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.

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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.
Introduction

Salicylic acid (SA) is a plant hormone required for full resistance against biotrophic pathogens. It is important in two essential defence processes: basal resistance and systemic acquired resistance (SAR, Vlot, Klessig & Park 2008). Basal resistance is triggered by compatible pathogens that do not trigger gene for gene resistance and produce disease (Jones & Dangl 2006). Thus, plants with less SA are more susceptible to compatible pathogens, and plants with more SA (either by genetic engineering or exogenous applications) are more resistant (Reviewed by Nawrath, Métraux & Genoud 2005). SAR is the resistance that is established in non-inoculated leaves of a plant that has previously been inoculated with a pathogen, generally requiring cell death (Reviewed by Vlot et al. 2008). SA is necessary but not sufficient for SAR.

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

SA is the only plant hormone where a receptor has not been described yet (Nawrath et al. 2005). It is plausible that its perception is through indirect means, like redox balances (see below). NPR1 (Non expresser of Pathogenesis Related proteins 1) is the only gene that has been found to have a profound
impact on SA signalling when mutated (Cao et al. 1994, Delaney, Friedrich & Ryals 1995, Glazebrook, Rogers & Ausubel 1996, and Shah, Tsui & Klessig 1997). There are two families of NPR1 interactors: TGAs (Zhang et al. 1999) and NIMINs (Weigel et al. 2001). Only when knocking out several genes (TGAs, Zhang et al. 2003), or expressing dominant members (NIMINs, Weigel, Pfitzner & Gatz 2005), is a measurable phenotype observed in SA signalling.

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

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

There are five paralogs of NPR1 in Arabidopsis (www.arabidopsis.org). Blade On Petiole1 (BOP1) and BOP2 have an important role in development (Ha et al. 2007; McKim et al. 2008). NPR3 and NPR4 are reported to have a role in defence (Liu et al. 2005, Zhang et al. 2006), but no function for NPR2 has been described yet. In this context, we proposed to screen for genotypes that do not recognize SA; applications of BTH on separate dates produce a small plant in the case of wild type Arabidopsis, while npr1 plants look unaffected (Canet et al. 2010). This prompted us to do a structure-function analysis of a full range of
npr1 alleles. The implications of their phenotype lead us to test also the
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 µmol m⁻² 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 (Tornero & 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 µM SA (in the form of sodium salicylate, S3007 SIGMA, St Louis, USA), and 350 µM 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 µM 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, (Boyesc, 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
references N31400, N31402, N31404, N21995, N21998, N21991, N23153, N84450, and N31100 were obtained from NASC (www.arabidopsis.info) and directly screened. The insertions \textit{npr1-63} (N831344, Alonso \textit{et al}. 2003), \textit{npr1-71} (N163245, Sundaresan \textit{et al}. 1995), and \textit{npr2-2} (N622643), were obtained from NASC, and \textit{npr1-70} (ET5232) from CSHL (www.cshl.edu). The insertions were followed to homozygosis by PCR, and their exact position checked by sequence.

\textbf{Sequence and Statistical Analysis of the Mutations.}

PCRs of the NPR1 gene were done with the oligonucleotides TP412 (5’ TGCTTCTTCATATCTCACCACCATCTCG 3’) and TP413 (5’CACCTTACACGCCCACTCAGTGTTCTCG 3’) (SIGMA). Four independent reactions were pooled and sequenced with internal oligonucleotides. The analysis of the mutations was done with Excel software (Microsoft, Redmond, USA) and custom Perl scripts. The data for the conservation of each AA in the NPR1 paralogs was obtained from CLUSTAW (Chenna \textit{et al}. 2003). The data for the different AA characteristics or mutations was downloaded from www.genome.jp/aaindex, and the background was considered to be all the possible mutations or only the EMS-induced mutations. A T-test was performed between the background and the mutations experimentally obtained, followed by a Benjamini-Hochberg False Discovery Rate correction (Benjamini & Hochberg 1995) with p<0.05.
Results

Searching for Mutations in SA Signalling.

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

A high-throughput screening was performed (Figure 1c) with several mutagens to obtain different types of mutations. Thus, besides using Ethyl Methane Sulfonate (EMS, Sega 1984), we also used Activation tagging, Gamma Rays, and Fast Neutrons (Table 1 *(Please insert Table 1 here)*). In the case of EMS, a mutagenesis was performed in the PR1:LUC background (Maleck et al. 2002). Since different mutant backgrounds could provide unusual results, several mutants or transgenic lines were used as parental lines for EMS mutagenesis.
There is no evidence that these mutants provided a different number or kind of mutations (see below). More than 3,600 plants per family were analyzed to ensure the recovery of all mutations with a 95% probability (Malmberg 1993). 377 plants that seemed unaffected by BTH were picked in the first selection (M2s), some of them from the same family of M1s. The progeny of these plants was retested with a mock treated pot of plants simultaneously with a BTH treated pot. 157 M3s were unaffected by BTH when compared with their mock treatment and with the Col-0 control. Figure S1 shows a picture of one candidate. The 157 candidates were crossed with npr1-1, and their F1s tested with BTH as described in Figure 1. Throughout this third selection of candidates, it was decided to discard those with a weaker phenotype. With such a restrictive criterion, 93 candidates remained. Their F2s were tested with BTH and SA as described in Figure 1 (with the exception of NahG background, where growth on SA plates is not informative). Eventually, counting only one allele per independent family when several were found, 43 alleles of npr1 were identified. These 43 alleles are hereafter named as a group as “new” alleles. The remaining 13 mutants that complement npr1-1 will be described elsewhere.

The New npr1 Alleles Share some Phenotypes.

Once the npr1 alleles were identified visually, a more detailed quantification was made. We used the percentage of fresh weight (PFW), that is, fresh weight of plants treated with 350 µM BTH divided by fresh weight of mock treated plants, expressed in percentage. Figure 2a shows that Col-0 has a low PFW, while
npr1-1 is unaffected. Since all the new alleles behaved similarly, only the first nine are presented. There is some variation in the PFW, but there are no reproducible differences with npr1-1.

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

SA perception is sometimes checked by the expression of Pathogen Related proteins (PRs, Van Loon *et al.* 1994). In fact, some npr1 alleles were identified by the lack of PR2 (Cao *et al.* 1994) or PR1 (Shah *et al.* 1997) expression in inductive conditions. This last protein is a reliable marker of plant distress, so a western blot was performed with the new npr1 alleles (Figure 2c) detecting PR1. Mock treated plants show no PR1 expression (data not shown). In contrast, upon *Pto* infection, Col-0 showed a strong signal three days after inoculation, while npr1-1 showed no signal at all. From the new npr1 alleles, only npr1-20 and npr1-41 consistently produced some PR1 protein, always in lower levels than Col-0 (Figure 2c shows the first nine alleles and npr1-41). The levels of PR1 in these two alleles are not correlated with a significant defence 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
mutate AAs to a different extent (Sega 1984), and a significant amount of the mutations recovered are produced by EMS, an additional calculation was made. This time only the canonical point mutations caused by EMS (22) were compared with all the AAs that could be mutated by EMS in NPR1 (413). Again, no bias was detected (data not shown).

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

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

Fourthly, we also analyzed whether the mutations were clustered. Figure 3a shows the localization of the mutations in the backbone of the protein. It is obvious that the mutations are not clustered in any domain, but were more frequent in a small region, between the ankyrin repeats and the nuclear 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. 2002b).
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
expected results (Figure S3a), so if there is any difference between the alleles, it is not detectable to the naked eye. In order to have a proper control, \( npr1 \)-1 was crossed with Laer-0 three successive times with marker selection. Four independent lines were selected in the F2 of the third cross for \( npr1 \)-1 homozygosis, and all four behave similarly to \( npr1 \)-1 in response to BTH (Figure S3b). Therefore, only one line was included in the experiments, named as \( npr1 \)-1L (Figure 5b). The effect of BTH in the biomass of the null \( npr1 \) alleles set them far apart from the other alleles (Figure 5b), but when SA or BTH treatment is followed by \( Pto \) growth, the differences are smaller, but still significant (Student’s T test, Figure 5c). Qualitatively, the alleles are also intermediates in their behaviour on MS-SA plates (Figure 5d; there are no differences between Col-0 and Laer-0 in these plates, data not shown). As in \( npr1 \)-1 and in most of the alleles presented here, the expression of PR1 in inductive conditions is not detectable in \( npr1 \)-70 and \( npr1 \)-71 (data not shown).

NPR1 Paralogs

The conclusions from the previous results are that the null alleles exist and express an intermediate phenotype. Hence the lethality hypothesis to explain the lack of null alleles in our screen is not valid, and the results are consistent with a partial redundancy in SA signalling. So the question that arises is the identification of these protein(s). The simplest explanation is that proteins with a similar sequence perform similar functions. In other words, the paralogs of \( NPR1 \) could have a role in SA signalling. There are five paralogs of \( NPR1 \) in Arabidopsis, and the knockouts of these genes in a \( NPR1 \) wt background do not
have a strong impact on SA signalling (Canet et al. 2010). Regarding NPR2, our initial characterization was carried out with npr2-1, which has a T-DNA in an intron. Since this could be a functional allele, a second allele, npr2-2, with a T-DNA inserted in the first exon was used (Figure S4). Crosses of npr1-70 and npr1-71 with npr2-2, npr3 npr4, and bop1 bop2 were carried out. The resulting F2s are a mixture of ecotypes, so we analyzed by weight across F2 pools to take into account the ecotype variation. The BTH treatment guarantees that the null alleles are selected, and by picking up the biggest plants in each family any additional effect of the paralogs will be detected (if such effect exists). Figure 6a shows these F2s, including the F2 with Col-0 as a baseline. All the F2s of npr1 nulls with paralogs have more biomass than the F2s with Col-0. To further confirm this result, F2 plants were selected by BTH and PCR to found the double mutants between npr1-70 and the paralogs. 8 independent lines of npr1-70 npr2-2 were characterized, along with 8 independent introgressions of npr1-70 in Col-0 as a control. Figure 6b shows the maximal and minimal values of each set of lines. A T-test with the pooled values of all the lines indicates that NPR2 has an impact in SA perception (p≈3E-5). This impact is also found in the other paralogs, from which 4 (npr1-70 npr3, npr1-70 npr4 npr1-70 bop2) and 2 (npr1-70 bop1) independent lines were selected. The degree of certainty is not as strong as in npr2, but it is still significant (npr3, p≈4E-3; npr4, p≈5E-3; bop2, p≈5E-3; bop1 p≈0.04). Therefore, all the paralogs have a measurable function in SA perception in an npr1 null background. The increase in biomass is small, and yet the five paralogs explain the difference between npr1 nulls and alleles like npr1-1.
Discussion

Genes Required for SA Signalling.

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

We cannot discard the idea that the model used, loss of biomass upon BTH treatment, is limiting the scope of mutants obtained. Our previous work done with the model suggests that, upon BTH treatment, one branch stops the growth of the plant ("scorched earth defence"), while another produces active defences against the pathogen (Canet \textit{et al.} 2010). Therefore, we could lose mutants that are impaired in pathogen response, but not in biomass response. In any case, the mutants in SA recognition itself would be enriched, even if we do not gain knowledge about the branch that stops the pathogen. Regarding the possible bias in the mutants, previous screenings using pathogens (Glazebrook \textit{et al.} 1996) or gene expression (Cao \textit{et al.} 1994; Shah \textit{et al.} 1997) gave only \textit{npr1} alleles, so it seems that we have not introduced any bias, but expanded the
identification of npr1 alleles and other genes. It is also important to notice that all the new npr1 alleles obtained with BTH are not responsive to SA in terms of pathogen growth and in growth in MS-SA plates. Therefore, and for the data here presented, a screen for lack of response to BTH leads to mutants that do not perceive SA.

The Mutations in npr1 are Clustered.

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

The results obtained with the marker PR1 were unexpected (Figure 2c), since there are two alleles that produce a low but detectable amount of this defence marker in all the experiments. The case of npr1-20 is unique, since the nucleotide mutation leads to a prediction of a conserved change (Valine to Methionine), but experimentally there is evidence for a different splicing (Figure 3c). The mutation could produce a fraction of NPR1 mRNA with the right splicing and a conserved change which would explain PR1 production.

Regarding npr1-41, the mutation is a change from Glutamic acid to Lysine, a change from acidic to basic AA that is recovered in other mutated AAs (Table 2). The only particular feature of this allele is that it is located in the ankyrin repeats, where few mutations are localized. A mutation like this in one of the
ankylin repeats may compromise the overall function of the protein, but not abolish the induction of PR1.

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

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

We searched in the databases for alleles of NPR1 (thus without selection) that could differentiate between the two explanations; lethality vs. redundancy. Two of these alleles have an insertion very early in the gene and they can be taken to homozygosis (Figure 5a). Furthermore, their phenotype in terms of weight is intermediate (Figure 5b). Although the intermediate phenotype is quantitative strong, it is close to the threshold of detection in a screening (Figure 1c), explaining why no null alleles were recovered in this screening. We speculate that only mutations that affect NPR1 and have a supplementary negative effect...
are recovered. This additional effect would be to inhibit other proteins that could work in the SA signalling pathway. In any case, lethality can be ruled out, and the results fit with a partial redundancy in SA perception. It is partial because the null alleles of NPR1 produce a quantitative strong effect, and npr1 alleles were 77% of the mutants recovered.

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

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

The existence of npr1 nulls with an intermediate phenotype reveals that other proteins besides NPR1 are acting in BTH perception at the same point. The mutations are enriched in AA conserved among the NPR1 clade, and are clustered in a conserved region (Figure 4c). This region coincides almost perfectly to a repression region described in NPR1 (AAs 463 to 513, Rochon et al. 2006). Although the repression region was not functional in SA stimulated
cells, the bias observed in the mutations could indicate a stronger repression, perhaps extending its function in SA stimulated cells.

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

The behaviour of the new npr1 alleles does not fit into conventional recessive or dominant negative alleles, but into a mixture of properties. When considering only NPR1, they are recessive, but when two copies of the allele are mutated, they interact with the paralogs as dominant negatives. A similar mechanism has
been described before for closely related paralogs. Five recessive alleles of $hsp90.2$ show a phenotype of loss of recognition of $avrRpm1$, while the knockout does not have any distinctive phenotype (Hubert et al. 2003; Hubert et al. 2009). The model proposed was that the HSP90s interact with RPM1, and a knockout is functionally replaced by the remaining paralogs. In this model, only mutations that abolish the function of HSP90.2 and interact negatively with other paralogs (HSP90s form heterodimers, Richter et al. 2001) are recovered. The fact that all the alleles recovered are in the same gene leads the authors to propose a certain specificity or preference. The analogies with NPR1 are not complete, but notable. The differences are that NPR1 nulls have a measurable phenotype and the number of NPR1 alleles recovered is one order of magnitude higher.

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

It is tempting to speculate with a correlation between the homology with NPR1 and the importance of the paralogs in BTH/SA perception. The order from more homology to less is $NPR2$, $NPR4$, $NPR3$, $BOP1$ and $BOP2$ (Liu et al. 2005). The F2 and F3 data of Figure 6 predict a role for all the paralogs with different degrees, from a strong one for $NPR2$, to a weaker, yet significant, for $BOP1$. 
There are several arguments in favour of a possible degree of functionality. Thus, *NPR2*, *NPR3* and *NPR4* are slightly induced upon biotic stress (like *NPR1*), while *BOP1* and *BOP2* are repressed (www.arabidopsis.org). Also, the clustering of the mutations in *NPR1* is stronger in a region that it is highly conserved with *NPR2*, *NPR3* and *NPR4*, but not with *BOP1* or *BOP2* (Figure 4c), so there is ample evidence for a predominant role of the three closer paralogs. Of course the model proposed does not exclude that other genes are required in BTH/SA perception, and they could be the mutations that complement npr1.
Acknowledgments

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References


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Table 1

<table>
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<tr>
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<td>157</td>
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Table 1. Mutants obtained in this screening. Seeds from plants treated with different mutagens (first column) were selected by BTH and then by size as described in Figure 1. The second column provides the number of independent families, and the third column is the number of seeds screened. The fourth column gives the number of mutants recovered as M2s and the fifth the number of mutants retested in M3 and selected to cross with npr1-1. The sixth column shows the mutants that still have a strong phenotype and the seventh the number of npr1 alleles found from independent families. EMS stands for ethyl methane sulfonate, T-DNAs for Activation tagging, Gamma for Gamma Rays, and FN for Fast Neutrons.
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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 µM 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 µM 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 µM 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 µM SA, or 350 µM 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, Tramtrack 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
represented as a bar (Y-axis). The schematic structure of NPR1 is represented in the X-axis for visualization of the domains. The dotted line shows where the distribution is not random (Poisson distribution, 3 alleles/50 AA p<0.05). (b) Distribution of point mutations, as described in (a). In this case, the dotted line is at 5 alleles/50 AA (Poisson distribution, p<0.05). (c) Distribution of mutations in a selected region (AA428 to 515) of NPR1. The mutations are showed over the wt AA, with the alignment of the NPR1 clade. The stop codons are marked with an asterisk, and the frameshifts (due to altered intron splicing) with a “«”. The repression region described by (Rochon et al. 2006) is marked with an arrow.

Figure 5.-Phenotype of null npr1s. (a) Three insertions (T-DNA and transposons) were characterized, and their localization is shown. Note that npr1-70 and npr1-71 are in background Laer-0. (b) Response to BTH in terms of fresh weight, as in Figure 2a. npr1-1L is an introgression of npr1-1 in the background Laer-0. (c) Response to SA and BTH in terms of Pto growth, as in Figure 2b. The response of npr1-70 and npr1-71 to SA and BTH, although small, is statistically significant (T-test, p<0.05). (d) Response to SA in MS-SA plates. Col-0, npr1-1, npr1-70, and npr1-71 as in Figure 1b.

Figure 6.-Role of the paralogs in response to BTH. (a) F2s of the indicated genotypes were analyzed as in Figure 2a, but selecting the biggest plants in each family. The baseline is the F2 of the null npr1 alleles with Col-0. (b) Double mutants between npr1-70 and the paralogs were selected by phenotype and PCR in F2, and their progeny tested as in Figure 2a. Only the maximal and
minimal values are showed from 8 (Col-0 and npr2-2) and 4 (npr3) independent
lines. (c) Similar to b, but showing the response of the rest of paralogs. 4 (npr4
and bop2) and 2 independent lines (bop1) were analyzed. For each double
mutant, the pooled values of all the tested lines were found to be significantly
higher than the pooled values of all the npr1-70 (Col-0) lines (T-test, p<0.05).
1 Figures
Supporting information.

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

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

-Figure S3. Introgression of $npr1\text{-}1$ in Laer-0.

-Figure S4. T-DNA insertions in $NPR2$
(a) BTB/POZ | Ankyrin | NLS

(b) Bar graph showing percentage of FW (%FW) for different genotypes:
- Col-0
- npr1-1
- npr1-63
- Laer-0
- npr1-1L
- npr1-70
- npr1-71

(c) Graph showing Log (ctu/plant) for different treatments:
- Mock
- SA
- BTH

(d) Petri dishes showing colony growth:
- Col-0
- npr1-1
- npr1-70
**Supporting Information**

**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.
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


