

Pathogen stress increases somatic recombination frequency in *Arabidopsis*

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Evolution is based on genetic variability and subsequent phenotypic selection. Mechanisms that modulate the rate of mutation according to environmental cues, and thus control the balance between genetic stability and flexibility, might provide a distinct evolutionary advantage^{1–4}. Stress-induced mutations stimulated by unfavorable environments, and possible mechanisms for their induction, have been described for several organisms^{2–4}, but research in this area has mainly focused on microorganisms. We have analyzed the influence of adverse environmental conditions on the genetic stability of the higher plant *Arabidopsis thaliana*. Here we show that a biotic stress factor—attack by the oomycete pathogen *Peronospora parasitica*—can stimulate somatic recombination in *Arabidopsis*. The same effect was observed when plant pathogen-defense mechanisms were activated by the chemicals 2,6-dichloroisonicotinic acid (INA) or benzothiadiazole (BTH), or by a mutation (*cim3*). Together with previous studies of recombination induced by abiotic factors, these findings suggest that increased somatic recombination is a general stress response in plants. The increased genetic flexibility might facilitate evolutionary adaptation of plant populations to stressful environments.

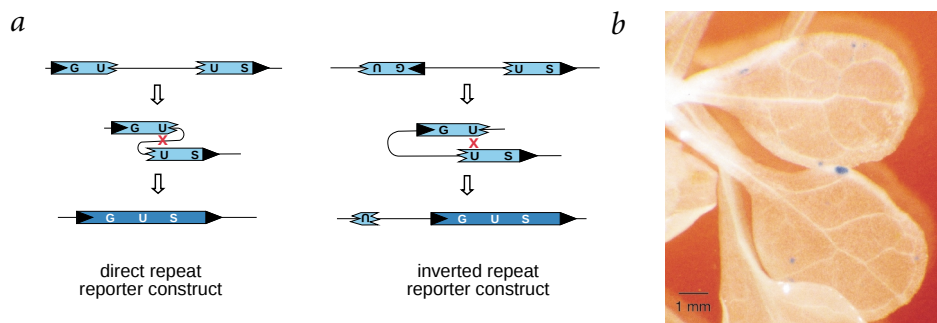
In plants, somatic recombination events are an important cause of genetic variability⁵, and, as plants lack a predetermined germ line, may also affect genetic composition of the progeny⁶. A variety of genotoxic factors, such as DNA-damaging chemicals, γ -irradiation and UV-B irradiation^{7–10}, stimulate somatic recombination, an

important pathway for the repair of DNA lesions in plants⁵. In addition, abiotic stress factors like heat and increased salinity seem to increase somatic recombination^{7,8}. It is not known, however, whether biotic stress affects recombination and whether increased recombination might therefore be a general stress response in plants. We examined whether exposure to pathogens, a major biotic stress factor for plants, affects plant genome stability. To measure somatic recombination in whole plants, we used *Arabidopsis* lines carrying reporter transgenes that allowed visual detection of recombination events (Fig. 1)^{8–12}. By testing populations of genetically identical plants grown under different conditions, this quantitative assay allowed us to determine the average recombination frequency per plant at the reporter gene locus and thus measure the influence of pathogen stress on genome stability.

We sprayed seedlings of *Arabidopsis* recombination reporter line 651 (ref. 8) with a spore suspension of *P. parasitica* isolate EMWA¹³, which resulted in localized necrotic lesions at the points of attempted penetration but no further proliferation of the pathogen, as detected by microscopic observation (data not shown). This suggests the activation of plant defense mechanisms^{14–16}. There was no macroscopic difference in appearance or growth of infected or uninfected plants. The infected plants showed a significant ($P < 0.01$) 1.8-fold increase in the average number of recombination sectors per plant (Fig. 2a), however, and a clear shift towards higher numbers of recombination sectors in individual plants (Fig. 2b).

Fig. 1 Detection of somatic recombination events in *Arabidopsis thaliana*.

a, Structure of reporter constructs. A DNA fragment carrying two inactive segments of a disrupted β -glucuronidase (GUS) reporter gene (*uidA*; indicated by light blue bars) sharing a stretch of identical DNA sequence (U) is introduced as a transgene into the plant genome. The sequence repeat on these reporter constructs can be arranged in either direct or inverted orientation. In both cases, homologous recombination (indicated by a red 'X') between the repeats restores an active reporter gene (dark blue bar)^{8–12}. The frequency of such events is affected by the structure of the reporter gene and the genomic integration site¹². **b**, Detection of recombination events within the reporter transgene. Histochemical staining of *Arabidopsis* recombination reporter plants for GUS activity reveals blue sectors of cells in which a functional reporter gene has been restored by a somatic recombination event. The size and shape of these recombination sectors is determined by the location and timing of recombination events and can vary from small sectors comprising a single or few cells to large sectors involving whole organs^{8–12}. The absolute number of recombination sectors reflects the number of somatic recombination events at the reporter gene locus.



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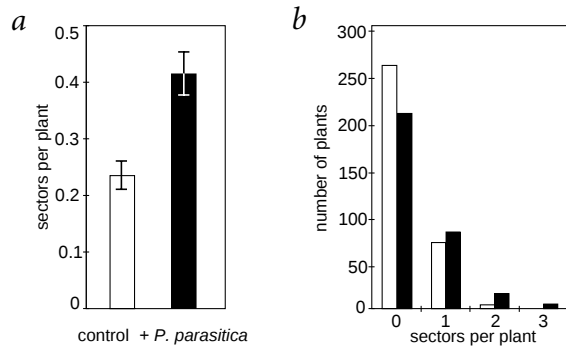


Fig. 2 Induction of somatic recombination in *Arabidopsis* by *P. parasitica* infection. **a**, Frequency of somatic recombination sectors per plant in soil-grown seedlings of *Arabidopsis* line 651 sprayed with water (control) or *P. parasitica*-isolate EMWA spores¹³. Error bars indicate s.e.m.. The numbers of recombination sectors per plant were 0.23 ± 0.025 (mean $\pm$ s.e.m.; $n=309$) for the control and 0.41 ± 0.038 ($n=332$) for the *P. parasitica*-infected plants; the difference between these means was significant ($P<0.01$). **b**, Distribution of recombination events within the plant population. White bars indicate control plants; black bars, plants infected by *P. parasitica*. We found no plants with more than two recombination sectors in the control population.

Once pathogens are recognized, different signaling pathways are activated in plants to transduce this stimulus¹⁷. One pathway involves salicylic acid as an important intermediate. Accumulation of salicylic acid is required for activation of several pathogenesis-related (PR) genes as well as for secondary defense responses that result in systemic immunity against pathogen attack^{15,17,18}. The same responses are stimulated by external application of salicylic acid analogs INA¹⁵ and BTH¹⁹. We sprayed two different *Arabidopsis* recombination reporter lines with water or solutions of INA or BTH. The latter treatments efficiently induced the pathogen-stress response (Fig. 3a). Seedlings from lines 651 and 11 (refs 8,11) that carry the homologous sequences of the recombination reporter transgene arranged as inverted or direct repeats, respectively, showed a 1.5-fold (INA) and 2.5-fold (BTH), or 7-fold (INA) and 4.6-fold (BTH), increase in the number of recombination sectors (Fig. 3b). Thus, chemical inducers of the salicylic acid-dependent pathogen response pathway stimulate homologous recombination in recombination reporter transgenes of different structure and at different positions in the plant genome.

To further confirm a crosstalk between the defense signal-transduction pathway and the recombination machinery of plants, we used a new mutant, *cim3* (ref. 16), that we recovered from a screen²⁰ of a mutagenized *A. thaliana* population for constitutive expression of the pathogen-defense marker gene (*PR-1*) in the absence of pathogen attack (Fig. 4a). *cim3* plants have constitutively increased levels of salicylic acid and increased resistance against infection with *P. parasitica* (data not shown), but do not show spontaneous necrotic lesions and do not differ phenotypically from wildtype plants. Genetic characterization of this mutant showed that it carries a single, dominant mutation responsible for the phenotype. We crossed two *Arabidopsis* lines with different recombination reporter transgenes with homozygous *cim3* mutants and wildtype plants and scored the recombination frequency in the resulting F1 plants,

which were hemizygous with respect to both the dominant *cim3* mutation and one copy of the recombination reporter construct. We observed a clear 4–5-fold increase in the frequency of recombination sectors in both progeny populations carrying the *cim3* allele (Fig. 4b).

Our results thus show an increase in the frequency of somatic recombination events in *Arabidopsis* after pathogen infection or induction of pathogen-defense responses by chemicals or a mutation. We saw this increase in several plant lines carrying reporter constructs at different positions in the genome; that the basal recombination frequencies of the lines differed is attributable to different reporter-gene structures and genomic position effects¹². This suggests that induction of somatic recombination by stress is a general effect that may influence homologous sequences throughout the plant genome.

Double-strand breaks and other forms of DNA damage are known to stimulate recombination in plants efficiently^{5,8}. Increases of DNA-damaging reactive oxygen intermediates^{17,21} and nuclease activities^{14,22} by pathogen stress have been described. Direct or indirect regulation of specific recombination factors by external stress^{10,23} may also be involved in increased somatic recombination. In addition, in many organisms increased transcription of a locus stimulates somatic recombination in the vicinity²⁴. Global transcriptional changes after pathogen stress affect about 1 in 25 *Arabidopsis* genes²⁵, and thus might affect somatic recombination at many places in the genome. The evolutionary significance of an increased somatic recombination rate after exposure to stress may lie in elevated genetic variability in the progeny, as stable genetic changes occurring in somatic tissue can be transmitted to the next generation⁶. Indeed, we previously observed an increased number of *Arabidopsis* progeny plants carrying a completely recombined reporter construct after induction of somatic recombination by UV-B irradiation of the parental plant generation¹⁰. Possible substrates for somatic recombination events in plants are the large number of disease-resistance genes involved in the perception of pathogen attack that are spread in clusters throughout the genome^{26,27}. Their evolution and the generation of novel dis-

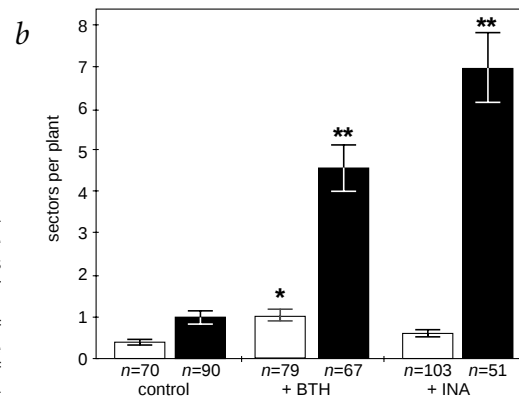
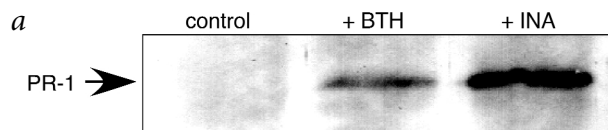


Fig. 3 Induction of plant pathogen-defense response and somatic recombination by salicylic-acid analogs. **a**, Expression of the PR-1 marker protein for pathogen stress-response was assayed by immunoblotting after spraying plants growing under sterile conditions with water (control), INA¹⁵ or BTH¹⁹. **b**, Frequency of somatic recombination sectors per plant (mean $\pm$ s.e.m.) in seedlings of *Arabidopsis* reporter lines 651 (black bars) and 11 (white bars) growing under sterile conditions after chemical treatment; the number of plants tested (n) is indicated. The single asterisk indicates a significant difference from the control ($P<0.05$); two asterisks indicate a highly significant difference ($P<0.01$). Because of the different growth conditions required for the assays (soil versus sterile culture), recombination frequencies differ from those shown in Fig. 2.

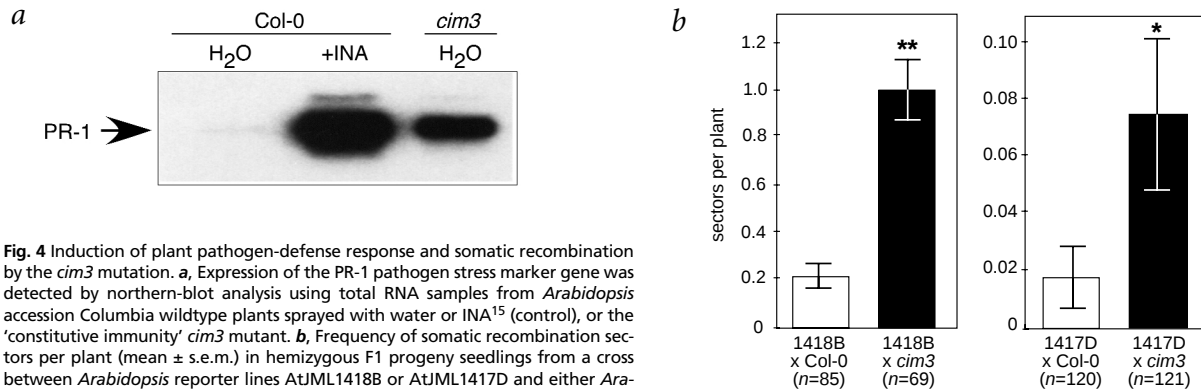


Fig. 4 Induction of plant pathogen-defense response and somatic recombination by the *cim3* mutation. **a**, Expression of the PR-1 pathogen stress marker gene was detected by northern-blot analysis using total RNA samples from *Arabidopsis* accession Columbia wildtype plants sprayed with water or INA¹⁵ (control), or the 'constitutive immunity' *cim3* mutant. **b**, Frequency of somatic recombination sectors per plant (mean ± s.e.m.) in hemizygous F1 progeny seedlings from a cross between *Arabidopsis* reporter lines AtJML1418B or AtJML1417D and either *Arabidopsis* accession Columbia wildtype plants (control) or Columbia plants homozygous with respect to the dominant *cim3* mutation. The number of plants tested (*n*) is indicated. Statistical evaluation and symbols are as in Fig. 3.

ease-resistance specificities has often been associated with recombination events^{28,29}, but the timescale and mechanism of these events is not known. Modulation of the rate of genetic change by environmental stress may be important for the long-term adaptation of populations to changing environments¹⁻³. It is tempting to speculate that somatic recombination events stimulated by pathogen stress and their transmission to the progeny might be involved in the evolution of plant R-gene clusters and new pathogen specificities.

Methods

Recombination reporter lines. The *A. thaliana* accession C24 lines 651 and 11 carry recombination reporter transgenes with two segments of the β -glucuronidase gene, sharing 566 bp of sequence homology arranged as an inverted repeat or 1,213 bp as a direct repeat, respectively. These plant lines have been described previously⁸. We produced new *A. thaliana* recombination reporter lines in the same genetic background as the *cim3* mutant (*A. thaliana* accession 'Columbia') by agrobacterium-mediated floral-dip transformation of wildtype *A. thaliana* Columbia plants with the β -glucuronidase recombination reporter constructs pCHNDC4 or pCHNIC4 (ref. 30). These carry a 618-bp sequence repeat in direct or inverted orientation. Transgenic lines AtJML1418B and AtJML1417D are homozygous with respect to the insertion of a single copy of pCHNDC4 or pCHNIC4, respectively.

Somatic recombination assays. We determined recombination frequencies at reporter gene loci by histochemical staining for GUS activity in *Arabidopsis* seedlings, using the substrate 5-bromo-4-chloro-3-indolyl glucuronide, and subsequently removed chlorophyll by ethanol extraction^{8,11}. We then counted blue-staining GUS-positive recombination sectors under a standard dissecting binocular that allowed detection of stained sectors as small as a single cell. The mean recombination frequency per plant was derived by dividing the total number of blue sectors observed by the number of plants analyzed. We tested the significance of the difference between mean recombination frequencies from untreated control plant populations and treated populations using Student's *t*-test for independent variance.

Pathogen infection assays. We sprayed 2-wk seedlings of *Arabidopsis* recombination reporter line 651 growing on soil (about 50 plants per pot) with water (control) or a spore suspension of *P. parasitica* isolate EMWA, as described¹³, and scored recombination sectors in plants from 6 individual pots from each treatment after 17 d.

Chemical induction assays. We sprayed 2-wk seedlings of *Arabidopsis* recombination reporter lines 651 and 11 growing on sterile medium in 100-mm Petri dishes^{8,11} with 500 μ l of water (control), 500 μ M INA¹⁵ or 500 μ M BTH¹⁹. After 4 d, we took samples to detect expression of the PR-1 pathogen-response marker protein^{15,19} by immunoblotting with a polyclonal antiserum. We scored GUS-positive recombination sectors 14 d after the chemical treatment.

Crossing experiments with the *cim3* mutant. We used pollen of *Arabidopsis* recombination reporter lines AtJML1418B or AtJML1417D to fertilize *Arabidopsis* Columbia plants homozygous for the *cim3* mutation, or wild-type control plants. To identify individuals hemizygous with respect to the recombination reporter construct, we sowed F1 seeds on sterile medium with 10 μ g ml⁻¹ hygromycin. We scored recombination sectors in these plants after 2 wk.

Acknowledgments

We thank C. Ramos for technical assistance, I. Kovalchuk and J. Molinier for critical comments on the manuscript, and D. Brubacher for help with data analysis. BTH was a gift from H. Kessmann, and K. Lawton supplied PR-1 antiserum. This work was supported by the NOVARTIS Research Foundation, by a grant from the European Union/Swiss Federal Office for Education and Science (to B.H.), a grant from the Swiss National Science Foundation (to J.P.M.) and an EMBO long-term fellowship (to J.M.L.).

Competing interests statement

The authors declare that they have no competing financial interests.

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