New Phytologist

Letters

Using a single transgenic event to infer fitness effects in crop–weed hybrids: a reply to the Letter by Grunewald & Bury (2014)

Grunewald & Bury (2014; in this issue of *New Phytologist*, pp. 367–369) criticize our recent peer-reviewed paper (Wang *et al.*, 2014; in this issue of *New Phytologist*, pp. 679–683), stating that we 'unnecessarily harm the sensitive debate on GM crops.' We will not focus on this politically charged topic here, but we do want to address scientific questions about our study of transgenic crop–weed (*Oryza sativa* and *O. sativa* f. *spontanea*) hybrids of rice. Grunewald and Bury propose that an insertion effect that stimulated tiller formation in the EP3 crop parent of these hybrids offers a more convincing explanation of our results than direct effects of a transgene for over-expressing 5-enolpyruvoylshikimate-3-phosphate synthase (*epsps*) (our central hypothesis). In our study, the genetically engineered (GE) segregants had significantly greater expression of *epsps* and produced significantly greater amounts of the enzyme of EPSPS than the non-GE segregants, as expected (Wang *et al.*, 2014). As discussed later, we doubt that an insertion effect could account for the clear and significant increases in our transgenic lines in terms of enhanced fecundity, greater tryptophan concentrations in leaves, and other traits, which are more parsimoniously explained by transgenic over-expression of *epsps* and its key role in the shikimic acid pathway (Wang *et al.*, 2014). Tryptophan is produced by this pathway and is a precursor of growth hormones (auxin) and secondary metabolites that play a role in plant defense (e.g. Maeda & Dudareva, 2012). Tryptophan is just one of many products of the shikimic acid pathway, which can account for as much as 35% of a plant's biomass (e.g. Franz *et al.*, 1997).

To review, our two crop parental rice lines (Fig. 1) were the inbred line Minghui-86 and a transgenic rice line (EP3), which was obtained by transforming Minghui-86 (Su *et al.*, 2008; Lu *et al.*, 2014 (this issue of *New Phytologist*, pp. 363–366); Wang *et al.*, 2014). Thus, these lines differed only in the absence or presence of a single-copy insertion of the transgenic construct and possible unknown side-effects of transformation. Su *et al.* (2008) and our Supporting Information Table S1 (Wang *et al.*, 2014) showed that EP3 produced significantly more tillers and panicles per plant than Minghui-86. This suggests a direct effect of the transgene on plant growth and reproduction in EP3. We do not understand why Grunewald and Bury do not even acknowledge this explanation under 'option (2)' of their letter. Instead, they assume that the superior performance of EP3 was due to a linked sequence that 'putatively stimulates tiller formation' and was 'the result of the insertion' rather than expression of the *epsps* transgene (see Grunewald & Bury, 2014, Fig. 1). All of their arguments against our hypothesis hinge on this assumption. Although we did not consider the point (similar to their assumption) to be the major cause of the enhanced fecundity, we included this caveat in our paper: '… we assume that the over-production of EPSPS and the downstream differences that we observed between GE plants and their non-GE counterparts were attributable to the over-expression of the modified transgene, epsps, rather than other tightly linked genes from the cultivated parent, although this possibility cannot be ruled out entirely.' (Wang *et al.*, 2014).

In a field experiment, we also found that EP3-derived F_1 crop–weed progeny had significantly more tillers, panicles, and seeds than those derived from Minghui-86. With the exception of the transgene, which was deliberately engineered to over-express *epsps*, overall differences in the crop-specific alleles found in these two types of F_1 progeny should be negligible, barring the type of major insertion effect postulated by Grunewald and Bury. Remarkably, the GE F_1 plants produced up to 50% more seeds per plant than non-GE controls in the mixed competition treatment (Wang *et al.*, 2014, Table S3).

Our evidence for an association between over-expressing *epsps* and strong, heritable increases in fecundity in both the EP3 parent and EP3-derived F_1 progeny is consistent with other traits measured in subsequent generations (Wang *et al.*, 2014). In GE F_3 plants, we found greater leaf concentrations of tryptophan, an aromatic amino acid produced by the shikimic acid pathway downstream from EPSPS (Herrmann, 1995), compared to non-GE F_3 plants. These GE plants also exhibited greater photosynthetic rates and greater percent seed germination than their non-GE counterparts. In the F_2 generation, GE plants produced 48–125% more seeds than the non-GE controls. Theoretically, any of the fitness-related effects that we documented in the F_2 and F_3 generations could have been influenced by non-GE crop alleles that were linked on the same chromosome as the transgene insertion site. However, these types of alleles, if present, would not have differed between the GE and non-GE controls in the *crop parents* or the *F_1 generation* (Fig. 1).

To bolster their argument that our results are simply an artifact of the insertion process and 'unrelated to the transgene', Grunewald and Bury claim that our paper contradicts 20 yr of experimental results involving comparisons between glyphosate-tolerant crops and their isogenic counterparts, citing a review paper in *AgBioForum* by Brookes & Barfoot (2006). Unfortunately, Brookes & Barfoot (2006) did not report empirical studies designed to test for an association between glyphosate resistance and increased yield in the absence of glyphosate, nor is it appropriate to group all types of genetic mechanisms for glyphosate resistance together. In soybean, we note that Owen *et al.* (2010)

© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust

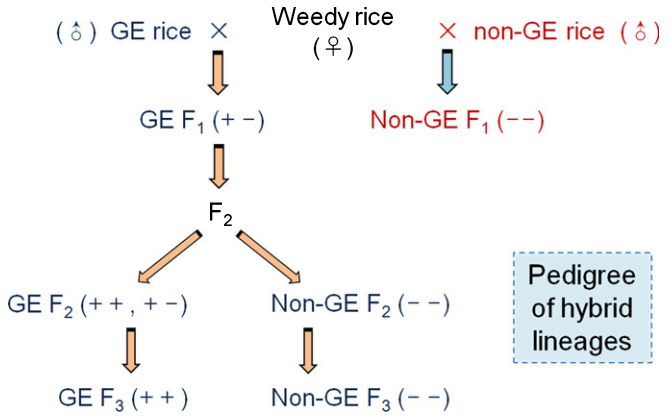


Fig. 1 The crossing procedure for obtaining hybrid lineages between transgenic or non-transgenic rice (*Oryza sativa*) and four weedy rice (*O. sativa* f. *spontanea*) accessions. The transgenic hybrid lineages segregated for the *epsps* transgene. Genetically engineered (GE) refers to plants with one (+ −) or two (+ +) copies of the *epsps* transgene. F_1 and F_2 plants were used in field experiments to test for differences between GE and non-GE controls in growth and fecundity; F_2 plants also were used to compare gene expression and EPSPS protein levels; the F_3 generation was used to test for differences in tryptophan concentrations, photosynthetic rates, and percent seed germination. From Wang *et al.* (2014).

reported greater yields of glyphosate resistant (CP4) cultivars in comparison to non-GE cultivars in the absence of glyphosate, but they attributed this benefit to the improved genetic background used to develop GE cultivars. For our purposes, a better transgenic event and experimental design is that of Zhou *et al.* (2003), who studied a transgenic event in wheat with two aroA:CP4 expression cassettes, one driven by the CaMV enhanced 35S promoter and another by the rice actin1 promoter, in comparison to the non-GE parent cultivar. Zhou *et al.* (2003) found that the grain yield of GE wheat plants was greater than that of the non-GE counterparts in 2000, and non-significant in 1999 and 2001, based on field experiments without glyphosate applications (see Zhou *et al.*, 2003, Table 5). It is possible that the two CP4 cassettes in GE wheat conferred over-production of EPSPS, although this was not addressed in their study. In the future, if data from sufficiently rigorous experiments of glyphosate-tolerant crops are available to public researchers, those involving transgenic events that confer over-production of EPSPS could be useful for testing our hypothesis.

In agreement with Grunewald and Bury, we note that the best way to test our hypothesis would have been to study crop–weed progeny derived from two or more insertion events for the transgene, but we were not able to carry out these studies due to various limitations (see Lu *et al.*, 2014). For any study of a single transgenic event, a combination of insertion-site changes and tissue culture-induced changes could result in heritable phenotypic effects that are independent of the transgene and may exhibit abnormal phenotypes (e.g. Alonso *et al.*, 2003; Filipecki & Malepszy, 2006; Neelakandan & Wang, 2012). Like many published studies of the fitness effects of particular transgenes (e.g. Stewart *et al.*, 1997; Snow *et al.*, 1999, 2003; Burke & Rieseberg, 2003; Guadagnuolo *et al.*, 2006; Laughlin *et al.*, 2009; Sasu *et al.*, 2009; Londo *et al.*, 2011; Xia *et al.*, 2011; Yang *et al.*, 2011, 2012), our research was based on a single transgenic event, which is far from ideal. Unfortunately, even single transgenic events that are under development for commercial applications are exceedingly difficult for ecologists to obtain (e.g. Dalton, 2002). Thus, although we agree that multiple events are needed to investigate fitness effects of transgenic traits, this is rarely feasible.

To conclude, we view our original publication as a key first step toward testing the novel hypothesis that over-production of EPSPS can stimulate growth and fecundity in crops and crop–weed progeny. To our knowledge, this hypothesis has not been addressed in the peer-reviewed literature or elsewhere. We have initiated further studies to test the generality of our findings by using multiple transgenic events in rice and *Arabidopsis thaliana*. Intriguingly, Klee *et al.* (1987) includes a photograph showing transgenic *Arabidopsis* seedlings that over-express endogenous *epsps* and are larger than wild-type seedlings. If our hypothesis is confirmed by further research, this would have broad implications for understanding and engineering a key enzyme (EPSPS) of the shikimic acid pathway. If we are not able to confirm this hypothesis, we plan to publish these findings accordingly, consistent with the scientific process.

Conflict of interest

The authors declare no financial interest in the use of herbicides nor in GE herbicide-resistant plants. Fudan University and Ohio State University are educational organizations that conduct research including GE plants for generating scientific knowledge.

Acknowledgements

The authors thank several anonymous reviewers for helpful comments and discussion.

Bao-Rong Lu[1]*, Allison A. Snow[2], Xiao Yang[1] and Wei Wang[1]

[1]Ministry of Education, Key Laboratory for Biodiversity Science and Ecological Engineering, Department of Ecology and Evolutionary Biology, Fudan University, Handan Road 220, Shanghai 200433, China;
[2]Department of Evolution, Ecology & Organismal Biology, Ohio State University, Columbus, OH, USA
(*Author for correspondence: tel +86 21 65643668; email brlu@fudan.edu.cn)

References

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R *et al.* 2003. Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301**: 653–657.

Brookes G, Barfoot P. 2006. Global impact of biotech crops: socio-economic and environmental effects in the first ten years of commercial use. *AgBioForum* **9**: 139–151.

Burke JM, Rieseberg LH. 2003. Fitness effects of transgenic disease resistance in sunflowers. *Science* **300**: 1250.

© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust

Dalton R. 2002. Superweed study falters as seed firms deny access to transgene. *Nature* **419**: 655.

Filipecki M, Malepszy S. 2006. Unintended consequences of plant transformation: a molecular insight. *Journal of Applied Genetics* **47**: 227–286.

Franz JE, Mao MK, Sikorski JA. 1997. *Glyphosate: a unique global herbicide.* Washington, DC, USA: American Chemistry Society.

Grunewald W, Bury J. 2014. Comment on 'A novel 5-enolpyruvoyl-shikimate-3-phosphate (EPSP) synthase transgene for glyphosate resistance stimulates growth and fecundity in weedy rice (*Oryza sativa*) without herbicide' by Wang *et al.* (2014) *New Phytologist* **202**: 367–369.

Guadagnuolo R, Clegg J, Ellstrand NC. 2006. Relative fitness of transgenic *vs.* non-transgenic maize × teosinte hybrids: a field evaluation. *Ecological Applications* **16**: 1967–1974.

Herrmann KM. 1995. The shikimate pathway: early steps in the biosynthesis of aromatic compounds. *Plant Cell* **7**: 907–919.

Klee HJ, Muskopf YM, Gasser C. 1987. Cloning of an *Arabidopsis thaliana* gene encoding 5-enolpyruvylshikimate-3-phosphate synthase sequence analysis and manipulation to obtain glyphosate-tolerant plants. *Molecular and General Genetics* **210**: 437–442.

Laughlin KD, Power AG, Snow AA, Spencer LJ. 2009. Risk assessment of genetically engineered crops: fitness effects of virus-resistant transgenes in wild *Cucurbita pepo*. *Ecological Applications* **19**: 1019–1101.

Londo JP, Bollman MA, Sager CL, Lee EH, Watrud LS. 2011. Changes in fitness-associated traits due to the stacking of transgenic glyphosate resistance and insect resistance in *Brassica napus* L. *Heredity* **107**: 328–337.

Lu B-R, Snow AA, Yang X, Wang W. 2014. Scientific data published by a peer-reviewed journal should be properly interpreted: a reply to the letter by Gressel *et al.* (2014). *New Phytologist* **202**: 363–366.

Maeda H, Dudareva N. 2012. The shikimate pathway and aromatic amino acid biosynthesis in plants. *Annual Review of Plant Biology* **63**: 73–105.

Neelakandan AK, Wang K. 2012. Recent progress in the understanding of tissue culture-induced genome level changes in plants and potential applications. *Plant Cell Reports* **31**: 597–620.

Owen MDK, Pedersen P, Bruin JL, De Stuart J, Lux J, Franzenburg D, Grossnickle D. 2010. Comparisons of genetically modified and non-genetically modified soybean cultivars and weed management systems. *Crop Science* **50**: 2597–2604.

Sasu MA, Ferrari MJ, Du D, Winsor JA, Stephenson AG. 2009. Indirect costs of a nontarget pathogen mitigate the direct benefits of a virus-resistant transgene in wild *Cucurbita*. *Proceedings of the National Academy of Sciences, USA* **106**: 19067–19071.

Snow AA, Andersen B, Jørgensen RB. 1999. Costs of transgenic herbicide resistance introgressed from *Brassica napus* into weedy *Brassica rapa*. *Molecular Ecology* **8**: 605–615.

Snow AA, Pilson D, Rieseberg LH, Paulsen M, Pleskac N, Reagon MR, Wolf DE, Selbo SM. 2003. A *Bt* transgene reduces herbivory and enhances fecundity in wild sunflowers. *Ecological Applications* **13**: 279–286.

Stewart CN, All JN, Raymer PL, Ramachandran S. 1997. Increased fitness of transgenic insecticidal rapeseed under insect selection pressure. *Molecular Ecology* **6**: 773–779.

Su J, Chen GM, Tian DG, Zhu Z, Wang F. 2008. A gene encodes 5-enolpyruvylshikimate-3-phosphate mutagenized by error-prone PCR conferred rice with high glyphosate-tolerance. *Molecular Plant Breeding* **6**: 830–836. (Chinese text with English abstract; available at: http://www.cnki.com.cn/Article/CJFDTotal-FZZW200805005.htm).

Wang W, Xia H, Yang X, Xu T, Si HJ, Cai XX, Wang F, Su J, Snow AA, Lu B-R. 2014. A novel 5-enolpyruvoylshikimate-3-phosphate (EPSP) synthase transgene for glyphosate resistance stimulates growth and fecundity in weedy rice (*Oryza sativa*) without herbicide. *New Phytologist* **202**: 679–683.

Xia H, Lu B-R, Xu K, Wang W, Yang X, Yang C, Luo J, Lai F, Ye W, Fu Q. 2011. Enhanced yield performance of *Bt* rice under target-insect attacks: implications for field insect management. *Transgenic Research* **20**: 655–664.

Yang X, Wang F, Su J, Lu B-R. 2012. Limited fitness advantages of crop-weed hybrid progeny containing insect-resistant transgenes (*Bt/CpTI*) in transgenic rice field. *PLoS ONE* **7**: e41220.

Yang X, Xia H, Wang W, Wang F, Su J, Snow AA, Lu B-R. 2011. Transgenes for insect resistance reduce herbivory and enhance fecundity in advanced generations of crop-weed hybrids of rice. *Evolutionary Applications* **4**: 672–684.

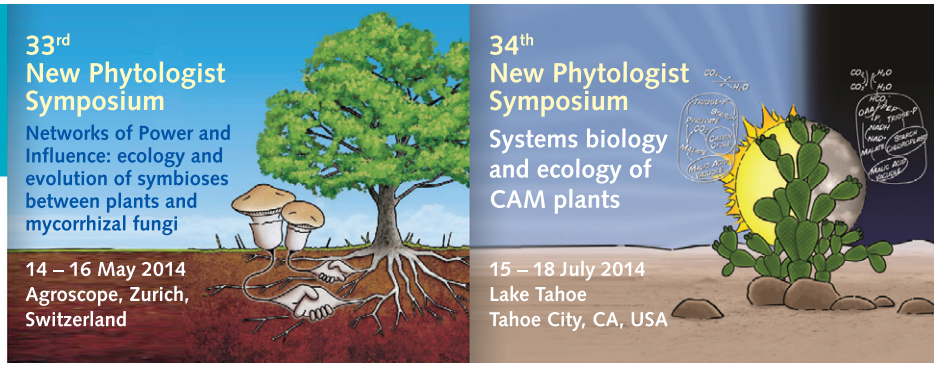
Zhou H, Berg JD, Blank SE, Chay CA, Chen G, Eskelsen SR, Fry JE, Hoi S, Hu T, Isakson PJ *et al.* 2003. Field efficacy assessment of transgenic Roundup Ready wheat. *Crop Science* **43**: 1072–1075.

Key words: EPSPS, genetically engineered rice, herbicide resistance, hybrid progeny, increased fecundity, insertion effect, transgene over-expression, weedy rice.

New Phytologist
Symposia for 2014
www.newphytologist.org/symposia

New Phytologist
next generation scientists

A special event for researchers in the early stages of their careers
29 – 30 July 2014
John Innes Conference Centre, Norwich UK



© 2014 The Authors
New Phytologist © 2014 New Phytologist Trust