Quantitative genetic analysis of salicylic acid perception in Arabidopsis

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Abstract

Salicylic acid (SA) is a phytohormone required for a full resistance against some pathogens in Arabidopsis, and *NPR1* (*Non-Expressor of Pathogenesis Related Genes 1*) is the only gene with a strong effect on resistance induced by SA which has been described. There can be additional components of SA perception that escape the traditional approach of mutagenesis. An alternative to that approach is searching in the natural variation of Arabidopsis. Different methods of analyzing the variation between ecotypes have been tried and it has been found that measuring the growth of a virulent isolate of *Pseudomonas syringae* after the exogenous application of SA is the most effective one. Two ecotypes, Edi-0 and Stw-0, have been crossed, and their F2 has been studied. There are two significant Quantitative Trait Loci (QTLs) in this population, and there is one QTL in each one of the existing mapping populations Col-4 x La*er*-0 and La*er*-0 x No-0. They have different characteristics: while one QTL is only detectable at low concentrations of SA, the other acts after the point of crosstalk with methyl jasmonate signalling. Three of the QTLs have candidates described in SA perception as *NPR1*, its interactors, and a calmodulin binding protein.

Keywords: Arabidopsis, Defence, Natural variation, Salicylic Acid

Abbreviations: SA (salicylic acid), *NPR1* (*Non-Expresor of Pathogenesis Related Genes 1*), QTL (Quantitative Trait Loci).

Introduction

Salicylic acid (SA, for a review, see Vlot et al. 2009) is a hormone with an impact on several areas of plant biology such as the induction of flowering (Rhoads and McIntosh 1992), and it is required for resistance against microbes, especially virulent pathogens like *Pseudomonas spp*. (Katagiri et al. 2002). *Arabidopsis thaliana* (Arabidopsis) is a well studied plant, and most of the knowledge on SA has been developed with Arabidopsis.

There are two biosynthetic genes (*EDS5*, Nawrath et al. 2002 and *SID2,* Wildermuth et al. 2001) regulated by SA itself and by other genes (e.g. EDS1, Wiermer et al. 2005), although mutations in these genes do not completely eliminate SA. For a severe depletion in the levels of SA, the transgenic plant *NahG* has to be used (Lawton et al. 1995). This plant overexpresses a salicylate hydroxylase from *Pseudomonas putida* thus converting SA to catechol (You et al. 1991). Regarding SA perception, NPR1 is the only protein that is known to be necessary for signal transduction (Pieterse and Van Loon 2004), although there is evidence pointing to a SA-dependent, NPR1-independent resistance (Desveaux et al. 2004).

In order to define the components of SA signalling, a number of mutant screenings have been performed, but more *NPR1* alleles are the only result reported so far (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997). The biochemical study of NPR1 has produced a number of proteins, although none of them is as relevant as NPR1 itself in terms of mutant phenotype of the corresponding gene. Thus, NPR1 interacts with proteins that regulate (NIMINs, Weigel et al. 2001) or carry on the signalling (TGAs, Zhang et al. 1999). In non inductive conditions NPR1 is reported to be located mainly in the cytoplasm. Upon SA perception, it is monomerized by a thioredoxin and migrates to the nucleus where it activates the genes that lead to resistance (Tada et al. 2008). This activation requires NPR1 degradation via proteosome (Spoel et al. 2009). An alternative model has been recently proposed where –at least in tobacco- NPR1 is always in the nucleus and is sensitive to SA, thus activating its function (Maier et al. 2011). SA has a negative crosstalk with at least methyl jasmonate (MeJA) (Genoud and Metraux 1999). Interestingly, applications of MeJA are capable of triggering a small resistance, and this resistance is dependent on NPR1 in its cytosolic form (Spoel et al. 2003).

Other approaches like transcriptomics (Pylatuik and Fobert 2005) and metabolomics (Hien Dao et al. 2009) have rendered a wealth of data, but have not produced any candidate different from the ones already described.

Our ongoing interest in SA perception has led us to inquire about it in the reported mutants in defence (Canet et al. 2010a) as well as in new screenings (Canet et al. 2010b). A resource that has not been used extensively to study SA is the natural variation of Arabidopsis. Arabidopsis is an almost perfect tool for natural variation studies (Alonso-Blanco and Koorneef 2000) since it has hundreds of ecotypes (also known as accessions or land races), and a good number of mapping populations (www.arabidopsis.org). The recombinant inbreed lines (RILs) constitute an important tool in natural variation. In this resource, the F2 plants from selected parents are taken from generation to generation until F8. The result is that the level of heterozygosity is negligible, allowing for the detection of quantitative trait loci (QTLs) with relatively few lines. Another advantage is that RILs are a stable resource so they have to be genotyped only once. Once a QTL is detected, the usual approach is to construct lines derived from RILs that share all the genome except that in the interval where the QTL is predicted to be. These *ad hoc* tools are called near isogenic lines (NILs, Alonso-Blanco et al. 2006).

There is extensive work dealing with the natural variation of the response of Arabidopsis to pathogens, like *Hyaloperonospora spp.* (Adam and Somerville 1996) or *Pseudomonas spp* (Perchepied et al. 2006). There is also work done that deals with the glucosinolate synthesis as a response to exogenous SA (Kliebenstein et al. 2002), and with the transcriptomic response to exogenous SA (van Leeuwen et al. 2007).

We have tried different approaches to best capture the natural variation of Arabidopsis in response to SA. The best option is to treat the plants directly with SA and then to inoculate them with virulent bacteria. With this method, we have found differences between ecotypes that give rise to four different QTLs. Two of them come from an *ad hoc* F2 population, and the other two from different RILs. The positions of the last two have been confirmed with NILs, thus allowing speculation about their different position in SA signalling on the basis of their phenotypes.

Materials and methods

Plant growth and inoculation

Arabidopsis thaliana (L.) Heynh. was sown and grown as described (Canet et al. 2010a), in phytochambers with days of 8 h at 21°C, 150 µmol m^{-2} s^{-1} and nights of 16 h at 19°C. All the ecotypes, mutants, and mapping populations were obtained from NASC (www.arabidopsis.info). When the population Edi-0 x Stw-0 was used, plants were grown as usual and after two weeks vernalized for six weeks at 4° C. After this treatment, plants were grown in normal conditions. 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 an empty pVSP61 was obtained from Dr. Dangl (University of North Carolina at Chapel Hill, NC, USA) and maintained as described (Ritter and Dangl 1996). The bacteria were grown, inoculated and measured as described (Tornero and Dangl 2001). Briefly, plants of 14 days were inoculated by spray with *Pto* at OD_{600}=0.1 with 0.02% Silwet L-77 (Crompton Europe Ltd, Evesham, UK). Three days later, the amount of colony forming units (cfu) per plant was quantified.

Chemical treatments

For measuring the effect in *Pto* growth, water, SA (in the form of sodium salicylate, S3007 Sigma, St Louis, MO, USA), 10 g/L fosetyl-al 80 (Aliette; Rhone-Poulenc, Lyon, France), and 35 or 350 µM BTH were applied by spray. Fosetyl was applied four days previous to pathogen inoculation, SA and BTH one day. SA was applied at 100 µM unless other concentration is stated. 100 µM methyl jasmonate (Sigma) was applied by spray one day previous to pathogen inoculation. For the measurement of SA *in planta*, three samples of 100 mg were frozen in liquid nitrogen. SA measurements were performed with the biosensor *Acinetobacter sp. ADPWH_lux* (Huang et al. 2005). For *in vitro* culture, plants were grown in MS media (Duchefa, Haarlem, The Netherlands) with 500 µM SA, and the growth of the plants evaluated every few days for three weeks (data not shown).

QTL mapping

Plants of Arabidopsis were treated with either mock or 100 µM SA as described above. The logarithm of cfu per plant and the genotype of each line were used as input for the program WinQTLCart 2.5 (Wang et al. 2007), that calculates the probability that a QTL is linked to a particular region of the genome. The populations analyzed were: Col-0 x Nd-1, 98 lines (Deslandes et al. 1998); Col-gl1 x Kas-1, 115 lines (Wilson et al. 2001); Cvi-1 x La*er*-2, 50 lines (Alonso-Blanco et al. 1998a); La*er*-0 x Sha-0, 114 lines (Clerkx et al. 2004); Bay-0 x Sha-0, 162 lines (Loudet et al. 2002); Col-4 x La*er*-0, 85 lines (Lister and Dean 1993); and La*er*-0 x No-0, 135 lines (Magliano et al. 2005). In the case of Edi-0 x Stw-0, 266 F2s and their parental were genotyped with iPLEX® in the CEGEN (Spanish National Genotyping Centre, www.cegen.org), with 24 markers (www.naturalvariation.org). Two additional SSLP markers were added to the map to complete the Chromosome II (Supplemental Fig. S1). The program GGT 2.0 (van Berloo 2008) was used for the selection of RILs and the STAIRs lines.

Benzothiadiazole (BTH) treatment and fresh weight determination

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. 2010a. Briefly, plants were treated with either mock or 350 µM BTH four times during two weeks, starting when the plants were one week old. Then, the fresh weight of each genotype was recorded in both treatments and expressed as percentage of the control fresh weight.

Statistical analysis

Figure 1 shows the average of three independent experiments to show which treatment is the most informative, and which ecotypes are in the extremes. Due to the number of ecotypes, the error bars are not shown. To compare the 49 ecotypes in a single figure, the value of Col-0 in mock was assigned a value of 100, and the rest of ecotypes were expressed in relation to Col-0. Thus, the value of a given ecotype would be the Log(cfu/plant) of the ecotype divided the Log(cfu/plant) of Col-0, expressed in percentage. To represent the resistance induced by the chemicals, in the last three panels the arbitrary value of 100 assigned to Col-0 represents the resistance induced by the chemicals. Thus, the value of a given ecotype would be the ratio of

the (Log(cfu/treated plant) minus Log(cfu/mock plant)) of the ecotype divided the (Log(cfu/treated plant) minus Log(cfu/mock plant)) of Col-0, expressed in percentage. In Figures 4 and 5, a t-student was performed, considering populations of equal variance, a single tailed test, and alpha equals 0.05. For the QTL mapping (Fig. 2, 3 and 7), two independent experiments were done, producing essentially the same result. The rest of experiments are done independently three times, and when all three experiments show the same information, one of them is shown. Each experiment consists in at least three measures, each measure with at least five plants sampled. The average and the standard deviation of the three measures are shown in the figures; two averages are statistically different if the error bars are not overlapping.

Results

Searching for natural variation in SA response among the ecotypes

We are interested in finding the genetic steps involved in salicylic acid (SA) perception. A system was proposed where no pathogen is involved, but this framework does not produce any relevant output in terms of natural variation (Canet et al. 2010a). For this reason, we have looked for the best conditions to maximize the differences among ecotypes by using chemicals that induce resistance through SA perception. As a representation of the natural variation of Arabidopsis, all measures were done in a nuclear core collection which maximizes the variation with a minimal number of genotypes (McKhann et al. 2004). Figure 1 shows the growth of *Pseudomonas syringae pv. tomato DC3000* (*Pto,* a virulent pathogen in most Arabidopsis ecotypes) upon different treatments. Three pots of each ecotype were treated with chemicals that induce resistance through SA perception, namely Fosetyl, (Molina et al. 1998), benzothiadiazole (BTH, an analogue of SA, Lawton et al. 1996), and SA itself (Nawrath et al. 2005), in addition to a mock-treated one. The four pots were then inoculated with *Pto*, and the logarithmic growth of the pathogen was measured in three independent experiments. In order to compare the 49 ecotypes in a single figure, the value of Col-0 in mock was assigned an arbitrary value of 100, and the rest of ecotypes were expressed in relation to Col-0 (Fig. 1a). This way, growth curves done in different days can be compared, since Col-0 was included in all experiments. To represent the resistance induced by the chemicals, in the last three panels the arbitrary value of 100 assigned to Col-0 represents the resistance induced by the chemicals, that is, the growth of *Pto* under mock treatment minus the growth of *Pto* under chemical treatment. Thus, a value of 50 indicates that an ecotype responds to a chemical inducing half of the resistance that Col-0 does. Correspondingly, a value of 200 implies that an ecotype responds twice as strongly as Col-0, again in relative terms. Note that we consider relative induction (mock minus chemical) in order to minimize the variation due to basal resistance and to maximize the variation due to the treatment. Figure 1b represents the response to Fosetyl; Fig. 1c shows the response to BTH, and Fig. 1d the response to SA. It is important to notice that these chemicals are used in concentrations that are not comparable: BTH is used at maximum concentration (350 µM, Lawton et al. 1996) while SA is used at a concentration close to the minimum dose that we can detect (100 µM, see below). From Fig. 1 it is clear that the treatment with 100 µM SA maximizes the natural variation of Arabidopsis with respect to

the other treatments. As a consequence, this treatment was used to find and evaluate possible QTLs.

The ecotypes Edi-0 and Stw-0 were chosen as parentals for establishing a mapping population due to their extreme phenotype regarding SA. Other ecotypes that are frequently used in research or that are parentals of RILs were also tested (data not shown). Figure 2a shows the ecotypes that were found to be relevant for the rest of this work (see below). *NPR1* is the only known gene required for SA perception, and so *npr1*-1 is included as a negative control (Dong 2004).

Searching for natural variation in SA response among the mapping populations

The cross between Edi-0 and Stw-0 was done and 266 F3s and the parental ecotypes challenged with *Pto* after the application of a treatment of 100 μM SA or a mock treatment (the measurement is destructive so the F2 is measured through 6 F3 individuals). Similarly, we tested all the RILs available at the beginning of this work (seven, see Methods). The Edi-0 x Stw-0 F2 produced the strongest variation (Fig. 2b, standard deviation of 87 vs. values of 73 to 62 in the rest of RILs of Fig. 2b and 2c). It is similar in shape to the one produced by the RIL Bay-0 x Sha-0 (Fig. 2b, Loudet et al. 2002). The distribution is quite different from the ones produced by the RILs Col-4 x La*er*-0 (Lister and Dean 1993) and La*er*-0 x No-0 (Magliano et al. 2005, both in Fig. 2c). While the populations in Fig. 2b show a bell-shaped distribution according to several QTLs, Fig. 2c shows two populations with a distribution far from normal, indicating that there are few and/or strong QTLs producing the variation in the phenotype.

In order to find the QTLs that explain this variation, the Edi-0 x Stw-0 F2 was genotyped with 26 markers (see Methods and Supplemental Fig. S1), and a QTL mapping approach was done with WinQTLCart (Wang et al. 2007). The threshold of the logs of the odds (LOD) was calculated at 0.05 significance with 1,000 permutations (Churchill and Doerge 1994). While there is no significant QTL with the mock treatment, there are two significant QTLs with the SA treatment, SAQ1 and SAQ2 (Fig. 3a). SAQ1 is located between 53.2 and 91.7 cM on Chr I (2 LOD support interval in all QTLs described). It explains 9% of the variation, and the Stw-0 allele has a slightly dominant effect (0.12). On the other hand, SAQ2 is located between 0 and 17.3 cM of Chr IV, explains 8% of the variation, and the Edi-0 allele has a dominant effect (0.19).

Out of the seven RILs tested, only three produced significant QTLs in the response to SA. In the RIL Bay-0 x Sha-0, there is a single QTL that co-localizes with one QTL in mock treatment

(Fig. 3b). Since the QTLs in response to *Pto* in this population have been already reported (Perchepied et al. 2006) and the QTL of SA response is likely to be one of them, we did not pursue it. The RILs Col-4 x La*er*-0 (Fig. 3c) and La*er*-0 x No-0 (Fig. 3d) have one single QTL with the SA treatment: SAQ3 and SAQ4, respectively. SAQ3 is seated between 14.8 and 22 cM of Chr. III and explains 18% of the variation produced by the response to SA, while SAQ4 is between 66.6 and 89.4 cM of Chr V and explains 15% of the variation. Regarding Col-4, there is no difference in SA perception with respect to Col-0 (data not shown), and that is the reason why Col-0 is shown in Fig. 2a.

Establishing near isogenic lines

These four QTLs pinpoint four different regions of the genome, so we continued with the characterization of all of them. For Edi-0 x Stw-0, a RIL is being established, (note that each generation requires six weeks of vernalization). For Col-4 x La*er*-0, we took advantage of the Stepped Aligned Inbred Recombinant Strains (STAIRs) lines (Koumproglou et al. 2002) already constructed between Col-0 and La*er*-0. After a careful selection, the result is a collection of lines that form a stepwise introgression of La*er*-0 in Col-0. The lines N9459 and N9464 (hereafter P59 and P64, respectively) were chosen, since they diverge in a small region that includes SAQ3 but share most of the genome (see Supplemental Fig. S2). Therefore, comparing these two lines implies ”mendelizing” SAQ3 and there is no need of the original parents.

For La*er*-0 x No-0, a conventional construction of NILs was carried out. The RIL174 was selected due to its content in No-0 (see Supplemental Fig. S3) and crossed with No-0, and the F1 was crossed again with No-0. Out of the resulting F2, the NIL N15 was selected, since it is heterozygous for SAQ4, but No-0 for most of the genome. Out of the progeny of N15, the line N15.15 was selected, which is homozygous La*er*-0 for SAQ4 but No-0 for most of the genome. On the other hand, the RIL132 was also selected (this time due to its content in La*er*-0, see Supplemental Fig. S3) and crossed with La*er*-0. Out of the resulting F2, the NIL N297 was kept, since it is heterozygous for SAQ4. From its progeny, the line N297.46 was selected (La*er*-0 in all the genome, except No-0 for the region containing SAQ4). Thus, by comparing No-0 vs. N15.15 and La*er*-0 vs. N297.46, we reduce the variation to the region containing SAQ4 (see Supplemental Fig. S3), and the characterization and mapping of the QTL is more accurate. By selecting N15 and N297 and analyzing their progeny, we have effectively constructed two mapping populations, thus mendelizing SAQ4.

Characterization of the QTLs in SA perception

Once all the corresponding NILs had been established, we sought to confirm the QTLs by checking the different behaviour between the NILs. Edi-0 and Stw-0 were included in these analyses, even though if they are not NILs, to document the difference between genotypes. In the case of La*er*-0 x No-0, we kept the two sets of introgression lines (La*er*-0 vs. N297.46 and No-0 vs. N15.15).

The first thing was to confirm if the NILs have indeed a measurable difference with respect to SA. Treatments with less than 50 µM of SA are not detectable in our hands, and even this concentration sometimes does not induce resistance in Col-0 (data not shown). When more than 500 µM of SA was applied, we got experiments with a strong resistance, but with some plants suffering from the phytotoxicity of SA (data not shown). Therefore, several concentrations of SA (from 50 µM to 500 µM), along with a mock treatment, were applied to plants before a *Pto* inoculation (Fig. 4a). The amount of growth in *Pto* reflects the response to SA. Edi-0 vs. Stw-0 and No-0 vs. N15.15 respond differentially to all four concentrations, La*er*-0 vs. N297.46 to three, and P59 and P64 to two concentrations. In all cases there is a differential behaviour with 100 µM, which is the concentration used for the mapping population, thus validating the QTLs. A similar experiment with plants grown in media containing SA did not produce any visible difference between the genotypes (data not shown).

Once the QTLs had been confirmed, the next step was to characterize their role in SA perception. The QTLs could make a difference in any number of the multiple steps required for SA to produce a measurable effect. We reasoned that the QTLs could be mechanistically located in the penetration, stability, accumulation or degradation of the exogenous SA, in short, in any step that could affect the amount of SA that reaches the cell. There is no difference in SA concentration in mock conditions (data not shown), and Fig. 4b shows SA contents measured one day after spraying the plants with 100 µM SA. An important part of SA is stored as glucoside (Nawrath et al. 2005), and therefore both free and total SA (free plus glucoside conjugated) were measured. *NahG* was included as a control, since *NahG* plants degrade the cytosolic SA (Lawton et al. 1995) and therefore its presence takes into account the SA that remains in the outside of the cell (Niederl et al. 1998), thus establishing a basal line. The result of Fig. 4b is that in terms of SA concentration one day after the SA treatment, there are no differences between the genotypes that define SAQ4. There is a small (but not significant) difference in the genotypes of SAQ3, and an unexpected difference in Edi-0 vs. Stw-0. In this

pair, Edi-0 has less SA (both free and total) than Stw-0 at the time of measuring, and yet Edi-0 shows a stronger response to SA in terms of stopping the pathogen. Therefore, none of the QTLs present a strong difference in the accumulation of SA that could explain the difference in the growth of the pathogen.

An important question in SA perception is the difference between SA and benzothiadiazole (BTH). BTH is a chemical analogue of SA, its effect *in planta* being stronger than that of SA and without its phytotoxicity. Fig. 5a shows the result of the application of 35 μM BTH to plants before a *Pto* inoculation. The concentration used is 35 μM because it increases the window of detection for small changes between close genotypes, while lower amounts of BTH do not produce robust results (data not shown). While Edi-0 vs. Stw-0 do not show a different behaviour, the other three comparisons show a considerable difference in response to BTH. There is an alternative way of assessing the response to BTH: by measuring the difference in plant fresh weight after several treatments (Canet et al. 2010a). When this alternative measurement of response was used, the differences were reduced in SAQ3 and SAQ4. In the case of Edi-0 vs. Stw-0, the response is even opposite to SA. Therefore, Edi-0 vs. Stw-0 discriminate SA from BTH, and SAQ3 and SAQ4 do not discriminate between these two analogues. Regarding this way of measuring SA perception, note that the BTH treatments used do not produce macroscopic cell death that could affect the fresh weight measurements (Canet et al. 2010a).

SA has a negative crosstalk with several hormones, among them MeJA (Genoud and Metraux 1999). If genotypes that have different SA perception show the opposite pattern for MeJA, it would imply that the QTL(s) are located in steps previous to (or even in) the point of crosstalk. Fig. 6 shows the result of treating plants with 100 μM MeJA prior to an inoculation with *Pto*. The differential response of the genotypes is significant (and opposite to the behaviour with SA) in SAQ3, but not in the rest of comparisons. In the case of Edi-0 vs. Stw-0 there is no difference between mock and treated plants, but in SAQ4 there is clear indication that the same trend seen with SA occurs with MeJA.

Fine mapping of the QTLs

The QTLs SAQ3 and SAQ4 were confirmed and further delimited with the creation of *ad hoc* populations. In the case of Col-0 vs. La*er*-0, an F2 population between P59 x P64 was created and genotyped. Out of 288 plants, 11 were recombinants in the interval (see Supplemental Fig.

S2). When SAQ3 was mapped with these lines, the actual position was shifted towards the telomere, between 8.1 and 12.2 cM (Fig. 7a), with the La*er*-0 allele being dominant over Col-0. In the case of La*er*-0 vs. No-0, two mapping populations were constructed. Out of the progeny of NIL15 (heterozygous for the interval and No-0 for the rest of the genome, see Supplemental Fig. S3), 96 plants were genotyped and 35 selected due to their informative genotype. From the progeny of NIL297 (heterozygous for the interval and La*er*-0 for the rest of the genome, see Supplemental Fig. S3), 96 plants were also genotyped, and 42 selected due to their informative genotype. When the descendants of NIL15 were analyzed, the previous QTL was divided into several (Fig. 7b, continuous line). The main QTL was also shifted towards the telomere, between 35 and 42.2 cM. The mapping of the descendants of NIL297 produced a QTL that overlaps with the main QTL produced by NIL15 (between 35 and 45 cM, Fig. 7b, discontinuous line), with the La*er*-0 allele being dominant.

Discussion

Finding the best system for SA perception in natural variation

Since our goal is to describe SA perception by using different approaches, we have tried to explore the underused natural variation of Arabidopsis. There are works that describe differences between ecotypes in their response to *Pto* (e.g. Perchepied et al. 2006; Fan et al. 2008) corresponding to our mock inoculations (Fig. 1a). Other reports deal with SA, but from the perspective of either its relationship with MeJA (Genoud and Metraux 1999) or the complex transcriptomic networks that differ from ecotype to ecotype (Genoud and Metraux 1999). A simplified system to explore the artificial variation (mutagenized populations) of Arabidopsis has been proposed, but it does not reveal any relevant difference in the RILs tested (Canet et al. 2010a). Since the simplified system did not work for natural variation, we searched for chemicals that trigger resistance against *Pto* and worked through SA perception. As shown in Fig. 1, the best option to maximize variation is 100 µM of SA itself, at least out of the doses and treatments used. Thus, SA is used in a concentration close to the minimum that we can robustly detect (Fig. 4a). It is interesting to note that the LOD score obtained (Fig. 3) was lower in comparison with other biological systems (e.g. flowering, Alonso-Blanco et al. 1998a or Werner et al. 2005). Our low LODs could be caused by the strong variation that the pathogen produces, even if its growth is measured in logarithmic scale. Note that we did not use Systemic Acquired Resistance (SAR) as a system (that is, one first inoculation that induces defence and a second one to measure the resistance, Vlot et al. 2008). Though this setup would have been likely to produce a strong variation among ecotypes, its application would have been difficult with more than a handful of genotypes at the same time.

Edi-0 vs. Stw-0

The SA treatment defines the ecotypes that are most diverse. Out of them, Edi-0 and Stw-0 were chosen. These ecotypes were selected because they share the same level of basal resistance (mock treatment in Fig. 2a) and are quite different in their response to SA. The result is that the population defines two QTLs which are specific for SA response, SAQ1 and SAQ2 (Fig. 3a). The opposite effect is observed in Bay-0 vs. Sha-0. These two ecotypes differ approximately in the same degree in the basal and SA-triggered resistance (Fig. 2a). Logically, the QTLs found are not specific of SA response, but in basal resistance (Fig. 3b).

The characterization of the F3s from the cross Edi-0 x Stw-0 unveiled a strong variation in the response to SA, even stronger than that of other mapping populations with only homozygous alleles due to their F8 state (Fig. 2b and c), so the variation is likely to increase when this population is taken through several generations (Alonso-Blanco et al. 2006).

The difference between ecotypes is maintained through all the concentrations of SA tested (Fig. 4a), so the differences are quite robust. When the concentration of SA is measured after an exogenous application, there is less SA in Edi-0 (Fig. 4b) even if it is the one that responds more to SA. This apparent contradiction could be explained by the fact that metabolism of SA is triggered by its perception. For example, the expression of *NPR1* is induced by SA and *npr1* alleles accumulate more SA, presumably because they are unable to trigger its degradation (Cao et al. 1997). In any case, the differences in SA accumulation do not explain any of the differences found between genotypes, so the four QTLs described herein are not due to differential intake or stability of SA (see below for a detailed account of SAQ3). Then, the logical conclusion is that the QTLs are located at some point between SA perception and the execution of the resistance.

The response to MeJA in Edi-0 vs. Stw-0 (Fig. 6) does not help to clarify the situation of the QTLs in the SA signal transduction. It could be due to differences among ecotypes besides the QTLs; while in other cases the comparisons are between NILs that share 90-94% of the genome (Supplemental Fig. S2 and S3), these are two wild type ecotypes that are quite divergent (McKhann et al. 2004). Therefore, the precise assessment of the differences in the MeJA signal transduction regarding SAQ1 and SAQ2 is postponed until a RIL is developed.

SA has several analogues that trigger resistance in plants, such as 2,6-dichloroisonicotinic acid (Uknes et al. 1992) and BTH (Lawton et al. 1996). We reasoned that it would be informative to compare the different genotypes with BTH (Fig. 5), and so it is in the case of Edi-0 vs. Stw-0: Fig. 5a shows that there is no significant difference in the resistance triggered by BTH. It is possible that BTH triggers too much resistance and we are not able to detect the differences. But the reduction of plant fresh weight triggered by BTH (Fig. 5b) shows that Stw-0 perceives BTH better than Edi-0, while Edi-0 perceives SA better than Stw-0 (Fig. 2a). There is only one precedent to this discrimination between these two chemicals, since the transgenic *NahG* degrades SA (and therefore does not react to it) but not BTH (Lawton et al. 1996). Since the difference between Edi-0 and Stw-0 is not in the intake or metabolism of SA, and it differentiates between two close chemicals, the simplest explanation is that the difference between ecotypes is in the receptor of SA itself.

There is no *bona fide* receptor for SA yet, but NPR1 is the strongest candidate. Mutations in NPR1 disrupt SA perception and none of the reported alleles differentiate between SA and BTH (Canet et al. 2010b). From the two QTLs found in the population Edi-0 x Stw-0, SAQ1 maps to a region of Chromosome I that includes *NPR1*. NPR1 presents a fair amount of polymorphisms, with 4 protein variants in the 96 ecotypes studied, while the average of defence response is 2.78 out of 96 ecotypes, and the background of the genome is 5.38 (Bakker et al. 2008). Therefore, it is plausible that polymorphisms in NPR1 are responsible for SAQ1. Nevertheless, there are other 51 genes labelled with the keywords “defence”, “salicylic”, or “systemic acquired resistance” in the mapping interval defined by SAQ1, and while 37 of them are resistance genes (TAIR, www.arabidopsis.org), unlikely to be responsible for SAQ1, there are other plausible candidates.

Among the genes that could be responsible for SAQ2, there are nine labelled with the keywords previously mentioned. Among them, the most interesting one is NIMIN1b (At4G01895). NIMIN proteins interact with NPR1 *in vitro* and *in planta* (Weigel et al. 2001), and overexpression of *NIMIN1* phenocopies *npr1* plants (Weigel et al. 2005). Therefore, a model has been proposed where NIMIN proteins are repressors of NPR1 activity (Weigel et al. 2005). Then, a simple and elegant model could be true, where the two proteins that produce SAQ1 and SAQ2, namely NPR1 and NIMIN1-like, would interact biochemically.

Col-0 vs. La*er*-0

The RIL Col-4 x La*er*-0 was one of the first mapping populations available (Lister and Dean 1993) and it has been genotyped with a large set of markers (Alonso-Blanco et al. 1998b). Note that, regarding the response to SA, we found no differences between Col-0 and Col-4 (data not shown). Although the difference between Col-0 and La*er*-0 in response to SA is not as strong as in other cases (Fig. 2a), the fact that these two ecotypes are the most used ones in Arabidopsis research granted a closer look. There is a strong transgression in the RIL population (Fig. 2c) which does not fit a normal distribution. This could be indicative of a small number of QTLs and indeed the result of mapping the population is a single QTL in Chromosome III (Fig. 3c). Taking advantage of the great work done with the STAIRs lines (Koumproglou et al. 2002), we were able to validate SAQ3 with newly generated lines from a different population, thus reducing the mapping interval (Fig. 7a).

Among the genes in this interval, there are no clear candidates labelled with the aforementioned keywords, and while there are QTLs of glucosinolates in response to SA described in the RIL Col-4 x La*er*-0 (Kliebenstein et al. 2002), none of them are close to SAQ3.

SAQ3 shows the negative interaction between SA and MeJA (Fig. 6), which indicates that the QTL is located before the point of crosstalk. This role has been proposed to be fulfilled by NPR1 or WRKY70 (Spoel et al. 2003 and Li et al. 2004, respectively), so SAQ3 would be relevant in the steps previous to these proteins. We interpret that the differential responses to MeJA proves that SAQ3 does not only respond to exogenous SA, but also to endogenous, physiological levels of SA. In this line of argument, SAQ3 is quite dependent on the SA dose used (Fig. 4a). The other pairs of genotypes differ at almost all the SA doses used, but P59 and P64 show the greatest difference at SA 50 µM, some difference at 100 µM, and no difference at the other two concentrations. This result suggests a process with two affinities. Thus, SAQ3 would be a quantitative trait gene with high affinity for SA in a process with another gene(s) with low affinity for SA. Regarding SA, such systems have been proposed in the influx and efflux SA carriers (Chen et al. 2001), although the measures of SA in Fig. 4b contradict this possibility. Perhaps further experiments in more advanced introgression lines could confirm the tendency of P59 having less total SA than P64. If this result were to be true, it would add more weight to the hypothesis of SAQ3 being an influx or efflux high affinity carrier. Alternatively, there could be a different process with two affinities that could explain the small difference in total SA but not that in free SA provoked by SAQ3. SAQ3 is detectable with high doses of BTH (Fig. 5). This fact could be due to the stability of BTH (Lawton et al. 1996), whereas SA is more readily metabolized, conjugated, or specifically transported (Nawrath et al. 2005).

La*er*-0 vs. No-0

The ecotypes La*er*-0 and No-0 are strongly different in their response to SA (Fig. 2a), and, like in the previous Col-4 x La*er*-0 RIL, the mapping population is not normally distributed (Fig. 2c). And as before, there is a single QTL, SAQ4 (Fig. 3d). In this case, there are no NILs available, so we constructed the corresponding lines. Two different lines were pursued, in order to test the effect of SAQ4 both in La*er*-0 and No-0 background. The mapping population derived from N15 (heterozygous for SAQ4, No-0 in most of the genome) reveals a clear, strong QTL that confirms SAQ4 and other small QTLs (continuous line in Fig. 7b). On the other hand, the mapping population derived from N297 confirms the position of the main QTL but it is not as strong as the population derived from N15 (discontinuous line in Fig. 7b). An explanation

could be that the background La*er*-0 is more responsive to SA even when SAQ4 is segregating (Fig. 4a and 5a), and this heightened response could partially mask the difference produced by the QTL. Thus, the difference between La*er*-0 and N297.46 is smaller than that between No-0 and N15.15 in several experiments (Fig. 4a and 5b). In the mapping interval defined in Fig. 7b, there is, among other candidates, a calmodulin-binding protein (CBP60g; At5G26920), a gene reported to participate in SA signalling (Wang et al. 2009).

SAQ4 is quite robust and is detectable under all the SA concentrations used (Fig. 4a). Although the pairs No-0 vs. N15.15 and La*er*-0 vs. N297.46 show significant differences, SAQ4 does not explain most of the difference in weight found between the ecotypes when BTH is applied (Fig. 5b, difference between La*er*-0 and No-0). This fact agrees with our previous report that no single QTL is significant in this population when the effect of the BTH on the weight of the plant is considered (Canet et al. 2010a).

The resistance triggered by MeJA in SAQ4 does not show negative crosstalk with SA (Fig. 6). The simplest explanation is that SAQ4 is located after NPR1 and/or WRKY70, since these are the candidates of this hormone interaction. But a closer look to Fig. 6 shows that the genomic region that includes SAQ4 reacts to MeJA as it does to SA. If indeed SAQ4 is responsible for both responses, it could imply that SAQ4 is at the last steps of the SA signal transduction, the execution of the resistance. The reason is that both signals, although antagonistic, reduce the growth of *Pto*. Therefore, it is plausible that the same effect is produced by the same genes, and hence that SAQ4 is located among those same genes.

Conclusions

To summarize (Fig. 8), the results of the genotypes and the candidate genes in the intervals aforementioned lead us to speculate that none of the QTLs are affecting SA stability or accumulation after exogenous application. SAQ3 could be a QTL in a high affinity process (like SA carriers or conjugation enzyme), while SAQ1 and SAQ2 could be polymorphisms in NPR1 and NIMIN1-like genes that would result in a difference in SA perception but not in BTH perception. Then, SAQ4 would be a polymorphism in the execution part of the defence. For example, a calmodulin binding protein that would sense the changes in cytosolic Ca^{2+} produced by the pathogen, integrating this information with the SA and MeJA signals. Of course these genes are named according to their homology or description, but there are alternatives both known and unknown for the mentioned genes. For example, there are

receptor-like proteins in the intervals of three of the QTLs which could be the genes responsible of the observed differences.

The defined QTLs are valuable in themselves and will also help to complement other approaches, such as the search for mutants lacking a response to SA (Canet et al. 2010b), or transcriptomics descriptions of the response to biotic stress (Bilgin et al. 2010).

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References

Adam L, Somerville SC (1996) Genetic characterization of five powdery mildew resistance loci in *Arabidopsis thaliana*. Plant J 9: 341-356

Alonso-Blanco C, El-Assal SE, Coupland G, Koornneef M (1998a) Analysis of natural allelic variation at flowering time loci in the Landsberg *erecta* and Cape Verde Islands ecotypes of *Arabidopsis thaliana*. Genetics 149: 749-764.

Alonso-Blanco C, Koorneef M (2000) Naturally occurring variation in Arabidopsis: an underexploited resource for plant genetics. Trends Plant Sci 5: 22-29

Alonso-Blanco C, Koornneef M, van Ooijen JW (2006) QTL analysis. Methods Mol Biol 323: 79-99

Alonso-Blanco C, Peeters A, Koornneef M, Lister C, Dean C, van den Bosch N, Pot J, Kuiper M (1998b) Development of an AFLP based linkage map of Ler, Col and Cvi *Arabidopsis thaliana* ecotypes and construction of a Ler/Cvi recombinant inbred line population. Plant J 14: 259-271

Bakker EG, Traw MB, Toomajian C, Kreitman M, Bergelson J (2008) Low levels of polymorphism in genes that control the activation of defense response in *Arabidopsis thaliana*. Genetics 178: 2031-2043

Bilgin DD, Zavala JA, Zhu J, Clough SJ, Ort DR, Delucia EH (2010) Biotic stress globally downregulates photosynthesis genes. Plant Cell Environ 33: 1597–1613

Canet JV, Dobón A, Ibáñez F, Perales L, Tornero P (2010a) Resistance and biomass in Arabidopsis: a new model for salicylic acid perception. Plant Biotech J 8: 126-141

Canet JV, Dobón A, Roig A, Tornero P (2010b) Structure-function analysis of *npr1* alleles in Arabidopsis reveals a role for its paralogs in the perception of salicylic acid. Plant Cell Environ 33: 1911-1922

Cao H, Bowling SA, Gordon AS, Dong X (1994) Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell 6: 1583-1592

Cao H, Glazebrook J, Clarke JD, Volko S, Dong X (1997) The Arabidopsis *NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell 88: 57-63

Clerkx EJ, El-Lithy ME, Vierling E, Ruys GJ, Blankestijn-De Vries H, Groot SP, Vreugdenhil D, Koornneef M (2004) Analysis of natural allelic variation of Arabidopsis seed germination and seed longevity traits between the accessions Landsberg erecta and Shakdara, using a new recombinant inbred line population. Plant Physiol. 135: 432-443

Chen HJ, Hou WC, Kuc J, Lin YH (2001) Ca^{2+}-dependent and Ca^{2+}-independent excretion modes of salicylic acid in tobacco cell suspension culture. J Exp Bot 52: 1219-1226

Churchill GA, Doerge RW (1994) Empirical threshold values for quantitative trait mapping. Genetics 138: 963-971

Delaney TP, Friedrich L, Ryals JA (1995) Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance. Proc Natl Acad Sci USA. 92: 6602-6606

Deslandes L, Pileur F, Liaubet L, Camut S, Can C, Williams K, Holub E, Beynon J, Arlat M, Marco Y (1998) Genetic characterization of RRS1 a recessive locus in *Arabidopsis thaliana* that confers resistance to the bacterial soft rot pathogen, *Ralstonia solanacearum*. Mol Plant-Microbe Interact 11: 659-667

Desveaux D, Subramaniam R, Despres C, Mess J-N, Levesque C, Fobert PR, Dangl JL, Brisson N (2004) A "whirly" transcription factor is required for salicylic acid-dependent disease resistance in Arabidopsis. Developmental Cell 6: 229-240

Dong X (2004) *NPR1*, all things considered. Curr Opin Plant Biol 7: 547-552

Fan J, Crooks C, Lamb C (2008) High-throughput quantitative luminescence assay of the growth *in planta* of *Pseudomonas syringae* chromosomally tagged with *Photorhabdus luminescens luxCDABE*. Plant J 53: 393-399
Genoud T, Metraux JP (1999) Crosstalk in plant cell signaling: structure and function of the genetic network. Trends Plant Sci 4: 503-507
Glazebrook J, Rogers EE, Ausubel FM (1996) Isolation of Arabidopsis mutants with enhanced disease susceptibility by direct screening. Genetics 143: 973-982
Hien Dao TT, Puig RC, Kim HK, Erkelens C, Lefeber AW, Linthorst HJ, Choi YH, Verpoorte R (2009) Effect of benzothiadiazole on the metabolome of *Arabidopsis thaliana*. Plant Physiol Biochem 47: 146-152
Huang WE, Wang H, Zheng H, Huang L, Singer AC, Thompson I, Whiteley AS (2005) Chromosomally located gene fusions constructed in *Acinetobacter sp.* ADP1 for the detection of salicylate. Environ Microbiol 7: 1339-1348
Katagiri F, Thilmony S, He SY (2002) The *Arabidopsis thaliana-Pseudomonas syringae* Interaction. The Arabidopsis book. American Society of Plant Biologists. doi: 10.1199/tab.0111. Rockville, MD, USA
Kliebenstein DJ, Figuth A, Mitchell-Olds T (2002) Genetic architecture of plastic methyl jasmonate responses in *Arabidopsis thaliana*. Genetics 161: 1685-1696
Koumproglou R, Wilkes TM, Townson P, Wang XY, Beynon J, Pooni HS, Newbury HJ, Kearsey MJ (2002) STAIRS: a new genetic resource for functional genomic studies of Arabidopsis. Plant J 31: 355-364
Lawton K, Weymann K, Friedrich L, Vernooij B, Uknes S, Ryals J (1995) Systemic acquired resistance in Arabidopsis requires salicylic acid but not ethylene. Mol Plant-Microbe Interact 8: 863-870
Lawton KA, Friedrich L, Hunt M, Weymann K, Delaney T, Kessmann H, Staub T, Ryals J (1996) Benzothiadiazole induces disease resistance in Arabidopsis by activation of the systemic acquired resistance signal transduction pathway. Plant J 10: 71-82
Li J, Brader G, Palva ET (2004) The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. Plant Cell 16: 319-331
Lister C, Dean C (1993) Recombinant inbred lines for mapping RFLP and phenotypic markers in Arabidopsis thaliana. Plant J 4: 745-750
Loudet O, Chaillou S, Camilleri C, Bouchez D, Daniel-Vedele F (2002) Bay-0 x Shahdara recombinant inbred line population: a powerful tool for the genetic dissection of complex traits in Arabidopsis. Theor Appl Genet 104: 1173-1184
Magliano TM, Botto JF, Godoy AV, Symonds VV, Lloyd AM, Casal JJ (2005) New Arabidopsis recombinant inbred lines (Landsberg erecta x Nossen) reveal natural variation in phytochrome-mediated responses. Plant Physiol 138: 1126-1135
Maier F, Zwicker S, Hückelhoven A, Meißner M, Funk J, Pfitzner AJP, Pfitzner UM (2011) NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS1 (NPR1) and some NPR1-related proteins are sensitive to salicylic acid. Mol Plant Pathol 12:73-91
McKhann HI, Camilleri C, Bérard A, Bataillon T, David JL, Reboud X, Le Corre V, Caloustian C, Gut IG, Brune D (2004) Nested core collections maximizing genetic diversity in *Arabidopsis thaliana*. Plant J 38: 193-202
Molina A, Hunt MD, Ryals JA (1998) Impaired fungicide activity in plants blocked in disease resistance signal transduction. Plant Cell 10: 1903-1914
Nawrath C, Heck S, Parinthawong N, Metraux JP (2002) *EDS5*, an essential component of salicylic acid-dependent signaling for disease resistance in Arabidopsis, is a member of the MATE transporter family. Plant Cell 14: 275-286

Nawrath C, Métraux JP, Genoud T (2005) Chemical signals in plant resistance: salicylic acid. In: Tuzun S, Bent E (eds) Multigenic and Induced Systemic Resistance in Plants. Springer US, Dordrecht, Netherlands, pp. 143–165

Niederl S, Kirsch T, Riederer M, Schreiber L (1998) Co-permeability of 3H-labeled water and 14C-labeled organic acids across isolated plant cuticles. Investigating cuticular paths of diffusion and predicting cuticular transpiration. Plant Physiol 116: 117-123

Perchepied L, Kroj T, Tronchet M, Loudet O, Roby D (2006) Natural variation in partial resistance to *Pseudomonas syringae* is controlled by two major QTLs in *Arabidopsis thaliana*. PLoS ONE. 1: e123

Pieterse CM, Van Loon LC (2004) NPR1: the spider in the web of induced resistance signaling pathways. Curr Opin Plant Biol 7: 456-464

Pylatuik JD, Fobert PR (2005) Comparison of transcript profiling on Arabidopsis microarray platform technologies. Plant Mol Biol 58: 609-624

Rhoads DM, McIntosh L (1992) Salicylic acid regulation of respiration in higher plants: alternative oxidase expression. Plant Cell 4: 1131-1139

Ritter C, Dangl JL (1996) Interference between two specific pathogen recognition events mediated by distinct plant disease resistance genes. Plant Cell 8: 251-257

Shah J, Tsui F, Klessig DF (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. Mol Plant-Microbe Interact 10: 69-78

Spoel SH, Koornneef A, Claessens SM, Korzelius JP, Van Pelt JA, Mueller MJ, Buchala AJ, Metraux JP, Brown R, Kazan K, Van Loon LC, Dong X, Pieterse CM (2003) NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell 15: 760-770

Spoel SH, Mou Z, Tada Y, Spivey NW, Genschik P, Dong X (2009) Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. Cell 137: 860-872

Tada Y, Spoel SH, Pajerowska-Mukhtar K, Mou Z, Song J, Wang C, Zuo J, Dong X (2008) Plant immunity requires conformational charges of NPR1 via S-nitrosylation and thioredoxins. Science 321: 952-956

Tornero P, Dangl JL (2001) A high throughput method for quantifying growth of phytopathogenic bacteria in *Arabidopsis thaliana*. Plant J 28: 475-481

Uknes S, Mauch-Mani B, Moyer M, Potter S, Williams S, Dincher S, Chandler D, Slusarenko A, Ward E, Ryals J (1992) Acquired resistance in Arabidopsis. Plant Cell 4: 645-656

van Berloo R (2008) GGT 2.0: versatile software for visualization and analysis of genetic data. J Hered 99: 232-236

van Leeuwen H, Kliebenstein DJ, West MA, Kim K, van Poecke R, Katagiri F, Michelmore RW, Doerge RW, St Clair DA (2007) Natural variation among *Arabidopsis thaliana* accessions for transcriptome response to exogenous salicylic acid. Plant Cell 19: 2099-2110

Vlot AC, Dempsey DMA, Klessig DF (2009) Salicylic acid, a multifaceted hormone to combat disease. Annual Review of Phytopathology 47: 177-206

Vlot AC, Klessig DF, Park SW (2008) Systemic acquired resistance: the elusive signal(s). Curr Opin Plant Biol 11: 436-442

Wang L, Tsuda K, Sato M, Cohen JD, Katagiri F, Glazebrook J (2009) Arabidopsis CaM binding protein CBP60g contributes to MAMP-induced SA accumulation and is involved in disease resistance against *Pseudomonas syringae*. PLoS Pathog 5: e1000301

Wang S, Basten CJ, Zeng Z-B (2007) Windows QTL Cartographer. Department of Statistics, North Carolina State University, Raleigh, NC, USA

Weigel RR, Bauscher C, Pfitzner AJ, Pfitzner UM (2001) NIMIN-1, NIMIN-2 and NIMIN-3, members of a novel family of proteins from Arabidopsis that interact with NPR1/NIM1, a key regulator of systemic acquired resistance in plants. Plant Mol Biol 46: 143-160
Weigel RR, Pfitzner UM, Gatz C (2005) Interaction of NIMIN1 with NPR1 modulates PR gene expression in Arabidopsis. Plant Cell 17: 1279-1291
Werner JD, Borevitz JO, Warthmann N, Trainer GT, Ecker JR, Chory J, Weigel D (2005) Quantitative trait locus mapping and DNA array hybridization identify an FLM deletion as a cause for natural flowering-time variation. Proc Natl Acad Sci USA 102: 2460-2465
Wiermer M, Feys BJ, Parker JE (2005) Plant immunity: the *EDS1* regulatory node. Curr Opin Plant Biol 8: 383-389
Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature 414: 562-565
Wilson IW, Schiff CL, Hughes DE, Somerville SC (2001) Quantitative trait loci analysis of powdery mildew disease resistance in the *Arabidopsis thaliana* accession kashmir-1. Genetics 158: 1301-1309
You IS, Ghosal D, Gunsalus IC (1991) Nucleotide sequence analysis of the *Pseudomonas putida PpG7* salicylate hydroxylase gene (*nahG*) and its 3'-flanking region. Biochemistry 30: 1635-1641
Zhang Y, Fan W, Kinkema M, Li X, Dong X (1999) Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. Proc Natl Acad Sci USA 96: 6523-6528

Supplementary Information

Fig. S1.-Markers used in the F2 between Edi-0 and Stw-0.

Fig. S2.-STAIRs lines used in this work.

Fig. S3.-RILs of La*er*-0 x No-0 selected and NIL generation.

Figure legends

Fig. 1 Natural variation among the ecotypes of Arabidopsis in response to induced resistance. Chemicals that induce resistance through salicylic acid (SA) perception were applied to a collection of ecotypes (McKhann et al. 2004). One day later, *Pseudomonas syringae pv. tomato DC3000* (*Pto*) was inoculated and its growth measured three days later in a logarithmic scale. For each genotype and treatment, three samples, with 5 plants per sample were taken. In order to represent in a single figure all the ecotypes tested, the average value of three independent experiments were expressed in arbitrary units relative to Col-0 being 100. **a** Mock treatment. **b** 10 g/L fosetyl. **c** 350 µM Benzothiadiazole (BTH). **d** 100 µM SA. In the case of the chemical treatments (b, c, and d), the values correspond to the induced resistance (mock treated minus chemical treated) of each ecotype related to the same value (mock treated minus chemical treated) in Col-0. Rubezhnoe stands for Rubezhnoe-1, N7 P. for N7 Pinguba, N6 K. for N6 Karelian, Sampo M. for Sampo Mountain, and N13 K. for N13 Konchezero

Fig. 2 Variation among some ecotypes and their progeny in response to SA. **a** Growth of *Pto* in selected ecotypes after a mock or 100 µM SA treatment, as described in Fig. 1. In the "Y" axis, logarithm of colony forming units per plant. This experiment was done three times with similar results. **b** Frequency distribution of the genotypes tested with SA. F2s from a cross Edi-0 x Stw-0 and RILs from Bay-0 x Sha-0 (Loudet et al. 2002). In the "Y" axis, percentage of genotypes for a given value of Log(cfu/plant), while in the "X" axis, Log(cfu/plant) after SA treatment for a given percentage of genotypes. The number indicates the maximal value (intervals of 0.5 Logs). **c** idem with the RILs Col-4 x La*er*-0 and La*er*-0 x No-0

Fig. 3 QTLs among some ecotypes in response to mock and SA pretreatment and *Pto* growth. Plants were treated with either mock or 100 µM SA, as described in Fig. 1. The output shown is the likelihood of a QTL (in logarithm of odds; LOD, in the Y axis) in a particular region of the genome (X axis). The horizontal line shows the threshold of significance. The continuous lines show the QTLs for SA treatment, and the dotted line the QTLs for mock treatment. The Chromosomes of Arabidopsis are delimited by vertical lines and named with roman numbers. The populations analyzed were **a** F3s of Edi-0 x Stw-0. **b** RILs of Bay-0 x Sha-0. **c** RILs of Col-4 x La*er*-0. **d** RILs of La*er*-0 x No-0

Fig. 4 Differential response to SA in selected genotypes. **a** Growth of *Pto* in some genotypes after a mock or different SA treatments (50, 100, 250 and 500 µM), as described in Fig. 1. **b**

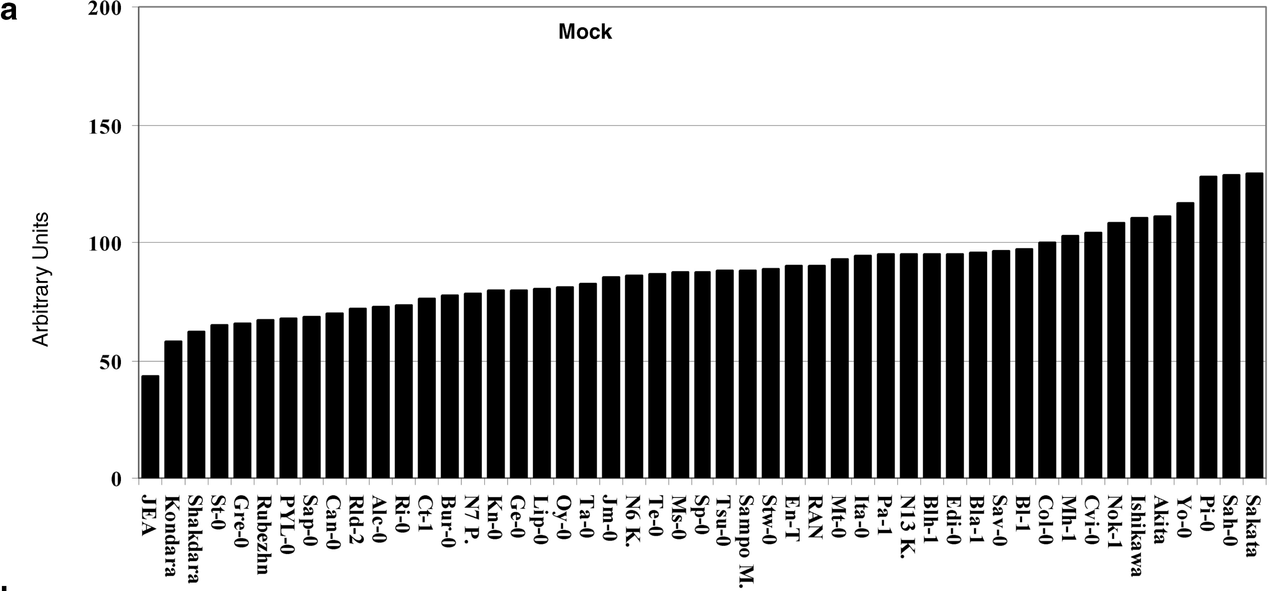
Accumulation of SA after 100 μM SA treatment. Plants were treated as in Fig. 1, and one day after the treatment, their concentration of SA, both "Free" and "Total" was measured. *NahG* is included as a control, taking into account the SA that remains on the surface of the plant. There is a significant difference between Edi-0 and Stw-0 in both free and total SA, but not between the rests of pairs (t-student, $P < 0.05$). These experiments were done three times with similar results

Fig. 5 Differential response to BTH in selected genotypes. **a** Growth of *Pto* in some genotypes as described in Fig. 1, after a mock or 35 μM BTH treatment. In the BTH treatment, there is no significant difference between Edi-0 and Stw-0, while it is significant in each of the other pairs (t-student, $P < 0.05$). **b** Plants were treated with either mock or 350 μM BTH four times, their fresh weight (FW) recorded, and the ratio between the BTH and mock treated plants represented (average and SD of 15 plants in three groups of five). This experiment was done three times with similar results

Fig. 6 Differential response to methyl jasmonate in selected genotypes. Growth of *Pto* in some genotypes after a mock or 100 μM methyl jasmonate (abbreviated as "MeJA") treatment as described in Fig. 1. This experiment was done three times with similar results

Fig. 7 Refined QTLs mapping. **a** Col-0 x La*er*-0; eleven recombinants were selected from an F2 originated by P59 x P64. **b** La*er*-0 x No-0; 35 recombinants were selected from the line N15, heterozygous for the QTL (continuous line) and 42 recombinants from the line N297 (dotted line). In both cases, the plants were genotyped with markers described in Supplemental Fig. S2, and their QTLs mapped as described in Fig. 3 with 100 μM SA and *Pto*

Fig. 8 A putative model of the QTLs described in this work. SA is perceived by NPR1 in its nuclear localization. NIMINs proteins interact with NPR1, repressing its activity. TGAs also interact with NPR1, inducing the expression of genes that eventually will have an impact in the growth of *Pto*. There is a negative crosstalk between SA and methyl jasmonate, and this hormone also requires NPR1 (but in its cytosolic form) to trigger a small resistance against *Pto*. The ellipses point to the positions that may correspond to the QTLs described, on the basis of the previous experiments and the genes in the mapping intervals. See the text for references of the mentioned genes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Kn-0
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Oy-0
Ta-0
Jm-0
N6 K.
Te-0
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Tsu-0
Sampo M.
Stw-0
En-T
RAN
Mt-0
Ita-0
Pa-1
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Blh-1
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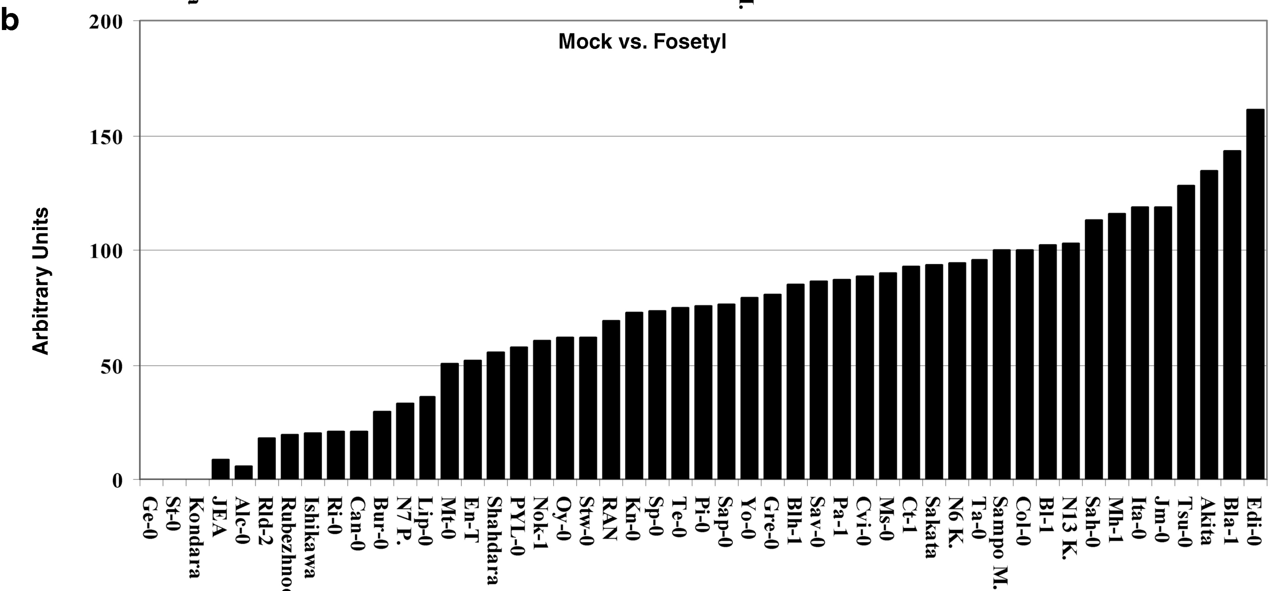
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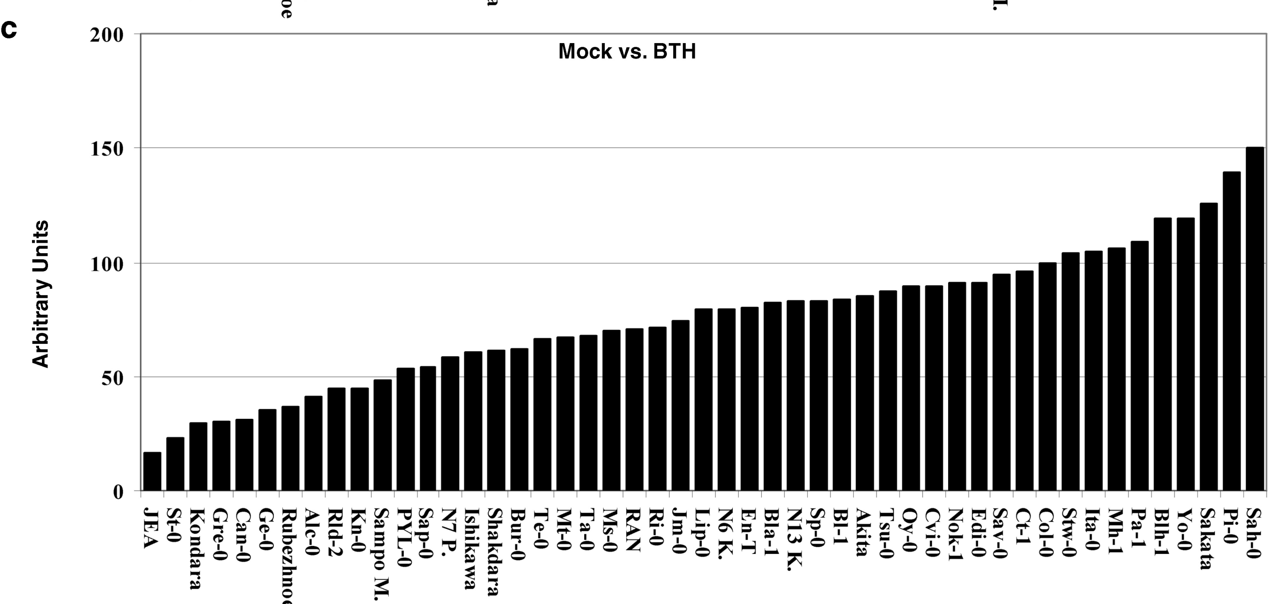
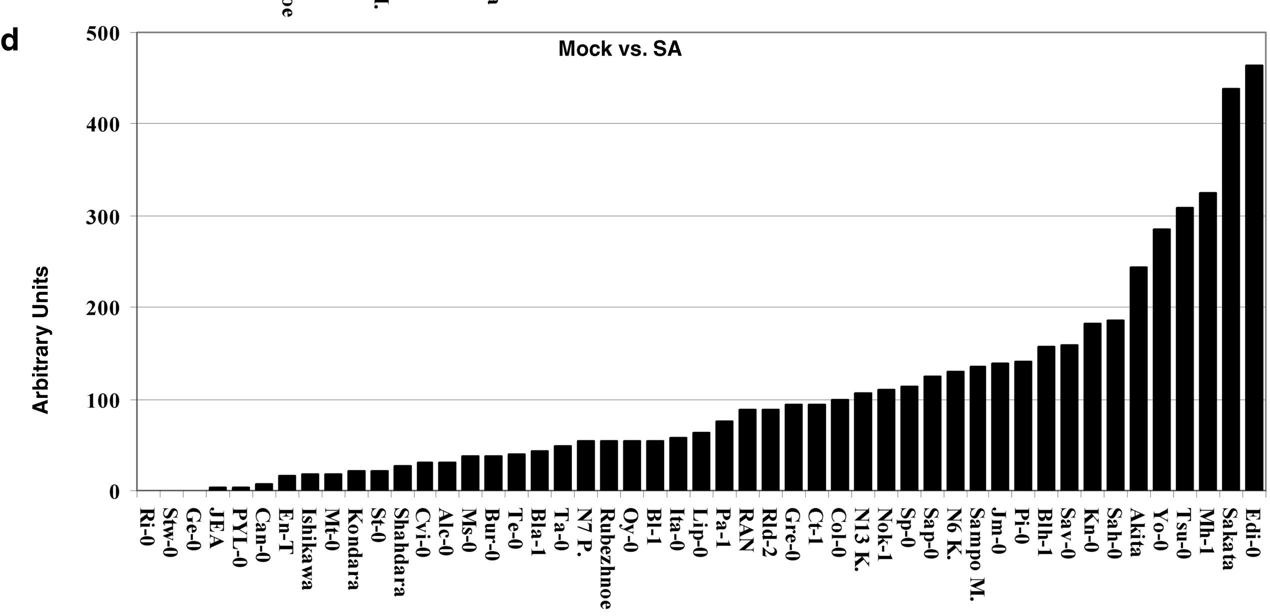
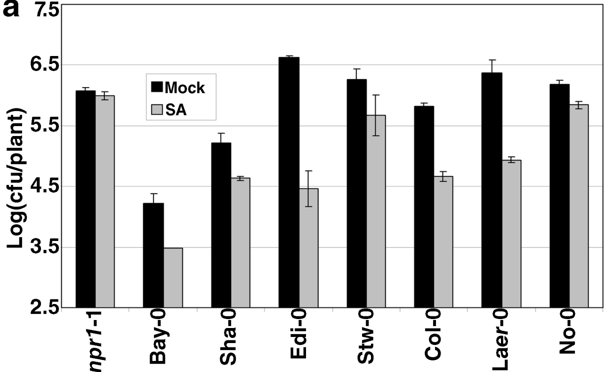
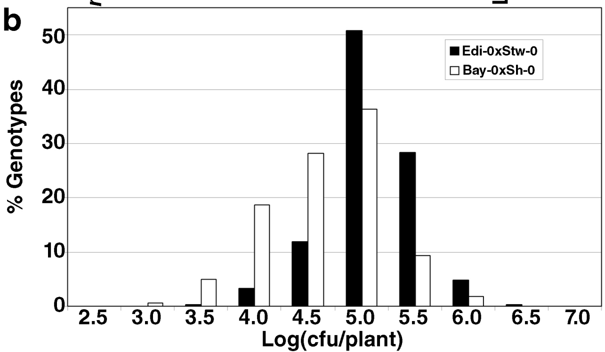
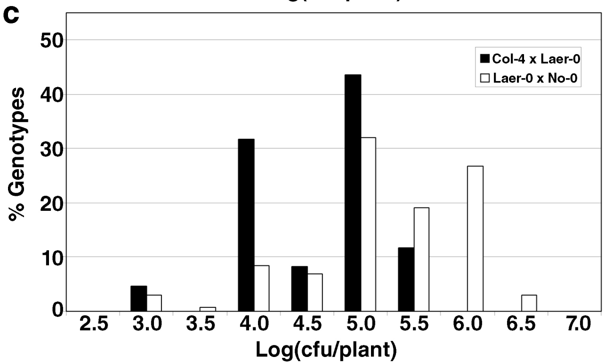
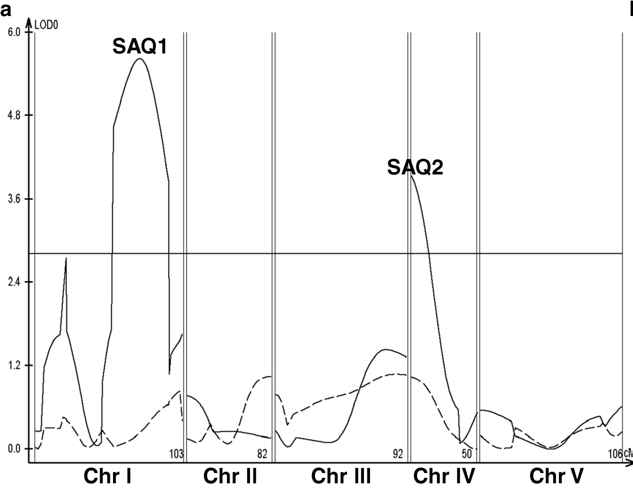
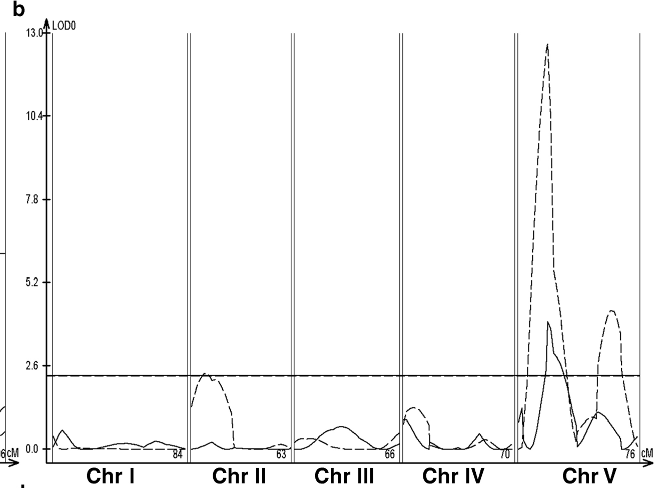
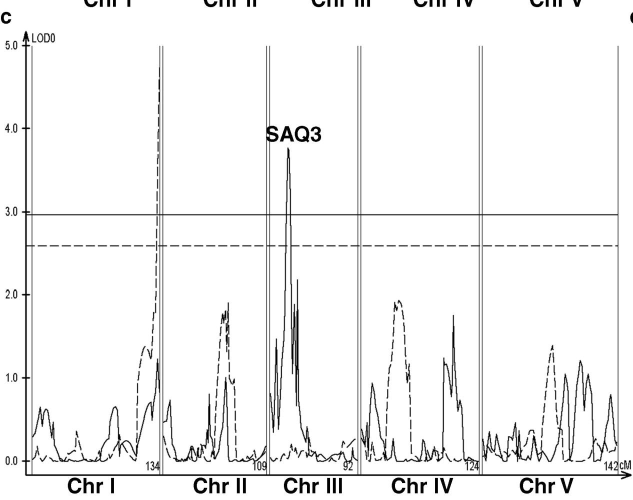
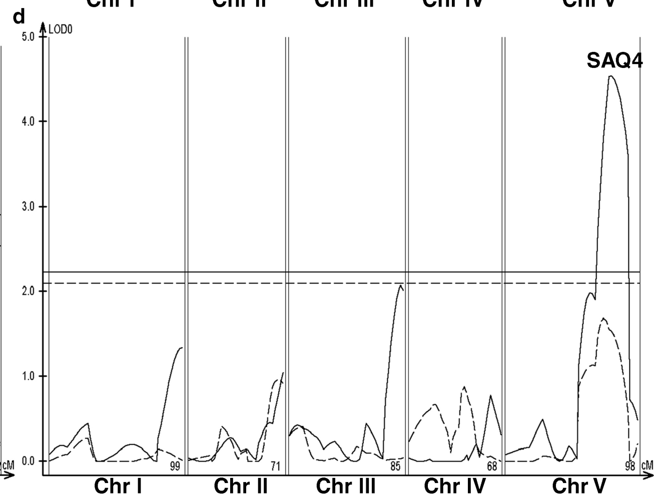
Bur-0
N7 P.
Lip-0
Mt-0
En-T
Shahdara
PYL-0
Nok-1
Oy-0
Stw-0
RAN
Kn-0
Sp-0
Te-0
Pi-0
Sap-0
Yo-0
Gre-0
Blh-1
Sav-0
Pa-1
Cvi-0
Ms-0
Ct-1
Sakata
N6 K.
Ta-0
Sampo M.
Col-0
Bl-1
N13 K.
Sah-0
Mh-1
Ita-0
Jm-0
Tsu-0
Akita
Bla-1
Edi-0


c
200
150
100
50
0
Mock vs. BTH
Arbitrary Units
JEA
St-0
Kondara
Gre-0
Can-0
Ge-0
Rubezhnoe
Alc-0
Rld-2
Kn-0
Sampo M.
PYL-0
Sap-0
N7 P.
Ishikawa
Shahdara
Bur-0
Te-0
Mt-0
Ta-0
Ms-0
RAN
Ri-0
Jm-0
Lip-0
N6 K.
En-T
Bla-1
N13 K.
Sp-0
Bl-1
Akita
Tsu-0
Oy-0
Cvi-0
Nok-1
Edi-0
Sav-0
Ct-1
Col-0
Stw-0
Ita-0
Mh-1
Pa-1
Blh-1
Yo-0
Sakata
Pi-0
Sah-0


d
500
400
300
200
100
0
Mock vs. SA
Arbitrary Units
Ri-0
Stw-0
Ge-0
JEA
PYL-0
Can-0
En-T
Ishikawa
Mt-0
Kondara
St-0
Shahdara
Cvi-0
Alc-0
Ms-0
Bur-0
Te-0
Bla-1
Ta-0
N7 P.
Rubezhnoe
Oy-0
Bl-1
Ita-0
Lip-0
Pa-1
RAN
Rld-2
Gre-0
Ct-1
Col-0
N13 K.
Nok-1
Sp-0
Sap-0
N6 K.
Sampo M.
Jm-0
Pi-0
Blh-1
Sav-0
Kn-0
Sah-0
Akita
Yo-0
Tsu-0
Mh-1
Sakata
Edi-0


a
Mock
SA
Log(cfu/plant)
7.5
6.5
5.5
4.5
3.5
2.5
npr1-1
Bay-0
Sha-0
Edi-0
Stw-0
Col-0
Laer-0
No-0

b
Edi-0xStw-0
Bay-0xSh-0
% Genotypes
50
40
30
20
10
0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
Log(cfu/plant)

c
Col-4 x Laer-0
Laer-0 x No-0
% Genotypes
50
40
30
20
10
0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
Log(cfu/plant)


a
LOD0
SAQ1
SAQ2
6.0
4.8
3.6
2.4
1.2
0.0
103
82
92
50
106
cM
Chr I
Chr II
Chr III
Chr IV
Chr V

b
LOD0
13.0
10.4
7.8
5.2
2.6
0.0
84
63
66
70
76
cM
Chr I
Chr II
Chr III
Chr IV
Chr V

c
LOD0
SAQ3
5.0
4.0
3.0
2.0
1.0
0.0
134
109
92
124
142
cM
Chr I
Chr II
Chr III
Chr IV
Chr V

d
LOD0
SAQ4
5.0
4.0
3.0
2.0
1.0
0.0
99
71
85
68
98
cM
Chr I
Chr II
Chr III
Chr IV
Chr V

a

Log(cfu/plant)

7.5
6.5
5.5
4.5
3.5
2.5

Mock
SA 50
SA100
SA250
SA500

Edi-0
Stw-0
P59
P64
L*aer*-0
N297.46
No-0
N15.15

b

ng/g FW

3000
2000
1000
0

Free
Total

NahG
Edi-0
Stw-0
P59
P64
L*aer*-0
N297.46
No-0
N15.15

a

Mock
BTH

Log(cfu/plant)

7.5
6.5
5.5
4.5
3.5
2.5

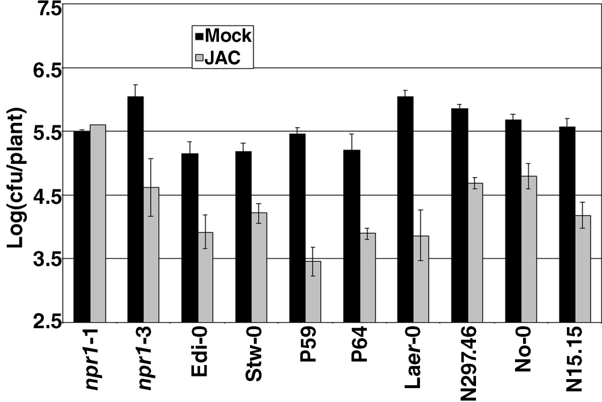
npr1-1
Edi-0
Stw-0
P59
P64
L*aer*-0
N297.46
No-0
N15.15

b

%FW

100
25

npr1-1
Edi-0
Stw-0
P59
P64
L*aer*-0
N297.46
No-0
N15.15



7.5
6.5
5.5
4.5
3.5
2.5
Log(cfu/plant)
Mock
JAC
npr1-1
npr1-3
Edi-0
Stw-0
P59
P64
Laer-0
N297.46
No-0
N15.15

a

LOD0

4.0
3.2
2.4
1.6
0.8
0.0

SAQ3

135 110 93 125 142 cM

Chr I Chr II Chr III Chr IV Chr V

b

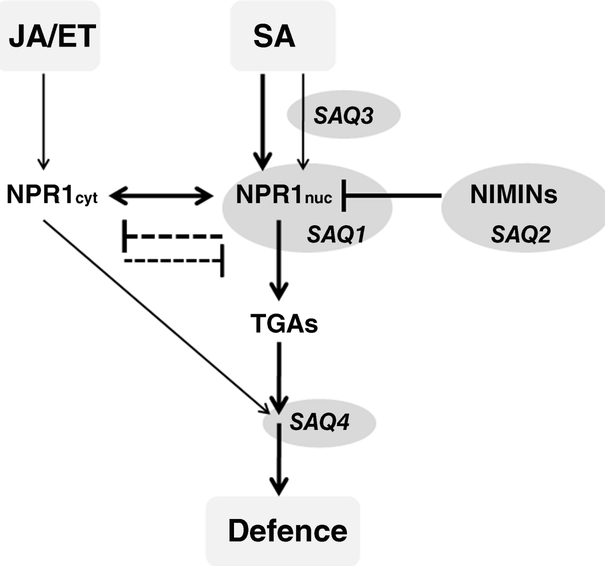
LOD0

5.0
4.0
3.0
2.0
1.0
0.0

SAQ4

135 110 93 125 142 cM

Chr I Chr II Chr III Chr IV Chr V


JA/ET
SA
SAQ3
NPR1cyt
NPR1nuc
NIMINs
SAQ1
SAQ2
TGAs
SAQ4
Defence

Quantitative genetic analysis of salicylic acid perception in Arabidopsis

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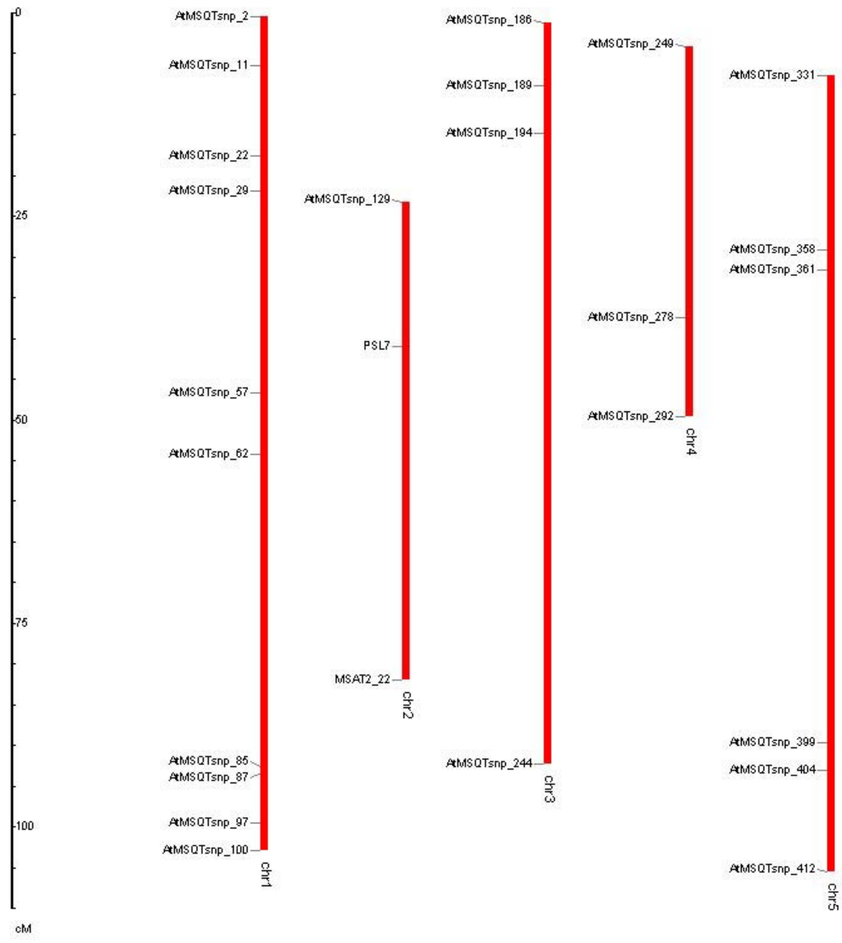

Fig. S1 Markers used in the F2 between Edi-0 and Stw-0. The markers described in www.naturalvariation.org as polymorphic for Edi-0 vs. Stw-0 were used to genotype with iPLEX® in the CEGEN (Spanish National Genotyping Centre, www.cegen.org). Two additional SSLP markers were added to complete the chromosome II. These figures were done with the program GGT 2.0 (van Berloo 2008)

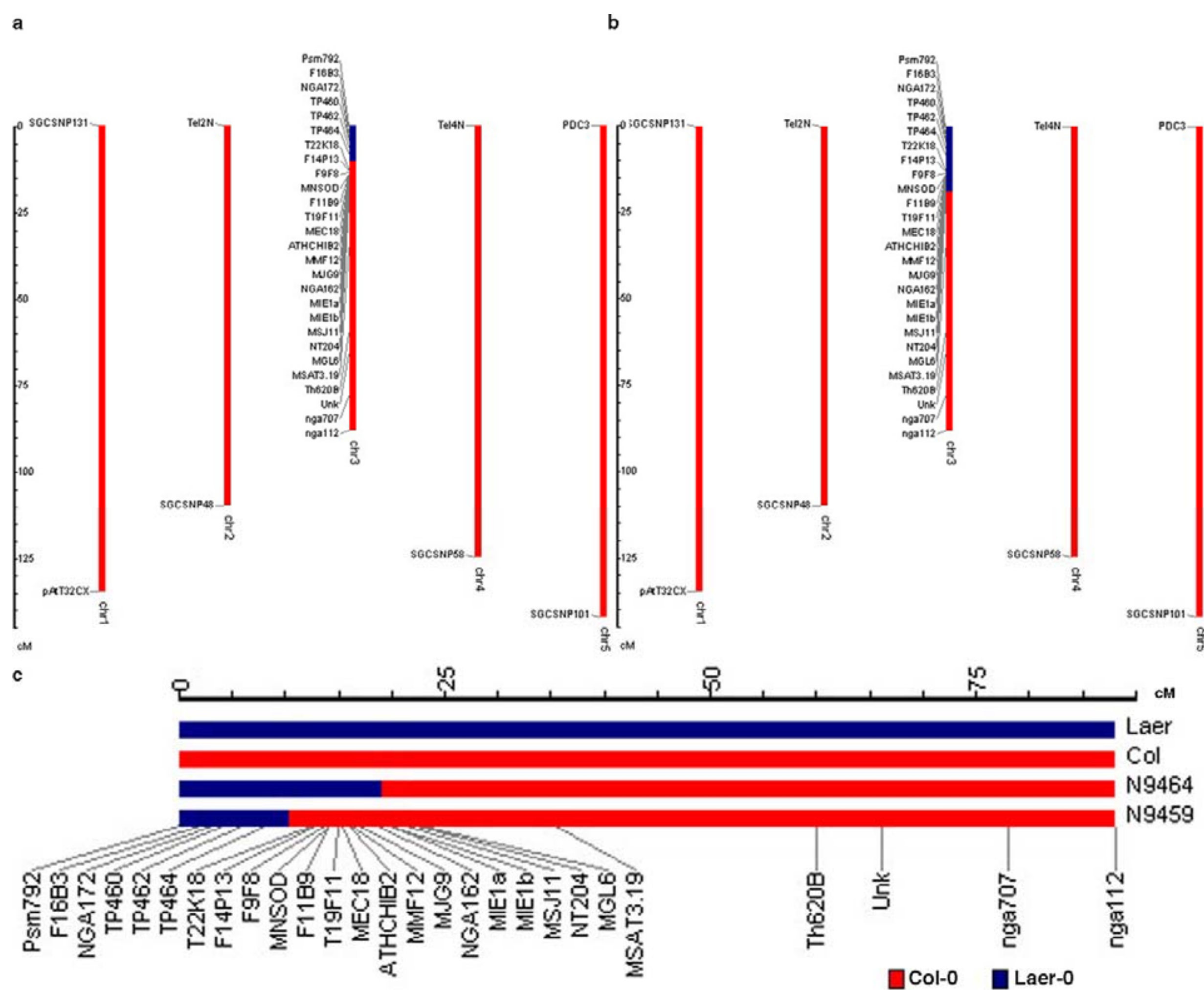

Fig. S2 STAIRs lines used in this work. The following STAIRs (Koumproglou et al. 2002) lines were genotyped with the indicated markers**: a** N9459 (abbreviated in the main text as P59) and **b** N9464 (P64 in the main text). **c** detail of Chromosome III in both lines, along Col-0 and La*er*-0. Note that our result differs slightly from what is available at www.arabidopsis.info

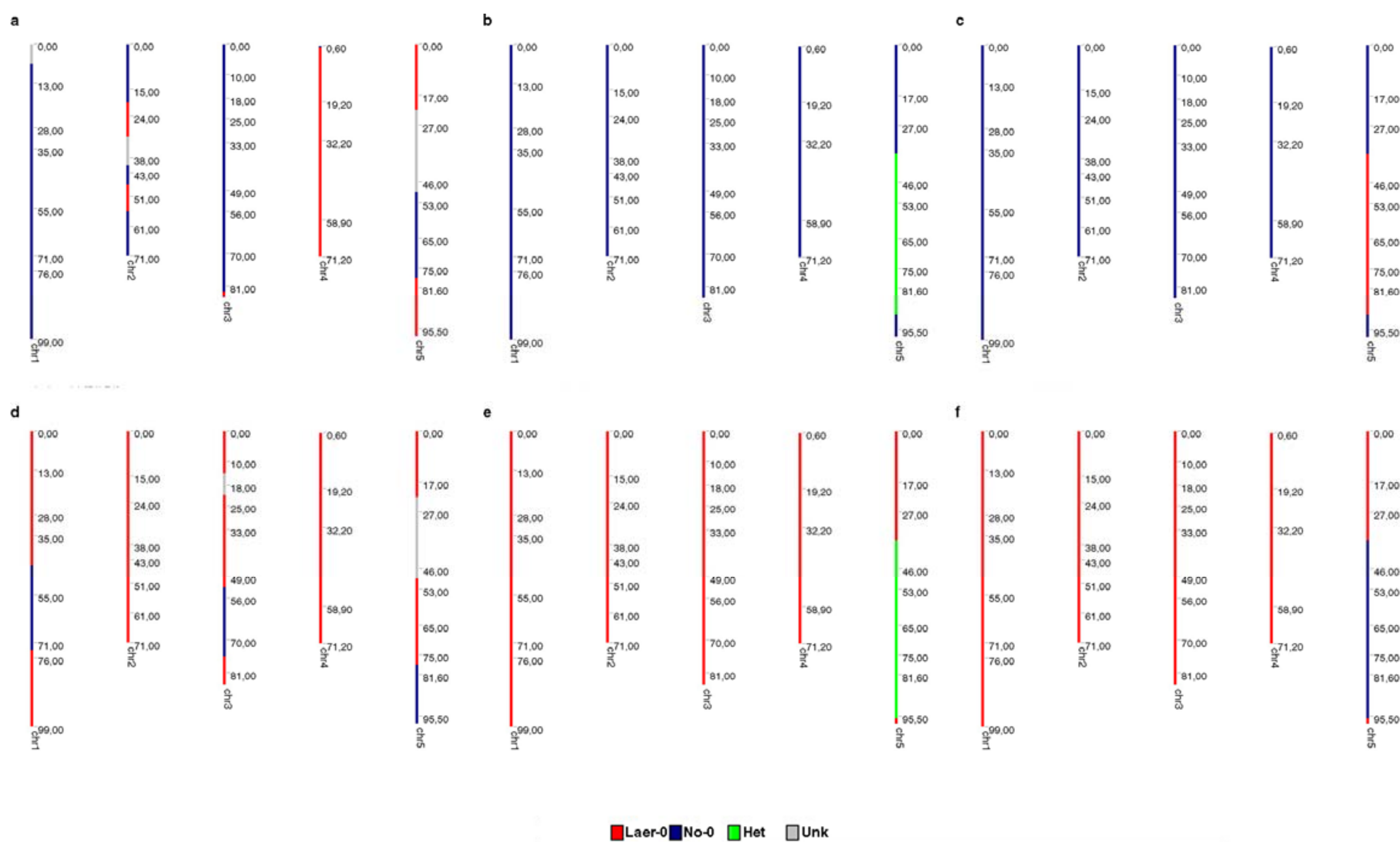

Fig. S3 RILs of La*er*-0 x No-0 selected and NIL generation. From the RIL La*er*-0 x No-0 (Magliano et al. 2005), the RIL 174 (**a**) was crossed with No-0 twice, and from that population, the NIL N15 (**b**) was selected. From the progeny of N15, the NIL N15.15 (**c**) was selected. In the other hand, the RIL 132 (**d**) was crossed with La*er*-0, and from that population, the NIL N297 (**e**) was selected. From the progeny of N297, the NIL N297.46 (**f**) was selected

References

Koumproglou R, Wilkes TM, Townson P, Wang XY, Beynon J, Pooni HS, Newbury HJ, Kearsey MJ (2002) STAIRS: a new genetic resource for functional genomic studies of Arabidopsis. Plant J 31: 355-364

Magliano TM, Botto JF, Godoy AV, Symonds VV, Lloyd AM, Casal JJ (2005) New Arabidopsis recombinant inbred lines (Landsberg erecta x Nossen) reveal natural variation in phytochrome-mediated responses. Plant Physiol 138: 1126-1135

van Berloo R (2008) GGT 2.0: versatile software for visualization and analysis of genetic data. J Hered 99: 232-236