. 2 Oxicam compounds inhibit SA-dependent immunity. **a** Chemical structures of salicylic acid (SA), tenoxicam (TNX), meloxicam (MLX), and piroxicam (PRX). **b, c** Col-0 WT plants were syringe-infiltrated with (+) or without (-) 1×10^5 cfu/mL *Pto* suspension in the presence (+) or absence (-) of 100 μ M TNX for 24 hours. *PR1* (**b**) and *PDF1.2* (**c**) transcript levels were measured by qRT-PCR with cDNA prepared from each of the samples. The expression values of individual genes were normalized against *ACT2*. Data are shown as means $\pm$ SE of three independent experiments, each with three replicates. Different letters indicate significant differences (Tukey-Kramer test, $p < 0.05$). **d** Col-0 WT seedlings were treated with TNX for 1 hour before treatment with 100 μ M SA (+) or water (-) for 24 hours. *PR1* transcript levels were measured by qRT-PCR. Expression values were normalized against *ACT2*. Data are shown as means $\pm$ SE of three independent experiments each with three replicates. Different letters indicate significant differences (Tukey-Kramer test, $p < 0.05$). **e** *PR1p:GUS* seedlings were treated with indicated concentration of oxicam derivatives (TNX, MLX, or PRX) or 0.5% DMSO control for 1 hour before treatment with 100 μ M SA (+) or water (-). GUS activity was measured after 24 hours. Data are shown as means $\pm$ SE of three biological replicates. Asterisks indicate significant differences from 100 μ M SA alone (Dunnett test, $p < 0.05$). Experiments were performed three times with similar results. **f** Col-0 WT, *sid2-2*, and *npr1-1* plants were syringe-infiltrated with 1×10^5 cfu/mL of *Pto* suspension in 10 mM $MgCl_2$ and 0.25% DMSO in the presence (+) or absence (-) of 100 μ M TNX. Bacterial growth was determined 3 days-post-inoculation. Data are shown as means $\pm$ SE ($n = 6$). Different letters indicate significant differences (Tukey-Kramer test, $p < 0.05$). Experiments were performed three times with similar results.

Fig. 3 TNX broadly affects SA-induced NPR1-dependent genes. **a** Volcano plots representing global changes in gene expression by TNX treatment in Col-0 WT. Red and blue dots indicate significantly up-regulated ($FDR < 0.001$ and $\log_2$ fold change (FC) > 1) and down-regulated genes ($FDR < 0.001$ and $\log_2FC < -1$), respectively. **b** Gene ontology (GO) enrichment analysis of TNX-induced genes (upper panel) and TNX-repressed genes (lower panel). The top 10 significantly enriched biological process GO terms are shown with their $-\log_{10}FDR$. **c** Venn diagram showing the number of SA-inducible genes whose expression levels were reduced in the *npr1* mutant (NPR1-dependent) and/or co-treatment with TNX (TNX-sensitive). **d** Heat map depicting $\log_2$ (fold change) of SA-inducible and NPR1-dependent genes in Col-0 WT or *npr1* mutant treated with SA or SA+TNX. Genes were categorized into three groups based on expression patterns as described in the Results section. Red and green colors indicate up- and down-regulated expression levels, respectively, compared with mock-treated plants. **e** Scatter plot showing expression levels of SA-inducible and NPR1-dependent genes in SA- and SA+TNX-treated Col-0 WT. The x- and y-axis represent the fold changes between SA-treated and mock-control (SA vs mock) and SA+TNX-treated and mock-control (SA+TNX vs mock), respectively. Blue, red, and orange dots represent group I, II, and III genes, respectively. The arrow-head indicates the spot of *PR1*.

Fig. 4 TNX inhibits nuclear NPR1 accumulation in response to SA. **a** *NPR1p:NPR1-YFP* seedlings were treated with 100 μ M oxamic acid derivatives (TNX, MLX, or PRX) or 0.5% DMSO control for 1 hour before 100 μ M SA (+) or water (-) treatment for 24 hours. YFP fluorescence and chlorophyll autofluorescence are shown in yellow and magenta, respectively. Bar = 20 μ m. **b** Protein extracts of *NPR1p:NPR1-YFP* seedlings treated with SA and/or oxamic acid derivatives as in (a) were resolved by SDS-PAGE and analyzed by immunoblotting using anti-NPR1 and anti-PR1 antibodies. CBB staining is shown as a loading control. **c** Protein extracts of Col-0 WT or *npr3-1npr4-3* seedlings treated with SA and/or TNX as in (a) were resolved by SDS-PAGE and analyzed by immunoblotting using anti-NPR1 antibody. CBB staining is shown as a loading control.

Fig. 5 TNX inhibits SA-induced glutathione pool redox state changes. a, b Col-0 WT seedlings were treated with 100 μ M TNX (+) or 0.25% DMSO control (-) for 1 hour before 100 μ M SA (+) or water (-) treatment for 24 hours, and total glutathione (**a**) and GSH/GSSG ratio (**b**) were determined. NT represents non-treated controls. Error bars represent means $\pm$ SE ($n > 4$). Asterisks indicate significant differences from DMSO control (Dunnett test, $p < 0.05$). Experiments were performed three times with similar results. **c** *35Sp:NPR1-GFP* seedlings were treated with SA and/or TNX as in (**a**). Proteins were extracted and labeled with 2k PEG-Maleimide (PEG-Mal). The labeled proteins were resolved by SDS-PAGE under reducing conditions and analyzed by immunoblotting using anti-NPR1 and anti-PR1 antibodies. Black and white arrowheads indicate non-labeled NPR1-GFP (calculated molecular mass: 93 kDa) and PEGylated NPR1-GFP, respectively.

Fig. 6 Inhibition of NPR1 oligomerization by TCA/acetone precipitation or by the application of a thiol blocking agent. a *35Sp:NPR1-GFP* seedlings were treated with 100 μ M TNX (+) or 0.5% DMSO control (-) for 1 hour before 100 μ M SA (+) or water (-) treatment for 24 hours. Protein extracts were prepared under reducing agent-free conditions. The proteins were resolved by SDS-PAGE under reducing (+DTT) or non-reducing (-DTT) conditions and analyzed by immunoblotting using anti-NPR1, anti-UGPase, and anti-PR1 antibodies. UGPase levels are shown as a loading control. **b** *35Sp:NPR1-GFP* seedlings were treated with (+) or without (-) 100 μ M SA for 24 hours. Protein extracts were prepared by TCA/acetone precipitation method. The proteins were resolved by SDS-PAGE under reducing (+DTT) or non-reducing (-DTT) conditions and analyzed by immunoblotting using anti-NPR1 and anti-PR1 antibodies. CBB staining is shown as a loading control. **c** Seedlings of *npr1-6*, Col-0 WT, *NPR1p:NPR1-YFP*, and *35Sp:NPR1-GFP* were treated with (+) or without (-) 100 μ M SA for 24 hours. Protein extracts were prepared using a buffer containing (+) or not containing (-) 20 mM *N*-ethylmaleimide (NEM). The proteins were analyzed as in (a). Black and white arrowheads indicate NPR1 and NPR1-GFP (or NPR1-YFP), respectively. L, ladder marker.

Salicylic acid derivatives

Acetaminosalol

Acetyl salicylic acid (Aspirine)

D-(-)-Salicin

Salicylic acid

Propionic acid derivatives

Fenopropfen

Flurbiprofen

Ibuprofen

Ketoprofen

Naproxen

Acetic acid derivatives

Diclofenac

Indomethacin

Sulindac

Oxicam derivatives

Isoxicam

Meloxicam

Piroxicam

Tenoxicam

Anthranilic acid derivatives

Mefenamic acid

Others

Epirizole

Valdecoxib

Supplementary Fig. 1 Chemical structure of NSAIDs identified by chemical screening.

Supplementary Fig. 2 TNX effect on bacterial growth. a, b Bacterial suspension of *Pseudomonas syringae* pv. *tomato* DC3000 (1×10^7 cfu/mL) in M9 minimal medium (a) or 10 mM MgCl₂ (b) supplemented with TNX were incubated at 28°C for 38 hours with continuous shaking at 160 rpm. Serial dilution of the cultures were spread onto LB agar media containing kanamycin and rifampicin and the number of colonies were counted. The bars represent the mean $\pm$ SE of three independent experiments.

Supplementary Fig. 3 Expression pattern of *PR1*, *WRKY18*, *WRKY39*, and *WRKY62* in the transcriptome analysis. Gene expression values obtained from transcriptome analysis (Supplementary Dataset 1) were normalized with that in mock-treated Col-0 WT. The bars represent the mean $\pm$ SD ($n = 3$).

Supplementary Fig. 4 Specific reaction of anti-NPR1 antibody. *Arabidopsis* seedlings of *npr1-6* mutant or Col-0 WT were treated with 100 μ M SA, and were sampled 24 hours after treatment. NPR1 protein was detected by immunoblotting using anti-NPR1 antibody. CBB staining were shown as a loading control.

Supplementary Fig. 5 Effect of IBF and IDM on SA-induced NPR1 accumulation. **a** *NPR1p:NPR1-YFP* seedlings were treated with 100 μ M NSAIDs (IBF or IDM) or 0.5% DMSO for 1 hour before 100 μ M SA (+) or water (-) treatment for 24 hours. Fluorescent image was obtained with a confocal laser microscope. YFP fluorescence and chlorophyll autofluorescence were shown in yellow and magenta, respectively. Bar = 20 μ m. **b** *NPR1p:NPR1-YFP* seedlings were treated with SA and/or NSAIDs as in (a). Protein extracts of the seedlings were resolved by SDS-PAGE and analyzed by immunoblotting using anti-NPR1 and anti-PR1 antibodies. CBB staining was shown as a loading control.

Supplementary Fig. 6 Expression pattern of *NPR1* in the transcriptome analysis. Expression value of *NPR1* obtained from transcriptome analysis (Supplementary Dataset 1) were normalized with that in mock-treated Col-0 WT. The bars represent the mean $\pm$ SD ($n = 3$).

a

b

Supplementary Fig. 7 Yeast two-hybrid assay for NPR1-NPR3/4 interaction in the presence of SA and/or TNX. a Yeasts expressing *NPR* genes fused with GAL4 activation domain (AD) or DNA-binding domain (BD) were spotted on synthetic dropout (SD) media lacking Leu and Trp (-LT) or Leu, Trp and His (-LTH) without or with 100 μ M SA and/or 100 μ M TNX. **b** Immunodetection of GAL4 AD or BD fused NPR proteins. Yeast strains were grown on SD (-LT) plates, and then fresh single colonies were grown in SD (-LT) liquid media for 1 day. Total proteins were extracted from the yeasts and subjected to immunoblot analysis using anti-HA and anti-Myc antibodies. CBB staining were shown as a loading control.

Supplementary Table 1 List of NSAIDs identified by chemical screening.

Chemical group	Chemical name	MicroSource	NPDepo
Salicylic acid derivatives	Acetaminosalol	+	
	Acetyl salicylic acid (Aspirine)		+
	D-(-)-Salicin		+
	Salicylic acid		+
Propionic acid derivatives	Fenoprofen	+	
	Flurbiprofen		+
	Ibuprofen	+	+
	Ketoprofen	+	+
	Naproxen*		+
Acetic acid derivatives	Diclofenac		+
	Indometacin		+
	Sulindac		+
Oxicam derivatives	Isoxicam	+	
	Meloxicam	+	
	Piroxicam		+
	Tenoxicam	+	
Anthranilic acid derivatives	Mefenamic acid		+
Others	Epirizole		+
	Valdecoxib	+	

*It was also present in the MicroSource library but its activity was slightly below the threshold in the first screening.