Memory of plant communications for priming anti-herbivore responses

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The emission of a specific blend of volatiles in response to *Mythimna separata* (herbivore-induced plant volatiles, HIPVs) plays a great ecological role by priming neighbouring plants. maize plants placed downwind of infested, conspecific plants showed reduced larval development not only immediately after exposure to HIPVs but also when receiver plants were tested after a time lag of up to 5 days, compared to those exposed to volatiles from uninfested plants and tested after the same time lag. The molecular basis of this plant memory was, in part, the similarly recalled expression of a Bowman-Birk type trypsin inhibitor (TI) gene, in a jasmonic acid induction-independent manner. Moreover, in the promoter region of TI, a suite of methylation sites was found to be demethylated by the HIPV treatment. These findings provide an innovative mechanism for the epigenetic basis of the memory of HIPV-mediated habituation for plant defence.

In response to herbivory, plants start to defend themselves against herbivores¹. Herbivore-induced plant volatiles (HIPVs), including a wide array of low molecular weight terpenes and green leaf volatiles, function as airborne signals within and between plants²–⁸. Such signals allow plants experiencing them, but themselves not under attack, to tailor their defences to their current and expected risk of herbivory. In some cases, these receiver plants do not show immediate changes in their level of defences, but respond more strongly and more rapidly when damaged by herbivores than non-receiver plants⁷–⁹. Such readying of a defence response, 'priming', is induced when volatiles emitted from clipped sagebrush (*Artemisia tridentata*) affect neighbouring *Nicotiana attenuata* plants by accelerating their production of trypsin proteinase inhibitors only after *Manduca sexta* larvae start to attack them¹⁰,¹¹. Priming also occurs in maize plants exposed to HIPVs emitted by other maize plants infested with the generalist herbivore *Spodoptera littoralis*¹² or the specialist *Mythimna separata*¹³,¹⁴. Exposure of maize plants to *S. littoralis*-induced volatiles does not activate genes that are responsive to wounding, jasmonic acid (JA) or caterpillar regurgitant, but primes the expression of these genes post-herbivory¹³. Exposure to the volatiles also enhances the emission in receiver plants of volatiles that can attract natural enemies of herbivores¹¹,¹⁴. These natural enemies can help plant indirect defences.

Field studies conducted by Richard Karban's group show the defensive consequences of responding to volatile cues emitted by experimentally damaged neighbours. Resistance was induced about four months after the volatile treatment was applied in May¹⁷,¹⁸. These findings prompted us to investigate how long receptor plants are able to store information conveyed by volatiles and how such information is recalled. Since HIPVs are emitted by damaged plants for only a few days after the onset of damage¹⁹, neighbouring plants can only receive volatile signals when emitter plants are damaged, or slightly later. Moreover, plants cannot be aware of how much later the herbivores will arrive. This issue is relevant to considerations of the ecological consequences for both emitters and receivers. For instance, plants should rapidly cease releasing HIPVs that attract parasitoids after the herbivore has left or become invulnerable¹⁰. If they do not, the cues will provide unreliable information and the parasitoids will be unable to track their hosts. Similarly, in the case of plant communications, receivers must pay costs for priming their defences to their current and expected risks. The appropriate time scale and the mode of sustaining priming should be linked to the cost performance of receiver plants.

In order to better understand the above issues, we assessed the memory abilities of maize plants placed downwind of conspecific plants infested with the specialist herbivore *M. separata* in controlled assay conditions. In response to *M. separata* attack, receiver maize plants emit high levels of HIPVs¹⁶. These include terpenoids (myrecene, (E)-β-ocimene, (E)-4,8-dimethyl-1,3,7-nonatriene, linalool, (E)-β-caryophyllene, (E)-α-bergamotene, (E)-β-farnesene and (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene), green leaf volatiles [(Z)-3-hexen-1-yl acetate]
and indole. Our results revealed the appropriate time period and possible mechanisms underlying the plants’ memory of HIPV stimuli for priming defences against herbivores. To reveal the possible mechanisms, we briefly focused on DNA methylation, a conserved epigenetic mechanism involved in many important biological processes, including gene regulation of stress responses in plants.

**Results**

**Appropriate time lag for HIPVs to impact the defence ability of receiver maize plants against herbivores.** Larvae of the common armyworm (*M. separata*) were applied onto HIPV-receiver maize plants after the receiver plants had been maintained post-volatile exposure for 0, 1, 5 or 10 days (Fig. 1). Larvae on HIPV-receivers had lower weight gain than did those on CV-receivers for the first three of these periods of post-exposure maintenance (Student’s t-test, *P* < 0.05). However, larvae on HIPV-receiver plants maintained for 10 days after exposure grew similarly to those on CV-receivers.

**Appropriate time lag for priming defence responses.** We analysed the transcription level of defence genes for Bowman-Birk type trypsin inhibitor (TI) and cysteine protease inhibitor (CPI) in leaves of HIPV-receiver and CV-receiver plants. These plants were maintained for 0, 5 or 10 days after exposure and then challenged with *M. separata* larvae or left unchallenged for an additional day (Fig. 2). TI expression was higher in infested CV-receivers compared with that in uninfested CV-receivers. The expression was 36 times higher in infested compared to uninfested plants maintained for no additional days, 23 times higher in those maintained for five days and 53 times in those maintained for 10 days. Moreover, upon infestation, the induction was increased 3.2 and 6.0 times in HIPV-receiver leaves compared with those in CV-receiver leaves after 0 and 5 days of post-exposure maintenance (Tukey-Kramer HSD test, *P* < 0.05). There was only very slightly increased expression...
(1.9 times), however, in HIPV-receiver plants maintained for 10 days when compared with those in CV-receiver plants maintained for the same period of time. Expression of CPI, the other defence gene examined, was very weakly increased by herbivore feeding to similar levels between HIPV- and CV-receiver leaves. This was in line with observation of the similarly increased accumulations of JA between HIPV-receiver and CV-receiver leaves in response to herbivory (Tukey-Kramer HSD test, \( P > 0.05 \); Fig. 3).

**Epigenetic modifications of the promoter region of TI.** To identify the mechanism of plant memory of HIPVs we assessed the methylation of appropriate defence genes. Such epigenetic modification of DNA is one of the most reliable strategies for priming defence induction\(^{22} \), especially because, in our case, JA (primary activator of herbivore-responsive genes\(^ {23} \)) is unlikely to be a possible effector (see Fig. 3). We therefore assessed cytosine (Cyt) methylation in the promoter region upstream of the predicted transcription start site of the TI gene in HIPV- or CV-receiver leaves. Twenty independent sample sequences from HIPV-receiver and CV-receiver leaves (10 sequences each) showed two constantly methylated Cyt (positions M1 (−354) and M3 (−345); Fig. 4), and a single or two methylated Cyt in one of the 20 sequences at positions M2 and M7-20. Intriguingly, at positions M4 and M5, larger numbers of non-methylated Cyt were detected in HIPV-receivers compared to CV-receivers (3 and 0 at M4 in HIPV-receivers and CV-receivers; and 8 and 4 at M5 in HIPV-receivers and CV-receivers, respectively).

**Discussion**

The priming effect of HIPVs on resistance against herbivores is memorised and stored by plants. The plant then recalls this memory when it is fed on by herbivores. The memory lasts for at least 5 days after exposure to HIPVs. The machinery of this memory might involve epigenetic regulation of DNA.

Genes for defensive compounds such as protease inhibitors play an essential role against herbivores\(^ {24,25} \). In the leaves of receiver maize plants, TI expression was not promoted directly by HIPVs but rather was recalled by the plant when it was later fed on. Moreover, the priming of TI expression was only 1.9 fold (HIPV-receivers vs CV-receivers) after 10 days of post-exposure maintenance, in contrast to the stronger priming at 0 day (3.2 fold) and 5 days (6.0 fold) of post-exposure maintenance.

![Figure 3](http://www.nature.com/scientificreports/) | The appropriate time lag for priming induced accumulation of JA in receiver plants. Endogenous JA levels were determined in leaves of HIPV-receiver and CV-receiver plants. They were then either treated by the addition of four *M. separata* larvae for an additional day or maintained for an additional day without a larva. Data represent the mean ± standard errors (\( n = 4–5 \)). There were no significant differences between HIPV-receivers and CV-receivers based on a Tukey-Kramer HSD test (\( P > 0.05 \)).

![Figure 4](http://www.nature.com/scientificreports/) | DNA methylation analysis of the TI gene. Distribution of DNA methylation in the promoter region upstream of the predicted transcription start site (355 bp) in the HIPV-receivers (HIPV1 to 10) and CV-receivers (CV1 to 10) (ten independent sequences each). Black and red circles in the map indicate methylated and non-methylated Cyt, respectively.
maintenance. These minor impacts might not have been sufficient for certain anti-herbivore phenotypes at 10 days. Altogether, we conclude that exposure to HIPV’s enables priming of the expression of defensive genes, including Ti, and that the HIPV information is stored for days, until the moment when recalled by the plant under the stimulus of herbivory.

Generally, since JA-mediated defence signalling is predominantly activated when plants are attacked by chewing herbivores, it might be expected that induced JA formation would be relevant to the HIPV-primed defence responses, as in (Z)-3-hexen-1-yl acetate-primed poplar receiver plants. Our results were inconsistent with that possibility, however, as we observed that both priming of Ti expression and memorizing of HIPV information in receiver leaves were independent of JA induction (Fig. 3). Nonetheless, we are unable to exclude the possibility that, rather than formation of JA, its signalling mode may be involved. Moreover, it cannot be doubted that jasmonates (JA-related compounds) are a master switch for participation of epigenetic regulation in plant communication. Epigenetic machinery for these demethylated sites are actually involved in the priming of defensive genes, including act1, and priming of an indirect plant defense in nature. Therefore, undoubtedly, our findings indicate the now appears to be a widespread phenomenon with strong potential for recipient plants by minimizing their costs of memorizing volatile information.

Methods

Plants and herbivores. Maize (Zea mays L. cv. Royal Dent) plants were grown in a greenhouse. Each individual plant was grown in a plastic pot in a growth chamber at 25°C, L:D 16:8 (natural + supplemental light). M. separata was transferred to our laboratory in 2001 from a culture reared at the National Institute of Sericultural and Entomological Science in Tsukuba, Ibaraki, Japan. The insects were reared on artificial diet (Insecta LFS, Nihon Nosan Kogyo Co. Ltd., Yokohama, Japan) in the laboratory in 25°C, L:D 16:8.

Inter-plant communication assays. Air-flow experiments were conducted in polypropylene open-flow tunnels (0.4 m wide, 0.8 m long and 0.4 m high). All the tunnels were open at both ends and a fan at one end produced a continuous air flow (0.3 m s⁻¹) from emitter to receiver plants through the tunnel. Uninfested maize plants (HIPV-receiver) were placed downwind of emitter plants treated with four artificial diet (Insecta LFS, Nihon Nosan Kogyo Co. Ltd., Yokohama, Japan) in the laboratory in 2001 from a culture reared at the National Institute of Sericultural and Entomological Science in Tsukuba, Ibaraki, Japan. Each individual plant was grown in a plastic pot in a growth chamber at 25°C, L:D 16:8. polypropylene open-flow tunnels (0.4 m wide, 0.8 m long and 0.4 m high). All the tunnels were open at both ends and a fan at one end produced a continuous air flow (0.3 m s⁻¹) from emitter to receiver plants through the tunnel. Uninfested maize plants (HIPV-receiver) were placed downwind of emitter plants treated with four artificial diet (Insecta LFS, Nihon Nosan Kogyo Co. Ltd., Yokohama, Japan) in the laboratory in 2001 from a culture reared at the National Institute of Sericultural and Entomological Science in Tsukuba, Ibaraki, Japan. Each individual plant was grown in a plastic pot in a growth chamber at 25°C, L:D 16:8. Norway. 452–512 (2000).

Bruin, J., Sabelis, M. W. & Dicke, M. Do plants tap SOS signals from their infested neighbours? Tree 10, 167–170 (1995).

Heil, M. & Silva Bueno, J. C. Within-plant signalling by volatiles leads to induction and priming of indirect plant defense in nature. Proc. Natl. Acad. Sci. USA 104, 5467–5472 (2007).

Engelbrecht, J., Alborn, H. T., Schmela, E. A. & Tumlinson, J. H. Airborne signals prime plants against insect herbivore attack. Proc. Natl. Acad. Sci. USA 101, 1781–1785 (2004).

Frost, C. J. et al. Priming defense genes and metabolites in hybrid poplar by the green leaf volatiles cis-3-hexenyl acetate. New Phytol. 180, 722–734 (2008).

Tschaplinski, T. J. et al. Priming by airborne signals boosts direct and indirect resistance in maize. Plant J. 49, 16–26 (2006).

Kessler, A., Halitschke, R., Diezel, C. & Baldwin, I. T. Priming of plant defense responses in nitrogen by airborne signaling between Artemisia tridentata and Nicotiana attenuata. Oecologia 148, 280–292 (2006).

1. Karban, R. & Baldwin, I. T. Induced responses to herbivory. (The University of Chicago Press, 1997).

2. Arimura, G. Explaining evolution of plant communication by airborne signals. Trends Ecol. Evol. 25, 137–144 (2010).

3. Heil, M. & Karban, R. Acquiring immunity to herbivory and allelopathy caused by airborne plant emissions. Phytochemistry 71, 1642–1649 (2010).

4. Ozawa et al. (2009)32.

5. Kessler, A., Halitschke, R., Diezel, C. & Baldwin, I. T. Priming of plant defense genes. Plant J. 38, 1781–1785 (2004).

6. Frost, C. J. et al. Priming defense genes and metabolites in hybrid poplar by the green leaf volatiles cis-3-hexenyl acetate. New Phytol. 180, 722–734 (2008).

7. Tschaplinski, T. J. et al. Priming by airborne signals boosts direct and indirect resistance in maize. Plant J. 49, 16–26 (2006).

8. Kessler, A., Halitschke, R., Diezel, C. & Baldwin, I. T. Priming of plant defense responses in nitrogen by airborne signaling between Artemisia tridentata and Nicotiana attenuata. Oecologia 148, 280–292 (2006).
13. Choh, Y. & Takabayashi, J. Herbivore-induced extrafloral nectar production in lima bean plants enhanced by previous exposure to volatiles from infested conspecifics. J. Chem. Ecol. 32, 2073–2077 (2006).
14. Muroi, A. et al. The composite effect of transgenic plant volatiles for acquired immunity to herbivory caused by inter-plant communications. PLoS One 6, e24594 (2011).
15. Karban, R., Baldwin, I. T., Baxter, K. J., Laue, G. & Felton, G. W. Communication between plants: induced resistance in wild tobacco plants following clipping of neighboring sagebrush. Oecologia 125, 66–71 (2000).
16. Ramadan, A., Muroi, A. & Arimura, G. Herbivore-induced maize volatiles serve as priming cues for resistance against post-attack by the specialist armyworm Mythimna separata. J. Plant Interact. 6, 155–158 (2011).
17. Karban, R. & Shiojiri, K. Self-recognition affects plant communication and defense. Ecol. Lett. 12, 502–506 (2009).
18. Karban, R., Shiojiri, K. & Ishizaki, S. An air transfer experiment confirms the role of volatile cues in communication between plants. Am. Nat. 176, 381–384 (2010).
19. Arimura, G., Huber, D. P. W. & Bohlmann, J. Forest tent caterpillars (Malacosoma disstria) induce local and systemic diurnal emissions of terpenoid volatiles in hybrid poplar (Populus trichocarpa x deltoides): cDNA cloning, functional characterization, and patterns of gene expression of (-)-germacrene D synthase, PtdTPS1. Plant J. 37, 603–616 (2004).
20. Puente, M. E., Kennedy, G. G. & Gould, F. The impact of herbivore-induced plant volatiles on parasitoid foraging success: a general deterministic model. J. Chem. Ecol. 34, 945–958 (2008).
21. Gratovil, C., Hemenly, A. S. & Ferreira, P. C. Genetic and epigenetic regulation of stress responses in natural plant populations. Biochim. biophys. Acta, in press (2011).
22. Holeski, L. M., Jander, G. & Agrawal, A. Transgenerational defense induction and epigenetic inheritance in plants. Trends Ecol. Evol. 27, 618–626 (2012).
23. Maffei, M. E., Arimura, G. & Mithofer, A. Natural elicitors, effectors and modulators of plant responses. Nat. Prod. Rep. 29, 1269–1368 (2012).
24. Royo, I. et al. Antisense-mediated depletion of a potato lipoygenase reduces wound induction of proteinase inhibitors and increases weight gain of insect pests. Proc. Natl. Acad. Sci. USA 96, 1146–1151 (1999).
25. Schlüter, U. et al. Recombinant protease inhibitors for herbivore pest control: a multitrophic perspective. J. Exp. Bot. 61, 4169–4183 (2010).
26. Arimura, G. et al. Effects of feeding Spodoptera littoralis on Lima bean leaves: IV. Diurnal and nocturnal damage differentially initiate plant volatile emission. Plant Physiol. 146, 965–973 (2008).
27. Goll, M. G. & Restor, T. H. Eukaryotic cytosine methyltransferases. Annu. Rev. Biochem. 74, 481–514 (2005).
28. Turner, I. J. & Matake, M. Methylation and demethylation of the Arabidopsis genome. Curr. Opin. Plant Biol. 14, 137–141 (2011).
29. Zemach, A., McDaniel, I. E., Silva, P. & Zilberman, D. Genome-wide evolutionary analysis of eukaryotic DNA methylation. Science 328, 916–919 (2010).
30. Heil, M. & Adame-Alvarez, R. M. Short signalling distances make plant communication a soliloquy. Biol. Lett. 6, 843–845 (2010).
31. Karban, R., Shiojiri, K., Huntzinger, M. & McCall, A. C. Damage-induced resistance in sagebrush: volatiles are key to intra- and interplant communication. Ecology 87, 922–930 (2006).
32. Ozawa, R. et al. Exogenous polyamines elicit herbivore-induced volatiles in lima bean leaves: involvement of calcium, H2O2 and jasmonic acid. Plant Cell Physiol. 50, 2183–2199 (2009).

Acknowledgments
This work was financially supported in part by Global COE Program A06 of Kyoto University; the Core-to-Core Program from the Japan Society for the Promotion of Science (JSPS) (No.20004); and a Grant-in-Aid for Scientific Research from JSPS (No. 24770019) to GA.

Author contributions
M.A., K.S., A.R., G.A. designed the study; M.A., K.S., A.R., G.A. performed the experiments and data analyses; G.A. wrote the manuscript.

Additional information
Competing financial interests: The authors declare no competing financial interests.
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How to cite this article: Ali, M., Sugimoto, K., Ramadan, A. & Arimura, G. Memory of plant communications for priming anti-herbivore responses. Sci. Rep. 3, 1872; DOI:10.1038/srep01872 (2013).