**Structure-Function Analysis of *npr1* Alleles in Arabidopsis
Reveals a Role for its Paralogs in the Perception of Salicylic
Acid.**

Running title: NPR1 alleles and NPR1 paralogs.

Juan Vicente Canet, Albor Dobón, Alejandra Roig, and Pablo Tornero.

Instituto de Biología Molecular y Celular de Plantas (IBMCP); Universidad
Politécnica de Valencia (UPV)-Consejo Superior de Investigaciones Científicas
(CSIC); Ciudad Politécnica de la Innovación (CPI), Ed. 8E; C/ Ingeniero Fausto
Elio s/n, 46022 Valencia, Spain.

All proofs and correspondence should be sent to:

Pablo Tornero

ptornero@ibmcp.upv.es, Phone: (+34)963879377 Fax (+34)963877859

IBMCP(UPV-CSIC) Camino de Vera s/n, 46022 Valencia (SPAIN)

Key words: Salicylic Acid, BTH, NPR1, Arabidopsis, defence

Abstract

Salicylic Acid (SA) is necessary for plant defence against some pathogens, and NPR1 is necessary for SA perception. Plant defence can be induced to an extreme by several applications of benzothiadiazole (BTH), an analogue of SA. Then, plants that do not perceive BTH grow unaffected, while wild type plants are smaller. This feature allows us to screen for mutants in *Arabidopsis thaliana* that show insensitivity to BTH in a high-throughput fashion. Most of the mutants are *npr1* alleles with similar phenotype in plant fresh weight reduction and pathogen growth upon SA or BTH application. No obvious null alleles were recovered in our screening, but most of the mutations are clustered in the carboxyl-terminal part of the protein. These facts have prompted a search for knockouts in the *NPR1* gene. Two of these KO alleles identified are null and have an intermediate phenotype. All the evidence lead us to propose a redundancy in BTH/SA perception, with the paralogs of *NPR1* taking part in this signalling. We show that the mutations recovered in the screening genetically interact with the paralogs preventing their function in BTH/SA signalling.

1 **Introduction**

2 Salicylic acid (SA) is a plant hormone required for full resistance against
3 biotrophic pathogens. It is important in two essential defence process; basal
4 resistance and systemic acquired resistance (SAR, Vlot, Klessig & Park 2008).

5 Basal resistance is triggered by compatible pathogens that do not trigger gene
6 for gene resistance and produce disease (Jones & Dangl 2006). Thus, plants
7 with less SA are more susceptible to compatible pathogens, and plants with
8 more SA (either by genetic engineering or exogenous applications) are more
9 resistant (Reviewed by Nawrath, Métraux & Genoud 2005). SAR is the
10 resistance that is established in non-inoculated leaves of a plant that has
11 previously been inoculated with a pathogen, generally requiring cell death
12 (Reviewed by Vlot *et al.* 2008). SA is necessary but not sufficient for SAR.

13 SA research is hampered by its chemical properties, since the exogenous
14 applications that produce a strong effect in (0.2-1 mM in *Arabidopsis thaliana*
15 (*Arabidopsis*)) are close to the phytotoxic ones (van Leeuwen *et al.* 2007). To
16 avoid the phytotoxicity, and with a clear biotechnological focus, several
17 analogues have been proposed, like 2,6-Dichloroisonicotinic acid (INA, Ward *et al.*
18 1991) and benzothiadiazole (BTH, Lawton *et al.* 1996). In the case of BTH,
19 applications as low as 0.12 mM have an effect, but applications as high as 1.2
20 mM are not phytotoxic (Lawton *et al.* 1996 and data not shown).

21 SA is the only plant hormone where a receptor has not been described yet
22 (Nawrath *et al.* 2005). It is plausible that its perception is through indirect
23 means, like redox balances (see below). *NPR1* (*Non expresser of Pathogenesis*
24 *Related proteins 1*) is the only gene that has been found to have a profound

1 impact on SA signalling when mutated (Cao *et al.* 1994, Delaney, Friedrich &
2 Ryals 1995, Glazebrook, Rogers & Ausubel 1996, and Shah, Tsui & Klessig
3 1997). There are two families of NPR1 interactors: *TGAs* (Zhang *et al.* 1999)
4 and *NIMINs* (Weigel *et al.* 2001). Only when knocking out several genes (*TGAs*,
5 Zhang *et al.* 2003), or expressing dominant members (*NIMINs*, Weigel, Pfitzner
6 & Gatz 2005), is a measurable phenotype observed in SA signalling.

7 When the plant is not under biotic stress, NPR1 is mainly present in the cytosol
8 as oligomers (Mou, Fan & Dong 2003). Upon SA perception, monomers are
9 released from the oligomers, and move into the nucleus. NPR1 is degraded by
10 the proteasome in the nucleus, as a requirement for defence activation (Spoel
11 *et al.* 2009).

12 SA is mechanistically involved in the release of NPR1 monomers by triggering
13 the expression of a thioredoxin (Tada *et al.* 2008). It is suggested that the fine
14 regulation of these events depends on the redox balance of the cell, a balance
15 that is altered when molecules such SA are more abundant (Mou *et al.* 2003).

16 There are five paralogs of *NPR1* in Arabidopsis (www.arabidopsis.org). *Blade*
17 *On Petiole1* (*BOP1*) and *BOP2* have an important role in development (Ha *et al.*
18 2007; McKim *et al.* 2008). *NPR3* and *NPR4* are reported to have a role in
19 defence (Liu *et al.* 2005, Zhang *et al.* 2006), but no function for *NPR2* has been
20 described yet. In this context, we proposed to screen for genotypes that do not
21 recognize SA; applications of BTH on separate dates produce a small plant in
22 the case of wild type Arabidopsis, while *npr1* plants look unaffected (Canet *et al.*
23 2010). This prompted us to do a structure-function analysis of a full range of

- 1 *npr1* alleles. The implications of their phenotype lead us to test also the
- 2 behaviour of the *NPR1* paralogs in SA perception.

Materials and Methods

Plant Growth and Inoculation.

Arabidopsis thaliana was sown and grown as described (Canet *et al.* 2010), in phytochambers with days 8 h at 21°C, 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and nights of 16 h. at 19°C. The treatments, inoculations, and sampling started 30 minutes after the initiation of the artificial day to ensure reproducibility. *Pseudomonas syringae pv. tomato* DC3000 (*Pto*) containing pVSP61 (empty vector) were maintained as described (Ritter & Dangl 1996). The bacteria were grown, inoculated and measured as described (Tornerio & Dangl 2001). For all the experiments, at least three independent treatments were performed (three independent sets of plants sown and treated on different dates).

BTH and Fresh Weight.

Benzothiadiazole (BTH, CGA 245704), in the form of a commercial product (Bion® 50 WG, a gift from Syngenta Agro S.A. Spain) was prepared in water for each treatment and applied with a household sprayer. The BTH treatments were done as described in Canet *et al.* 2010. For the screening of mutants, plants that seemed unaffected by BTH were rescued (First selection) and their progeny phenotyped both in mock and in BTH treatments (Second selection). A third selection consisted of a treatment of an F1 of each mutant with *npr1-1* along with its parents.

Western Blot.

Immunodetection of PR1 protein was carried out as described (Wang *et al.* 2005), using an Amersham ECL Plus Western Blotting Detection Reagent (GE HealthCare, Little Chalfont, UK). The second antibody was a 1:25000 dilution of Anti-Rabbit IgG HRP Conjugate (Promega, Madison, USA). Chemiluminescent signals were detected using a LA-3000 Luminescent Image Analyzer (Fujifilm Life Science, Stamford, USA).

Chemical Treatments.

For measuring the effect in *Pto* growth, water, 500 μ M SA (in the form of sodium salicylate, S3007 SIGMA, St Louis, USA), and 350 μ M BTH were applied by spray one day previous to pathogen inoculation. For in vitro culture, MS plates were prepared and sown as described (Canet *et al.* 2010) with or without 500 μ M SA. The results were evaluated between 10 and 14 days of growth.

Mutagenesis

Seeds were mutagenized with 0.15% ethyl methanesulfonate (M0880, SIGMA) for 8 hr, and M2 seed collected from ~ 100 M1 plants. For EMS, different backgrounds were used (*PR1:LUC*, Maleck *et al.* 2002; *rar1-21* and *rar1-21 ndr1-1*, Tornero *et al.* 2002b; *rpm1-1*, Grant *et al.* 1995; *NahG*, Lawton *et al.* 1995, *RPM1-MYC*, (Boyes, Nam & Dangl 1998) and others). Seeds of Col-0 treated with fast neutrons or with gamma ray were purchased (M1F-02-04 and M1G-02-02 respectively, Lehle seeds, Round Rock, TX, USA), and their progeny screened as with EMS. In the case of the activation tagging seeds, the

1 references N31400, N31402, N31404, N21995, N21998, N21991, N23153,
2 N84450, and N31100 were obtained from NASC (www.arabidopsis.info) and
3 directly screened. The insertions *npr1*-63 (N831344, Alonso *et al.* 2003), *npr1*-
4 71 (N163245, Sundaresan *et al.* 1995), and *npr2*-2 (N622643), were obtained
5 from NASC, and *npr1*-70 (ET5232) from CSHL (www.cshl.edu). The insertions
6 were followed to homozygosity by PCR, and their exact position checked by
7 sequence.

8 9 **Sequence and Statistical Analysis of the Mutations.**

10 PCRs of the NPR1 gene were done with the oligonucleotides TP412 (5'
11 TGCTTCTTCATATCTCACCACCACTCTCG 3') and TP413
12 (5'CACCTTACACGCCCACTCAGTGTTCTCT 3') (SIGMA). Four independent
13 reactions were pooled and sequenced with internal oligonucleotides. The
14 analysis of the mutations was done with Excel software (Microsoft, Redmond,
15 USA) and custom Perl scripts. The data for the conservation of each AA in the
16 NPR1 paralogs was obtained from CLUSTAW (Chenna *et al.* 2003). The data
17 for the different AA characteristics or mutations was downloaded from
18 www.genome.jp/aaindex, and the background was considered to be all the
19 possible mutations or only the EMS-induced mutations. A T-test was performed
20 between the background and the mutations experimentally obtained, followed
21 by a Benjamini-Hochberg False Discovery Rate correction (Benjamini &
22 Hochberg 1995) with $p < 0.05$.

1 **Results**

2 **Searching for Mutations in SA Signalling.**

3 An important question in the field of plant-pathogen interactions is if *NPR1* is the
4 only gene necessary for SA recognition. We used the model of BTH previously
5 described (Canet *et al.* 2010) to answer this question and to further characterize
6 the role of NPR1 by mutagenesis. Figure 1a shows the proof of concept of the
7 model in a screening setting. 3 seeds of *npr1-1* were mixed with c. 3600 seeds
8 of Col-0. After BTH treatments, the *npr1-1* plants are easily identified in a wild
9 type (wt) background (only a quarter of the experiment is shown in Figure 1a,
10 with a single *npr1-1*). Our previous work suggests that mainly *npr1* alleles would
11 appear in this kind of screening, so we set up the most difficult scenario. This
12 scenario would be to find lack of complementation in an F2 between *npr1-1*
13 (recessive) and a dominant mutation, or a proportion of 3 wt plants and 13 *npr1*-
14 like. Such a proportion is detectable (data not shown). To be sure that no
15 mutation is misclassified, MS plates with 500 μ M SA were used (Figure 1b).
16 Although not all the wt plants are recovered, they are easily identified as green
17 plants over a white background of *npr1-1* plants.

18 A high-throughput screening was performed (Figure 1c) with several mutagens
19 to obtain different types of mutations. Thus, besides using Ethyl Methane
20 Sulfonate (EMS, Segal 1984), we also used Activation tagging, Gamma Rays,
21 and Fast Neutrons (Table 1 (*Please insert Table 1 here*)). In the case of EMS, a
22 mutagenesis was performed in the *PR1:LUC* background (Maleck *et al.* 2002).
23 Since different mutant backgrounds could provide unusual results, several
24 mutants or transgenic lines were used as parental lines for EMS mutagenesis.

1 There is no evidence that these mutants provided a different number or kind of
2 mutations (see below). More than 3,600 plants per family were analyzed to
3 ensure the recovery of all mutations with a 95% probability (Malmberg 1993).
4 377 plants that seemed unaffected by BTH were picked in the first selection
5 (M2s), some of them from the same family of M1s. The progeny of these plants
6 was retested with a mock treated pot of plants simultaneously with a BTH
7 treated pot. 157 M3s were unaffected by BTH when compared with their mock
8 treatment and with the Col-0 control. Figure S1 shows a picture of one
9 candidate.

10 The 157 candidates were crossed with *npr1-1*, and their F1s tested with BTH as
11 described in Figure 1. Throughout this third selection of candidates, it was
12 decided to discard those with a weaker phenotype. With such a restrictive
13 criterion, 93 candidates remained. Their F2s were tested with BTH and SA as
14 described in Figure 1 (with the exception of *NahG* background, where growth on
15 SA plates is not informative). Eventually, counting only one allele per
16 independent family when several were found, 43 alleles of *npr1* were identified.
17 These 43 alleles are hereafter named as a group as “new” alleles. The
18 remaining 13 mutants that complement *npr1-1* will be described elsewhere.

20 **The New *npr1* Alleles Share some Phenotypes.**

21 Once the *npr1* alleles were identified visually, a more detailed quantification was
22 made. We used the percentage of fresh weight (PFW), that is, fresh weight of
23 plants treated with 350 μ M BTH divided by fresh weight of mock treated plants,
24 expressed in percentage. Figure 2a shows that Col-0 has a low PFW, while

1 *npr1-1* is unaffected. Since all the new alleles behaved similarly, only the first
2 nine are presented. There is some variation in the PFW, but there are no
3 reproducible differences with *npr1-1*.

4 It is plausible that a selection by BTH based on size could produce mutations
5 that still recognize BTH in terms of *Pseudomonas syringae pv tomato* DC3000
6 (*Pto*) growth. Similarly, a lack of response to BTH does not necessarily imply a
7 lack of response to SA and vice versa (e.g. *NahG*, Lawton *et al.* 1995). At least
8 for the new *npr1* alleles, four phenotypes (response to BTH in PFW, response
9 to BTH in *Pto* growth, response to SA in *Pto* growth, and tolerance to SA in
10 vitro) were absent in the same mutants (Figure 2b and data not shown). Since
11 all the new alleles behaved similarly, only the first nine are presented.

12 SA perception is sometimes checked by the expression of Pathogen Related
13 proteins (PRs, Van Loon *et al.* 1994). In fact, some *npr1* alleles were identified
14 by the lack of *PR2* (Cao *et al.* 1994) or *PR1* (Shah *et al.* 1997) expression in
15 inductive conditions. This last protein is a reliable marker of plant distress, so a
16 western blot was performed with the new *npr1* alleles (Figure 2c) detecting
17 *PR1*. Mock treated plants show no *PR1* expression (data not shown). In
18 contrast, upon *Pto* infection, Col-0 showed a strong signal three days after
19 inoculation, while *npr1-1* showed no signal at all. From the new *npr1* alleles,
20 only *npr1-20* and *npr1-41* consistently produced some *PR1* protein, always in
21 lower levels than Col-0 (Figure 2c shows the first nine alleles and *npr1-41*). The
22 levels of *PR1* in these two alleles are not correlated with a significant defence
23 response (Figure 2b and data not shown), likely because the amount of protein

is low enough that a threshold is not reached, and/or because other defence molecules are required.

Sequence and Analysis of the Alleles

The next step was to sequence the *NPR1* gene in these alleles to identify the mutations. Table 2 shows the result of the sequence, presenting the nucleotide change, AA change, mutagen, and genetic background (*Please insert Table2 here*). There was a mutation in the structural gene in all the alleles, except in the case of *npr1-62*. All the attempts to amplify a part of this allele by PCR failed regardless of the pair of primers, or the origin of the DNA (data not shown), likely due to a large deletion or rearrangement. Some mutations are repeated two or three times. Although we cannot rule out that these repetitions are caused by contamination from one family to another, we consider them independent events based on our screening procedure described above. Note that some of these were isolated from different backgrounds, and we did not recover the *npr1-1* control (Table 2), even though it was present in all the screening batches.

The position and kind of mutation allow for a deep analysis, searching for correlations between the molecular data and the phenotype. Firstly, the simplest correlation would be a bias in the nature of the wt AA that has been mutated (e.g. more mutated polar AA than the average). There are 544 AA indices (www.genome.jp/aaindex/, V9.1), but no bias was found for any index when the values of the wt AA subject to mutation were compared with the average value all the AAs that make up NPR1 (see Methods for details). Since EMS is able to

1 mutate AAs to a different extent (Sega 1984), and a significant amount of the
2 mutations recovered are produced by EMS, an additional calculation was made.
3 This time only the canonical point mutations caused by EMS (22) were
4 compared with all the AAs that could be mutated by EMS in NPR1 (413). Again,
5 no bias was detected (data not shown).

6 Secondly, the nature of the change was considered. Thus, the mutations could
7 be biased in the properties of the new AAs (e.g. a trend towards more polar
8 AAs). There are 94 AA substitution matrices (www.genome.jp/aaindex/, V9.1),
9 but no bias was found. Note that we can only consider the EMS mutations that
10 produce a point mutation in order to have a background (all the possible new
11 AAs that EMS can produce in NPR1, 516) that can be contrasted with the
12 observable mutations.

13 Thirdly, the conservation of the AA could also mark the mutations. There are
14 five paralogs of *NPR1* in Arabidopsis, *NPR2*, *NPR3*, *NPR4*, *BOP1* and *BOP2*.
15 The predicted sequences of the proteins were aligned with CLUSTALW
16 (Chenna *et al.* 2003), which provides a numerical value for each AA based on
17 its conservation. The AAs mutated in the alleles recovered in this work (EMS
18 point mutations) are more likely to be conserved among the paralogs, in a
19 modest but significant fashion (Student's T test, $p \approx 0.03$).

20 Fourthly, we also analyzed whether the mutations were clustered. Figure 3a
21 shows the localization of the mutations in the backbone of the protein. It is
22 obvious that the mutations are not clustered in any domain, but were more
23 frequent in a small region, between the ankyrin repeats and the nuclear
24 localization signals (see below). Some of the mutations are in introns (Figure

3b), but since they are at the beginning or the end of an intron, they affect the splicing and the coding sequence. Figure 3c shows RT-PCR with primers designed to amplify exon junctions. *npr1-54* (also *npr1-23*, data not shown) does not splice the first intron properly, introducing a frameshift in the coding sequence. In *npr1-26* (also *npr1-29* and *npr1-43*, data not shown) the third intron is not spliced, again creating a frameshift. A peculiar case occurs with *npr1-20*. In this allele, the mutation is a change from Valine to Methionine in the position 501 (Table 2). Since this is a conservative change, the wt AA had to be a very important one or have an additional effect. Indeed, the mutated nucleotide is the first one of the fourth exon, and it does alter the splicing of the fourth exon (Figure 3c).

The stop codons obtained are not randomly distributed, but clustered in a small region. The coding sequence of NPR1 was divided into windows of 50 AAs (Tornero *et al.* 2002a), and the number of stop codons found in each window plotted (Figure 4a). The tenth window is enriched in stop codons, and the statistical significance of this enrichment can be calculated with a Poisson distribution (for 3 alleles/50 AA, $p \approx 0.042$). The frameshifts are not point mutations, nor stop codons, since there are additional AAs introduced before a stop codon is found. However, if the frameshifts were to be considered equal to the stop codons, the same window (plus an additional one between 150 to 200 AA) is significantly enriched (Figure S2a). Extending the argument, if we were to consider not only the *npr1* alleles described here, but also the ones obtained before (5 alleles between stops and frameshifts, Cao *et al.* 1994, Delaney *et al.*

1995, Glazebrook *et al.* 1996, and Shah *et al.* 1997), the result still holds (Figure S2b).

There are 25 point mutations in this screening. Surprisingly, there is no enrichment in the described domains of NPR1, but in the region between the ankyrin repeats and the NLS (Figure 4b, windows from AA 400 to 550). In this case, the probability of this clustering being a random event is lower than before (Poisson distribution for 7 alleles/50 AA, $p \approx 0.004$). As happened with the stop codons, the biased distribution still holds with the inclusions of point mutations from previous screenings (Figure S2c). The only difference is that two windows show mutations clustered instead of three, but with a higher significance ($p \approx 0.001$).

The last two points (conservation and localization of the mutations) are related. Thus, 68% of the point mutations are found in 15% of NPR1 (17 out of 25 mutations in the region between AAs 428 and 515). This region is highly conserved in NPR2, NPR3 and NPR4, but not with BOP1 and BOP2 (Figure 4c). Although there is no homology with a described domain, this region has been described as a repression region of NPR1 (Rochon *et al.* 2006).

Fifthly, there could be more quantitative information in the position. Since there is some variation in the strength of alleles in terms of PFW (Figure 2a), we searched for a correlation with the position. There is no significant correlation between stops and point mutations when ordered by phenotype (data not shown) and their localization in the protein.

***npr1* Null Alleles**

From the information presented we conclude two important points. The screening did not produce a clear *npr1* null allele, and the point mutations obtained are clustered. The logical step was to check if there was a question of lethality (i.e. NPR1 has an essential role and only mutations that do not alter that role are recovered) or redundancy (SA perception is accomplished by other proteins, and only mutations that affect NPR1 and other proteins are recovered). It follows that it would be a partial redundancy since the screening provided a majority of *npr1* alleles (43 out of 56 or 77%, considering only one mutant per M2 family). We reasoned that the existence of *npr1* null alleles would discern between these two possibilities, so a search in the databases for knockouts in genes was initiated. Three insertions that could be taken to homozygosis were identified at the time of the search (Figure 5a). *npr1*-63 is in Col-0 background, while *npr1*-70 and *npr1*-71 are in Laer-0 (see Methods for details). While *npr1*-63 could produce a protein with the two known domains, *npr1*-70 and *npr1*-71 contain transposon insertions at AA 55 and 40, respectively. Using the same set of primers of Figure 3c, no mRNA was detected in *npr1*-70 and *npr1*-71 (data not shown), so both were considered as null alleles.

The phenotype of response to BTH in terms of fresh weight is strikingly intermediate in *npr1*-70 and *npr1*-71 (Figure 5b), low enough to avoid selection in our screening. This intermediate response could be attributed to a difference in ecotypes, since both null alleles are in Laer-0 background. An F2 between *npr1*-1 and Laer-0 analyzed with BTH as in Figure 1a did not deviate from the

1 expected results (Figure S3a), so if there is any difference between the alleles,
2 it is not detectable to the naked eye. In order to have a proper control, *npr1-1*
3 was crossed with Laer-0 three successive times with marker selection. Four
4 independent lines were selected in the F2 of the third cross for *npr1-1*
5 homozygosis, and all four behave similarly to *npr1-1* in response to BTH (Figure
6 S3b). Therefore, only one line was included in the experiments, named as *npr1-1_L*
7 (Figure 5b). The effect of BTH in the biomass of the null *npr1* alleles set them
8 far apart from the other alleles (Figure 5b), but when SA or BTH treatment is
9 followed by *Pto* growth, the differences are smaller, but still significant
10 (Student's T test, Figure 5c). Qualitatively, the alleles are also intermediates in
11 their behaviour on MS-SA plates (Figure 5d; there are no differences between
12 Col-0 and Laer-0 in these plates, data not shown). As in *npr1-1* and in most of
13 the alleles presented here, the expression of PR1 in inductive conditions is not
14 detectable in *npr1-70* and *npr1-71* (data not shown).

16 **NPR1 Paralogs**

17 The conclusions from the previous results are that the null alleles exist and
18 express an intermediate phenotype. Hence the lethality hypothesis to explain
19 the lack of null alleles in our screen is not valid, and the results are consistent
20 with a partial redundancy in SA signalling. So the question that arises is the
21 identification of these protein(s). The simplest explanation is that proteins with a
22 similar sequence perform similar functions. In other words, the paralogs of
23 *NPR1* could have a role in SA signalling. There are five paralogs of *NPR1* in
24 Arabidopsis, and the knockouts of these genes in a *NPR1* wt background do not

1 have a strong impact on SA signalling (Canet *et al.* 2010). Regarding *NPR2*, our
2 initial characterization was carried out with *npr2-1*, which has a T-DNA in an
3 intron. Since this could be a functional allele, a second allele, *npr2-2*, with a T-
4 DNA inserted in the first exon was used (Figure S4). Crosses of *npr1-70* and
5 *npr1-71* with *npr2-2*, *npr3 npr4*, and *bop1 bop2* were carried out. The resulting
6 F2s are a mixture of ecotypes, so we analyzed by weight across F2 pools to
7 take into account the ecotype variation. The BTH treatment guarantees that the
8 null alleles are selected, and by picking up the biggest plants in each family any
9 additional effect of the paralogs will be detected (if such effect exists). Figure 6a
10 shows these F2s, including the F2 with Col-0 as a baseline. All the F2s of *npr1*
11 nulls with paralogs have more biomass than the F2s with Col-0. To further
12 confirm this result, F2 plants were selected by BTH and PCR to found the
13 double mutants between *npr1-70* and the paralogs. 8 independent lines of *npr1-*
14 *70 npr2-2* were characterized, along with 8 independent introgressions of *npr1-*
15 *70* in Col-0 as a control. Figure 6b shows the maximal and minimal values of
16 each set of lines. A T-test with the pooled values of all the lines indicates that
17 *NPR2* has an impact in SA perception ($p \approx 3E-5$). This impact is also found in
18 the other paralogs, from which 4 (*npr1-70 npr3*, *npr1-70 npr4* *npr1-70 bop2*) and
19 2 (*npr1-70 bop1*) independent lines were selected. The degree of certainty is
20 not as strong as in *npr2*, but it is still significant (*npr3*, $p \approx 4E-3$; *npr4*, $p \approx 5E-3$;
21 *bop2*, $p \approx 5E-3$; *bop1* $p \approx 0.04$). Therefore, all the paralogs have a measurable
22 function in SA perception in an *npr1* null background. The increase in biomass
23 is small, and yet the five paralogs explain the difference between *npr1* nulls and
24 alleles like *npr1-1*.

1 **Discussion**

2 **Genes Required for SA Signalling.**

3 The screening described in Figure 1 and Table 1 has provided us with a wealth
4 of information about the genes required for SA signalling. It is difficult to
5 compare different mutagenesis, even when the same mutagen at the same
6 dose was used. Nevertheless, and considering only EMS, there are 69 mutants
7 in 600 M2 families. In a similar selection, 110 mutants in the recognition of
8 *avrRpm1* were detected in 172 M2 families (Tornerio *et al.* 2002a), more than
9 five times the amount presented here. The main reason for the abundance of
10 mutations was the 95 alleles of *RPM1*, a gene that is deleted in some ecotypes
11 (Grant *et al.* 1998), so is not essential for the plant. Assuming that we recover
12 all the mutants (as in the proof of concept of Figure 1a), the low numbers of
13 mutants found indicate that lethality and/or redundancy limits their number.

14 We cannot discard the idea that the model used, loss of biomass upon BTH
15 treatment, is limiting the scope of mutants obtained. Our previous work done
16 with the model suggests that, upon BTH treatment, one branch stops the growth
17 of the plant ("scorched earth defence"), while another produces active defences
18 against the pathogen (Canet *et al.* 2010). Therefore, we could lose mutants that
19 are impaired in pathogen response, but not in biomass response. In any case,
20 the mutants in SA recognition itself would be enriched, even if we do not gain
21 knowledge about the branch that stops the pathogen. Regarding the possible
22 bias in the mutants, previous screenings using pathogens (Glazebrook *et al.*
23 1996) or gene expression (Cao *et al.* 1994; Shah *et al.* 1997) gave only *npr1*
24 alleles, so it seems that we have not introduced any bias, but expanded the

1 identification of *npr1* alleles and other genes. It is also important to notice that
2 all the new *npr1* alleles obtained with BTH are not responsive to SA in terms of
3 pathogen growth and in growth in MS-SA plates. Therefore, and for the data
4 here presented, a screen for lack of response to BTH leads to mutants that do
5 not perceive SA.

6 7 **The Mutations in *npr1* are Clustered.**

8 All the alleles recovered show a similar phenotype in weight and in *Pto* growth
9 upon defence induction (Figure 2a and b). Also, there is no discrimination of the
10 chemicals SA and BTH by the new *npr1* alleles. The main difference is the
11 phytotoxicity of SA (Figure 1b and data not shown), and the stronger resistance
12 triggered by BTH with respect to SA (Figure 2a).

13 The results obtained with the marker PR1 were unexpected (Figure 2c), since
14 there are two alleles that produce a low but detectable amount of this defence
15 marker in all the experiments. The case of *npr1*-20 is unique, since the
16 nucleotide mutation leads to a prediction of a conserved change (Valine to
17 Methionine), but experimentally there is evidence for a different splicing (Figure
18 3c). The mutation could produce a fraction of *NPR1* mRNA with the right
19 splicing and a conserved change which would explain PR1 production.
20 Regarding *npr1*-41, the mutation is a change from Glutamic acid to Lysine, a
21 change from acidic to basic AA that is recovered in other mutated AAs (Table
22 2). The only particular feature of this allele is that it is located in the ankyrin
23 repeats, where few mutations are localized. A mutation like this in one of the

1 ankyrin repeats may compromise the overall function of the protein, but not
2 abolish the induction of PR1.

3 The new *npr1* alleles are not biased in a considerable number of features
4 (www.genome.jp/aaindex). There is no preference for any property of AA to be
5 mutated from or to, including the cysteines that have been reported to be critical
6 for the oligomerization (two cysteines out of the nine tested by Mou *et al.* 2003).

7 More importantly, the known domains of NPR1 do not have more mutations
8 than the rest of the protein. Note that we assume that all the nucleotides have
9 an equal probability of being affected by the mutagen, and what changes is the
10 selection based on phenotype (lethal, wt, or mutant). In the case of EMS (86%
11 of the alleles) this is experimentally true, since all the regions of the genome
12 have a similar probability of being mutated. There is a local compositional bias,
13 but it does not produce any significant difference between triplets (Greene *et al.*
14 2003). Since the mutagens do not have a preference for any region, it follows
15 that there are a number of alleles that are not recovered. They could be
16 detrimental to the plant, or could produce a phenotype similar to the wt allele.

17 We searched in the databases for alleles of *NPR1* (thus without selection) that
18 could differentiate between the two explanations; lethality vs. redundancy. Two
19 of these alleles have an insertion very early in the gene and they can be taken
20 to homozygosis (Figure 5a). Furthermore, their phenotype in terms of weight is
21 intermediate (Figure 5b). Although the intermediate phenotype is quantitative
22 strong, it is close to the threshold of detection in a screening (Figure 1c),
23 explaining why no null alleles were recovered in this screening. We speculate
24 that only mutations that affect *NPR1* and have a supplementary negative effect

1 are recovered. This additional effect would be to inhibit other proteins that could
2 work in the SA signalling pathway. In any case, lethality can be ruled out, and
3 the results fit with a partial redundancy in SA perception. It is partial because
4 the null alleles of *NPR1* produce a quantitative strong effect, and *npr1* alleles
5 were 77 % of the mutants recovered.

6 The *npr1* null alleles are in Laer-0 background. We discarded the possibility that
7 the intermediate phenotype is due to the difference between ecotypes based on
8 several facts. There are no significant QTLs between Col-0 and Laer-0 in terms
9 of PFW (Canet *et al.* 2010). Besides, there is no observable variation of *npr1-1*
10 in Laer-0, with segregations that fit the expected value (Figure S3a), and the
11 introgression lines of *npr1-1* in Laer-0 behave similarly (Figure S3b). Lastly, the
12 F2 or F3s of the nulls with Col-0 show that, while there is variation due to
13 different lines isolated, there is no difference between ecotypes when the null
14 alleles are present (Figure 6a and 6b). Therefore, there is no intrinsic difference
15 between the ecotypes and there is no specific interaction of the alleles with one
16 ecotype.

18 **NPR1 Paralogs Have a Role in BTH Perception.**

19 The existence of *npr1* nulls with an intermediate phenotype reveals that other
20 proteins besides NPR1 are acting in BTH perception at the same point. The
21 mutations are enriched in AA conserved among the NPR1 clade, and are
22 clustered in a conserved region (Figure 4c). This region coincides almost
23 perfectly to a repression region described in NPR1 (AAs 463 to 513, Rochon *et al.*
24 *et al.* 2006). Although the repression region was not functional in SA stimulated

1 cells, the bias observed in the mutations could indicate a stronger repression,
2 perhaps extending its function in SA stimulated cells.

3 Thus, a simple model that can explain the bias in the mutations is that they
4 could enhance the mentioned repression region, locking the protein in a
5 repression state when it is close to the target DNA. The problem with this
6 hypothesis is that -while explaining the observed bias- it does not explain how
7 some mutations have a strong phenotype, without having a repression region
8 (i.e. stops and frameshifts). A second alternative is that a mutated *npr1* protein
9 could interact negatively with interactors of NPR1 reducing the availability of
10 these proteins. There are two families of NPR1 interactors described so far.
11 *NIMINs* are repressors of *NPR1* (Weigel *et al.* 2005), and there is no known
12 interaction with the *NPR1* paralogs, so there is no simple model that takes into
13 account all the requirements. The *TGAs*, on the other hand, are members of a
14 multigenic family (Jakoby *et al.* 2002), that also interact with NPR3, NPR4
15 (Zhang *et al.* 2006), BOP1, and BOP2 (Hepworth *et al.* 2005), so they seem a
16 clear candidate to be titred out by the *npr1* mutated proteins. A third option can
17 be postulated without requiring interactors. If the paralogs have the same
18 mechanisms of oligomerization as NPR1 does (Tada *et al.* 2008), an *npr1*
19 defective protein could interfere with the monomerization of the paralogs,
20 reducing their activity.

21 The behaviour of the new *npr1* alleles does not fit into conventional recessive or
22 dominant negative alleles, but into a mixture of properties. When considering
23 only *NPR1*, they are recessive, but when two copies of the allele are mutated,
24 they interact with the paralogs as dominant negatives. A similar mechanism has

1 been described before for closely related paralogs. Five recessive alleles of
2 *hsp90.2* show a phenotype of loss of recognition of *avrRpm1*, while the
3 knockout does not have any distinctive phenotype (Hubert *et al.* 2003; Hubert *et*
4 *al.* 2009). The model proposed was that the HSP90s interact with RPM1, and a
5 knockout is functionally replaced by the remaining paralogs. In this model, only
6 mutations that abolish the function of HSP90.2 and interact negatively with
7 other paralogs (HSP90s form heterodimers, Richter *et al.* 2001) are recovered.
8 The fact that all the alleles recovered are in the same gene leads the authors to
9 propose a certain specificity or preference. The analogies with NPR1 are not
10 complete, but notable. The differences are that NPR1 nulls have a measurable
11 phenotype and the number of NPR1 alleles recovered is one order of
12 magnitude higher.

13 Following a similar model, we propose that all the members of the *NPR1* clade
14 participate in the perception of BTH, with a strong preference for *NPR1*. This
15 model explains the results of the screening: the low number of *NPR1* alleles,
16 the lack of null alleles, and the clustering of the mutations (see below). There is
17 a precedent in this family of an allele with an analogous behaviour to the new
18 *npr1* alleles; *bop1-1* has a stronger phenotype than several *bop1* nulls (Ha *et al.*
19 2004).

20 It is tempting to speculate with a correlation between the homology with NPR1
21 and the importance of the paralogs in BTH/SA perception. The order from more
22 homology to less is *NPR2*, *NPR4*, *NPR3*, *BOP1* and *BOP2* (Liu *et al.* 2005).

23 The F2 and F3 data of Figure 6 predict a role for all the paralogs with different
24 degrees, from a strong one for *NPR2*, to a weaker, yet significant, for *BOP1*.

1 There are several arguments in favour of a possible degree of functionality.
2 Thus, *NPR2*, *NPR3* and *NPR4* are slightly induced upon biotic stress (like
3 *NPR1*), while *BOP1* and *BOP2* are repressed (www.arabidopsis.org). Also, the
4 clustering of the mutations in *NPR1* is stronger in a region that it is highly
5 conserved with *NPR2*, *NPR3* and *NPR4*, but not with *BOP1* or *BOP2* (Figure
6 4c), so there is ample evidence for a predominant role of the three closer
7 paralogs. Of course the model proposed does not exclude that other genes are
8 required in BTH/SA perception, and they could be the mutations that
9 complement *npr1*.

Acknowledgments

This work was supported by a BIO2006-02168 grant from Ministerio de Ciencia e Innovación (MICINN) of Spain to PT, a JAE-CSIC Fellowship to JVC and a FPI-MICINN to AD. We thank the English translation service of the Universidad Politécnica de Valencia. We appreciate the BTH provided by Syngenta. Thanks also to Jeff Dangl, Pablo Vera and José León for helpful comments on the manuscript.

References

- Alonso, J. M., A. N. Stepanova, T. J. Leisse, C. J. Kim, H. Chen, P. Shinn, D. K. Stevenson, J. Zimmerman, P. Barajas, R. Cheuk, *et al.* (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science*. **301**(5633): 653-7.
- Benjamini, Y. and B. Hochberg (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B* **57**(1): 289-300.
- Boyes, D. C., J. Nam and J. L. Dangl (1998). The *Arabidopsis thaliana* RPM1 disease resistance gene product is a peripheral plasma membrane protein that is degraded coincident with the hypersensitive response. *Proc. Natl. Acad. Sci., USA* **95**: 15849-15854.
- Canet, J. V., A. Dobón, F. Ibáñez, L. Perales and P. Tornero (2010). Resistance and biomass in *Arabidopsis*: a new model for Salicylic Acid perception. *Plant Biotechnology Journal* **8**: 126-141.
- Cao, H., S. A. Bowling, A. S. Gordon and X. Dong (1994). Characterization of an *Arabidopsis* Mutant That Is Nonresponsive to Inducers of Systemic Acquired Resistance. *Plant Cell* **6**(11): 1583-1592.
- Chenna, R., H. Sugawara, T. Koike, R. Lopez, T. J. Gibson, D. G. Higgins and J. D. Thompson (2003). Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res.* **31**(13): 3497-500.
- Delaney, T. P., L. Friedrich and J. A. Ryals (1995). *Arabidopsis* signal transduction mutant defective in chemically and biologically induced disease resistance. *Proc Natl Acad Sci U S A.* **92**(14): 6602-6.

1 Glazebrook, J., E. E. Rogers and F. M. Ausubel (1996). Isolation of *Arabidopsis*
2 mutants with enhanced disease susceptibility by direct screening. *Genetics* **143**:
3 973-982.

4 Grant, M. R., L. Godiard, E. Straube, T. Ashfield, J. Lewald, A. Sattler, R. W.
5 Innes and J. L. Dangl (1995). Structure of the *Arabidopsis RPM1* gene enabling
6 dual specificity disease resistance. *Science* **269**: 843-846.

7 Grant, M. R., J. M. McDowell, A. G. Sharpe, M. de Torres Zabala, D. J. Lydiate
8 and J. L. Dangl (1998). Independent deletions of a pathogen-resistance gene in
9 *Brassica* and *Arabidopsis*. *Proc. Natl. Acad. Sci., USA* **95**: 15843-15848.

10 Greene, E. A., C. A. Codomo, N. E. Taylor, J. G. Henikoff, B. J. Till, S. H.
11 Reynolds, L. C. Enns, C. Burtner, J. E. Johnson, A. R. Odden, *et al.* (2003).
12 Spectrum of chemically induced mutations from a large-scale reverse-genetic
13 screen in *Arabidopsis*. *Genetics*. **164**(2): 731-40.

14 Ha, C. M., J. H. Jun, H. G. Nam and J. C. Fletcher (2004). BLADE-ON-
15 PETIOLE1 encodes a BTB/POZ domain protein required for leaf
16 morphogenesis in *Arabidopsis thaliana*. *Plant Cell Physiol.* **45**(10): 1361-70.

17 Ha, C. M., J. H. Jun, H. G. Nam and J. C. Fletcher (2007). BLADE-ON-
18 PETIOLE 1 and 2 control *Arabidopsis* lateral organ fate through regulation of
19 LOB domain and adaxial-abaxial polarity genes. *Plant Cell.* **19**(6): 1809-25.
20 Epub 2007 Jun 29.

21 Hepworth, S. R., Y. Zhang, S. McKim, X. Li and G. W. Haughn (2005). BLADE-
22 ON-PETIOLE-dependent signaling controls leaf and floral patterning in
23 *Arabidopsis*. *Plant Cell.* **17**(5): 1434-48. Epub 2005 Apr 1.

1 Hubert, D. A., Y. He, B. C. McNulty, P. Tornero and J. L. Dangl (2009). Specific
2 Arabidopsis HSP90.2 alleles recapitulate RAR1 cochaperone function in plant
3 NB-LRR disease resistance protein regulation. *Proc Natl Acad Sci U S A*.
4 **106**(24): 9556-63. Epub 2009 Jun 1.

5 Hubert, D. A., P. Tornero, Y. Belkhadir, P. Krishna, A. Takahashi, K. Shirasu
6 and J. Dangl (2003). Cytosolic HSP90 associates with and modulates the
7 Arabidopsis RPM1 disease resistance protein. *EMBO J*. **22**(21): 5679-89.

8 Jakoby, M., B. Weisshaar, W. Droge-Laser, J. Vicente-Carbajosa, J.
9 Tiedemann, T. Kroj and F. Parcy (2002). bZIP transcription factors in
10 Arabidopsis. *Trends Plant Sci*. **7**(3): 106-11.

11 Jones, J. D. and J. L. Dangl (2006). The plant immune system. *Nature*.
12 **444**(7117): 323-9.

13 Lawton, K. A., L. Friedrich, M. Hunt, K. Weymann, T. Delaney, H. Kessmann, T.
14 Staub and J. Ryals (1996). Benzothiadiazole induces disease resistance in
15 Arabidopsis by activation of the systemic acquired resistance signal
16 transduction pathway. *Plant J* **10**(1): 71-82.

17 Lawton, K. A., K. Weymann, L. Friedrich, B. Vernooij, S. Uknes and J. Ryals
18 (1995). Systemic acquired resistance in Arabidopsis requires salicylic acid but
19 not ethylene. *Mol Plant Microbe Interact*. **8**(6): 863-70.

20 Liu, G., E. B. Holub, J. M. Alonso, J. R. Ecker and P. R. Fobert (2005). An
21 Arabidopsis NPR1-like gene, NPR4, is required for disease resistance. *Plant J*.
22 **41**(2): 304-18.

1 Maleck, K., U. Neuenschwander, R. M. Cade, R. A. Dietrich, J. L. Dangl and J.
2 A. Ryals (2002). Isolation and characterization of broad-spectrum disease-
3 resistant Arabidopsis mutants. *Genetics* **160**(4): 1661-71.

4 Malmberg, R. L. (1993). Production and Analysis of Plant Mutants, emphasizing
5 *Arabidopsis thaliana*. *Methods in Plant Molecular Biology and Biotechnology*. B.
6 R. Glick and J. E. Thompson, CRC Press, Inc.

7 McKim, S. M., G. E. Stenvik, M. A. Butenko, W. Kristiansen, S. K. Cho, S. R.
8 Hepworth, R. B. Aalen and G. W. Haughn (2008). The BLADE-ON-PETIOLE
9 genes are essential for abscission zone formation in Arabidopsis. *Development*.
10 **135**(8): 1537-46. Epub 2008 Mar 13.

11 Mou, Z., W. Fan and X. Dong (2003). Inducers of plant systemic acquired
12 resistance regulate NPR1 function through redox changes. *Cell*. **113**(7): 935-44.

13 Nawrath, C., J. P. Métraux and T. Genoud (2005). Chemical signals in plant
14 resistance: salicylic acid. . *Multigenic and Induced Systemic Resistance in*
15 *Plants*. S. Tuzun and E. Bent. Dordrecht, Netherlands., Springer US: pp. 143–
16 165.

17 Richter, K., P. Muschler, O. Hainzl and J. Buchner (2001). Coordinated ATP
18 hydrolysis by the Hsp90 dimer. *J Biol Chem*. **276**(36): 33689-96. Epub 2001 Jul
19 5.

20 Ritter, C. and J. L. Dangl (1996). Interference between two specific pathogen
21 recognition events mediated by distinct plant disease resistance genes. *Plant*
22 *Cell* **8**: 251-257.

23 Rochon, A., P. Boyle, T. Wignes, P. R. Fobert and C. Despres (2006). The
24 coactivator function of Arabidopsis NPR1 requires the core of its BTB/POZ

domain and the oxidation of C-terminal cysteines. *Plant Cell*. **18**(12): 3670-85. Epub 2006 Dec 15.

Sega, G. A. (1984). A review of the genetic effects of ethyl methanesulfonate. *Mutation Research* **134**: 113-142.

Shah, J., F. Tsui and D. F. Klessig (1997). Characterization of a salicylic acid-insensitive mutant (*sai1*) of *Arabidopsis thaliana* identified in a selective screen utilizing the SA-inducible expression of the *tms2* gene. *Molec. Plant-Microbe Interact.* **10**: 69-78.

Spoel, S. H., Z. Mou, Y. Tada, N. W. Spivey, P. Genschik and X. Dong (2009). Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. *Cell*. **137**(5): 860-72.

Sundaresan, V., P. Springer, T. Volpe, S. Haward, J. D. Jones, C. Dean, H. Ma and R. Martienssen (1995). Patterns of gene action in plant development revealed by enhancer trap and gene trap transposable elements. *Genes Dev.* **9**(14): 1797-810.

Tada, Y., S. H. Spoel, K. Pajerowska-Mukhtar, Z. Mou, J. Song, C. Wang, J. Zuo and X. Dong (2008). Plant immunity requires conformational changes of NPR1 via S-nitrosylation and thioredoxins. *Science*. **321**(5891): 952-6. Epub 2008 Jul 17.

Tornero, P., R. A. Chao, W. N. Luthin, S. A. Goff and J. L. Dangl (2002a). Large scale structure-function analysis of the Arabidopsis RPM1 disease resistance protein. *The Plant Cell* **14**: 435-450.

1 Tornero, P. and J. L. Dangi (2001). A high throughput method for quantifying
2 growth of phytopathogenic bacteria in *Arabidopsis thaliana*. *The Plant Journal*
3 **28**(4): 475-481.

4 Tornero, P., P. Merritt, A. Sadanandom, K. Shirasu, R. W. Innes and J. L. Dangi
5 (2002b). RAR1 and NDR1 contribute quantitatively to disease resistance in
6 *Arabidopsis*, and their relative contributions are dependent on the R gene
7 assayed. *Plant Cell* **14**(5): 1005-15.

8 van Leeuwen, H., D. J. Kliebenstein, M. A. West, K. Kim, R. van Poecke, F.
9 Katagiri, R. W. Michelmore, R. W. Doerge and D. A. St Clair (2007). Natural
10 variation among *Arabidopsis thaliana* accessions for transcriptome response to
11 exogenous salicylic acid. *Plant Cell*. **19**(7): 2099-110. Epub 2007 Jul 13.

12 Van Loon, L. C., W. S. Pierpoint, T. Boller and V. Conejero (1994).
13 Recommendations for naming plant pathogenesis-related proteins. *Plant Mol.*
14 *Biol. Rep.* **12**: 245-264.

15 Vlot, A. C., D. F. Klessig and S. W. Park (2008). Systemic acquired resistance:
16 the elusive signal(s). *Curr Opin Plant Biol.* **11**(4): 436-42. Epub 2008 Jul 9.

17 Wang, D., N. D. Weaver, M. Kesarwani and X. Dong (2005). Induction of protein
18 secretory pathway is required for systemic acquired resistance. *Science*.
19 **308**(5724): 1036-40.

20 Ward, E. R., S. J. Uknes, S. C. Williams, S. S. Dincher, D. L. Wiederhold, D. C.
21 Alexander, P. Ahl-Goy, J. P. Metraux and J. A. Ryals (1991). Coordinate Gene
22 Activity in Response to Agents That Induce Systemic Acquired Resistance.
23 *Plant Cell*. **3**(10): 1085-1094.

1 Weigel, R. R., C. Bauscher, A. J. Pfitzner and U. M. Pfitzner (2001). NIMIN-1,
2 NIMIN-2 and NIMIN-3, members of a novel family of proteins from Arabidopsis
3 that interact with NPR1/NIM1, a key regulator of systemic acquired resistance in
4 plants. *Plant Mol Biol* **46**(2): 143-60.

5 Weigel, R. R., U. M. Pfitzner and C. Gatz (2005). Interaction of NIMIN1 with
6 NPR1 modulates PR gene expression in Arabidopsis. *Plant Cell*. **17**(4): 1279-
7 91. Epub 2005 Mar 4.

8 Zhang, Y., Y. T. Cheng, N. Qu, Q. Zhao, D. Bi and X. Li (2006). Negative
9 regulation of defense responses in Arabidopsis by two NPR1 paralogs. *Plant J*.
10 **48**(5): 647-56. Epub 2006 Oct 31.

11 Zhang, Y., W. Fan, M. Kinkema, X. Li and X. Dong (1999). Interaction of NPR1
12 with basic leucine zipper protein transcription factors that bind sequences
13 required for salicylic acid induction of the PR-1 gene. *Proc Natl Acad Sci U S A*
14 **96**(11): 6523-8.

15 Zhang, Y., M. J. Tessaro, M. Lassner and X. Li (2003). Knockout analysis of
16 Arabidopsis transcription factors TGA2, TGA5, and TGA6 reveals their
17 redundant and essential roles in systemic acquired resistance. *Plant Cell*.
18 **15**(11): 2647-53. Epub 2003 Oct 23.

19

20

1 Tables

2 **Table 1**

<u>Mutagens</u>	<u>Families</u>	<u>M2s</u> <u>(x10E6)</u>	<u>First</u> <u>selection</u>	<u>Second</u> <u>selection</u>	<u>Third</u> <u>selection</u>	<u><i>npr1</i></u> <u>alleles</u>
EMS	600	2.16	166	104	69	37
T-DNAs	543	1.96	46	21	7	2
Gamma	140	0.50	95	13	7	2
FN	180	0.65	70	19	10	2
Total	1463	5.27	377	157	93	43

3

4 **Table 1. Mutants obtained in this screening.** Seeds from plants treated with

5 different mutagens (first column) were selected by BTH and then by size as

6 described in Figure 1. The second column provides the number of independent

7 families, and the third column is the number of seeds screened. The fourth

8 column gives the number of mutants recovered as M2s and the fifth the number

9 of mutants retested in M3 and selected to cross with *npr1-1*. The sixth column

10 shows the mutants that still have a strong phenotype and the seventh the

11 number of *npr1* alleles found from independent families. EMS stands for ethyl

12 methane sulfonate, T-DNAs for Activation tagging, Gamma for Gamma Rays,

13 and FN for Fast Neutrons.

1 **Table 2**

<u>Allele</u>	<u>Nt Mutation</u>	<u>AA mutation</u>	<u>Mutagen</u>	<u>Background</u>
<i>npr1-20</i>	G to A	V501M and splicing 4 th exon	EMS	<i>rar1-21</i>
<i>npr1-21</i>	C to T	Q384*	EMS	<i>rar1-21</i>
<i>npr1-22</i>	G to A	R544K	EMS	<i>PR1:LUC</i>
<i>npr1-23</i>	G to A	Splicing 2 nd exon	EMS	<i>PR1:LUC</i>
<i>npr1-24</i>	C to T	L497F	EMS	<i>PR1:LUC</i>
<i>npr1-25</i>	G to A	E443K	EMS	<i>PR1:LUC</i>
<i>npr1-26</i>	G to A	Splicing 4 th exon	EMS	<i>PR1:LUC</i>
<i>npr1-27</i>	G to A	D428N	EMS	<i>PR1:LUC</i>
<i>npr1-28</i>	C to T	R538*	EMS	<i>PR1:LUC</i>
<i>npr1-29</i>	G to A	Splicing 4 th exon	EMS	<i>PR1:LUC</i>
<i>npr1-30</i>	C to T	A451V	EMS	<i>PR1:LUC</i>
<i>npr1-31</i>	G to A	E449K	EMS	<i>PR1:LUC</i>
<i>npr1-32</i>	G to A	R432K	EMS	<i>PR1:LUC</i>
<i>npr1-33</i>	G to A	R432K	EMS	<i>PR1:LUC</i>
<i>npr1-34</i>	C to T	Q526*	EMS	<i>PR1:LUC</i>
<i>npr1-35</i>	G to A	C155Y	EMS	<i>PR1:LUC</i>
<i>npr1-36</i>	G to A	E449K	EMS	<i>PR1:LUC</i>
<i>npr1-37</i>	C to T	Q491*	EMS	<i>PR1:LUC</i>
<i>npr1-38</i>	G to A	R493K	EMS	<i>RPM1-MYC</i>
<i>npr1-39</i>	C to T	S512L	EMS	<i>rpm1-1</i>
<i>npr1-40</i>	G to A	C306Y	EMS	<i>rpm1-1</i>

<i>npr1-41</i>	G to A	E288K	EMS	<i>rpm1-1</i>
<i>npr1-42</i>	G to A	G504E	EMS	<i>rpm1-1</i>
<i>npr1-43</i>	G to A	Splicing 4 th exon	EMS	<i>NahG</i>
<i>npr1-44</i>	C to T	H80Y	EMS	<i>NahG</i>
<i>npr1-45</i>	C to T	Q 371*	EMS	<i>NahG</i>
<i>npr1-46</i>	C to T	L497F	EMS	<i>NahG</i>
<i>npr1-47</i>	C to T	L497F	EMS	<i>NahG</i>
<i>npr1-48</i>	C to T	L515F	EMS	<i>NahG</i>
<i>npr1-49</i>	C to T	Q491*	EMS	<i>NahG</i>
<i>npr1-50</i>	C to T	L274F	EMS	<i>NahG</i>
<i>npr1-51</i>	G to A	R432K	EMS	<i>rar1-21 ndr1-1</i>
<i>npr1-52</i>	Deletion	N210FS	EMS	<i>rar1-21 ndr1-1</i>
<i>npr1-53</i>	C to T	Q343*	EMS	<i>rar1-21 ndr1-1</i>
<i>npr1-54</i>	G to A	Splicing 2 nd exon	EMS	<i>rar1-21 ndr1-1</i>
<i>npr1-55</i>	C to T	Q491*	EMS	<i>rar1-21 ndr1-1</i>
<i>npr1-56</i>	G to C	A496P	EMS	<i>rar1-21 ndr1-1</i>
<i>npr1-57</i>	Deletion	Δ VDFML 164-168	T-DNA	Col-0
<i>npr1-58</i>	T to A	Y64N	T-DNA	Col-0
<i>npr1-59</i>	Deletion	V194*	Gamma	Col-0
<i>npr1-60</i>	C to T	P342S	Gamma	Col-0
<i>npr1-61</i>	Deletion	V194*	FN	Col-0
<i>npr1-62</i>	Unknown	Unknown	FN	Col-0

Table 2. *npr1* alleles obtained in this screening. The allele number (first column) is followed by the nucleotide mutation (second), the amino acid mutation (third), the mutagen used (fourth) and the background of each allele (fifth). Where a change in nucleotide produces a change in splicing, it is noted as “Splicing” and the exon affected in the amino acid column. “FS” stand for Frameshift. We could not get a PCR product for the *npr1*-62 allele. *rar1*-21 and *rar1*-21 *ndr1*-1 are described in Tornero *et al.* 2002b, *PR1:LUC* in Maleck *et al.* 2002, *rpm1*-1 in Grant *et al.* 1995, *RPM1-MYC* in Boyes *et al.* 1998 and *NahG* in Lawton *et al.* 1995.

Figure legends

Figure 1.-Screening for non-recognition of BTH. (a) Proof of concept in soil.

3 seeds of *npr1-1* were mixed with c. 3600 seeds of Col-0 and sown. Plants were treated with 350 μ M benzothiadiazole (BTH) four times, and the picture taken at 3 weeks of growth. Only a quarter of the experiment is shown, with a single *npr1-1* plant. **(b)** Proof of concept *in vitro*. Seeds of Col-0 and *npr1-1* were mixed in a proportion of 3 to 13, respectively, and sown in MS plates containing 500 μ M salicylic acid (SA). The picture was taken at 12 days of growth. The arrows point to wt plants. **(c)** Screening with mutagenized plants. An M2 family with a putative mutant is showed, at three weeks of growth.

Figure 2.-All the *npr1* alleles show a similar phenotype in response to SA

or BTH. (a) Plants were treated with either mock or 350 μ M BTH as described in Figure 1a, their weight recorded, and the ratio between the BTH and mock treated plants represented (average and SD of 15 plants in three groups of five). The ratio is expressed as percentage of fresh weight (FW). **(b)** Plants were pretreated with mock, 500 μ M SA, or 350 μ M BTH, inoculated with *Pseudomonas syringae* pv *tomato* DC3000 (*Pto*), and the growth of *Pto* measured three days later. The bacteria are measured in Logarithm of colony forming units per plant (Logs(cfu/plant)). **(c)** Plants were inoculated with *Pto*, and tissue was sampled three days later. The defence marker PR1 was detected by western blot. Only two alleles, *npr1-20* and *npr1-41*, show consistent expression of PR1. The arrow points to the expected size of PR1 (14 kDa). For simplicity, the first nine alleles are shown in (a) and (b) plus *npr1-41* in

(c). Col-0 is a positive control and *npr1-1* a negative one for response to SA and BTH.

Figure 3.- The distribution of mutations in *npr1* is biased. The localization of the mutations found is represented along the structure of NPR1. **(a)** Mutations in exons of *NPR1* found in this screening. The structure of NPR1 with its domains is shown, along with a 50 AA scale. BTB/POZ stands for Broad-Complex, Itramtrack and Bric-a-brac proteins, Pox virus and Zinc finger proteins. Ankyrin for Ankyrin Repeat Motifs (4 of them) and NLS for Nuclear Localization Signal. The arrows indicate point mutations, the asterisks stop codons, the letter “F” frameshift, and a triangle an internal deletion. **(b)** Mutations in introns of *NPR1* found in this screening. The structure of introns and exons of *NPR1* is shown, with the position of the mutations that alter the splicing. “Ex” stands for Exon. Note that *npr1-20* is in both (a) and (b), since it belongs to both categories. **(c)** RT-PCR of the mutations in introns. The picture shows an ethidium bromide agarose gel with its molecular weights in nucleotides on the left side. Specific PCR probes for each exon-exon junction were used (labelled Intron 1 to 3) in Genomic DNA, as a positive control; H₂O, as a negative control, and RTs from the labelled genotypes. *npr1-29*, *npr1-43*, and *npr1-26* have the same mutation, and only the last one is showed. Also, *npr1-23* and *npr1-54* share the same change and the second is showed.

Figure 4.- Statistics of the distribution of mutations. (a) Distribution of stop codons. In each 50 AA window (X- axis), the number of stop codons is

1 represented as a bar (Y-axis). The schematic structure of NPR1 is represented
2 in the X-axis for visualization of the domains. The dotted line shows where the
3 distribution is not random (Poisson distribution, 3 alleles/50 AA $p < 0.05$). **(b)**
4 Distribution of point mutations, as described in (a). In this case, the dotted line is
5 at 5 alleles/50 AA (Poisson distribution, $p < 0.05$). **(c)** Distribution of mutations in
6 a selected region (AA428 to 515) of NPR1. The mutations are showed over the
7 wt AA, with the alignment of the NPR1 clade. The stop codons are marked with
8 an asterisk, and the frameshifts (due to altered intron splicing) with a “«”. The
9 repression region described by (Rochon *et al.* 2006) is marked with an arrow.

10
11 **Figure 5.-Phenotype of null *npr1*s.** **(a)** Three insertions (T-DNA and
12 transposons) were characterized, and their localization is shown. Note that
13 *npr1-70* and *npr1-71* are in background *Laer-0*. **(b)** Response to BTH in terms
14 of fresh weight, as in Figure 2a. *npr1-1_L* is an introgression of *npr1-1* in the
15 background *Laer-0*. **(c)** Response to SA and BTH in terms of *Pto* growth, as in
16 Figure 2b. The response of *npr1-70* and *npr1-71* to SA and BTH, although
17 small, is statistically significant (T-test, $p < 0.05$). **(d)** Response to SA in MS-SA
18 plates. Col-0, *npr1-1*, *npr1-70*, and *npr1-71* as in Figure 1b.

19
20 **Figure 6.-Role of the paralogs in response to BTH.** **(a)** F2s of the indicated
21 genotypes were analyzed as in Figure 2a, but selecting the biggest plants in
22 each family. The baseline is the F2 of the null *npr1* alleles with Col-0. **(b)**
23 Double mutants between *npr1-70* and the paralogs were selected by phenotype
24 and PCR in F2, and their progeny tested as in Figure 2a. Only the maximal and

1 minimal values are showed from 8 (Col-0 and *npr2-2*) and 4 (*npr3*) independent
2 lines. **(c)** Similar to b, but showing the response of the rest of paralogs. 4 (*npr4*
3 and *bop2*) and 2 independent lines (*bop1*) were analyzed. For each double
4 mutant, the pooled values of all the tested lines were found to be significantly
5 higher than the pooled values of all the *npr1-70* (Col-0) lines (T-test, $p < 0.05$).

1 **Figures**

1 **Supporting information.**

2 -Figure S1. Screening for non-recognition of BTH.

3 -Figure S2. Extending the analysis of the distribution of mutations.

4 -Figure S3. Introgression of *npr1-1* in *Laer-0*.

5 -Figure S4. T-DNA insertions in *NPR2*

6

Supporting Information

BTH	+	+	+	-
	Col-0	<i>npr1-1</i>	<i>npr1-57</i>	<i>npr1-57</i>

Figure S1.-Screening for non-recognition of BTH. Picture of a candidate of the screening after the second selection. The original name of the candidate is AT26, and it was later confirmed to be an *npr1* allele (*npr1-57*). Also shown Col-0 as a positive control and *npr1-1* as a negative control. Note that the plants have no particular phenotype upon mock treatment.

Figure S2.-Extending the analysis of the distribution of mutations. (a)

Distribution of stop codons. In each 50 AA window (X- axis), the number of stop codons is represented as a bar (Y-axis). The schematic structure of NPR1 is represented in the X-axis for visualization of the domains. The difference with Figure 4a is that here we include also frameshifts and mutations in introns that alter the splicing. The dotted line shows where the distribution is not random.

(b) Similar to (a), including stops, frameshifts and mutations that alter the splicing found in other screenings (Cao *et al.* 1994, Delaney, Friedrich & Ryals 1995, Glazebrook, Rogers & Ausubel 1996, and Shah, Tsui & Klessig 1997). **(c)**

Distribution of point mutations. The difference with Figure 4b is that here we include point mutations found in other screenings.

(a)

F2 *npr1-1* x *Laer-0*

First experiment

	wt	mut	TOTAL		
Observed	163	58	221		
	wt	mut	Chi square	Maximum Chi square	
Expected	165.75	55.25	0.1825038	<	3.8415
	(x3/4)	(x1/4)			

Second experiment

	wt	mut	TOTAL		
Observed	150	52	202		
	wt	mut	Chi square	Maximum Chi square	
Expected	151.5	50.5	0.0594059	<	3.8415

(b)

Figure S3.-Introgression of *npr1-1* in *Laer-0*. (a) Chi square distribution of an F2 from *npr1-1* x *Laer-0*. Two independent experiments are shown, and in both cases, the distribution fits in a single recessive gene. (b) Characterization of four introgression lines as described in Figure 2a. *npr1-1* was crossed to *Laer-0*, the F1s selected by PCR marker for heterozygosis at the *npr1-1* locus, and then crossed again with *Laer-0*, repeating this process three times. Then, an F2 was selected by PCR marker for *npr1-1* homozygous plants. The four lines behaved similarly. These experiments were repeated two times with similar results.

Figure S4.-T-DNA insertions in *NPR2*. **(a)** Two T-DNA insertions are shown, along the structure of *NPR2*. *npr2-1* has been previously described (Canet *et al.* 2010). **(b)** Phenotype of PFW upon BTH treatments of *npr2-1* and *npr2-2*, as described in Figure 2a.

References

Canet, J. V., A. Dobón, F. Ibáñez, L. Perales and P. Tornero (2010). Resistance and biomass in Arabidopsis: a new model for Salicylic Acid perception. *Plant Biotechnology Journal* **8**: 126-141.

Cao, H., S. A. Bowling, A. S. Gordon and X. Dong (1994). Characterization of an Arabidopsis Mutant That Is Nonresponsive to Inducers of Systemic Acquired Resistance. *Plant Cell* **6**(11): 1583-1592.

Delaney, T. P., L. Friedrich and J. A. Ryals (1995). Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance. *Proc Natl Acad Sci U S A*. **92**(14): 6602-6.

Glazebrook, J., E. E. Rogers and F. M. Ausubel (1996). Isolation of Arabidopsis mutants with enhanced disease susceptibility by direct screening. *Genetics* **143**: 973-982.

Shah, J., F. Tsui and D. F. Klessig (1997). Characterization of a salicylic acid-insensitive mutant (*sai1*) of *Arabidopsis thaliana* identified in a selective screen utilizing the SA-inducible expression of the *tms2* gene. *Molec. Plant-Microbe Interact.* **10**: 69-78.