

# Fast Neutron Mutagenesis of Soybean (*Glycine soja* L.) Produces a Supernodulating Mutant Containing a Large Deletion in Linkage Group H

Artem E. Men,<sup>1,†</sup> Titeki S. Laniya,<sup>1,†</sup> Iain R. Searle,<sup>2,‡</sup> Inaki Iturbe-Ormaetxe,<sup>1</sup>  
Irma Gresshoff,<sup>3</sup> Qunyi Jiang,<sup>1</sup> Bernard J. Carroll,<sup>2</sup> and Peter M. Gresshoff<sup>1,\*</sup>

<sup>1</sup>Department of Botany (SOLS) and <sup>2</sup>Department of Biochemistry (SMMS/LAFS),  
The University of Queensland, St. Lucia, Brisbane QLD 4072, Australia

(Received: 24 July 2002. Revised/Accepted: 11 November 2002)

**ABSTRACT:** We describe for the first time the application of fast neutron mutagenesis to the genetic dissection of root nodulation in legumes. We demonstrate the utility of chromosomal deletion mutations through production of a soybean supernodulation mutant FN37 that lacks the internal autoregulation of nodulation mechanism. After inoculation with microsymbiont *Bradyrhizobium japonicum*, FN37 forms at least 10 times more nodules than the wild type *G. soja* parent and has a phenotype identical to that of chemically induced allelic mutants *nts382* and *nts1007* (*NTS1* locus). Reciprocal grafting of shoots and roots confirmed systemic shoot control of the FN37 nodulation phenotype. RFLP/PCR marker pUTG132a and AFLP marker UQC-IS1 which are tightly linked to *NTS1* allowed the isolation of BAC contigs delineating both ends of the deletion. The genetic/physical distance ratio in the *NTS1* region is 279 kb/cM. The deletion is estimated to be about 460 kb based on the absence of markers and bacterial artificial chromosomes (BAC) ends as well as genetic and physical mapping. Deletion break points were determined physically and placed within flanking BAC contigs.

**Keywords:** BAC, Deletion, Map-Based Cloning, Soybean, Symbiosis, Nodulation, Genomics.

of ethyl methan sulphon ate(EMS) induced mutants [4-7]. Several single recessive Mendelian alleles (nts382, nts1007, En6500) that were altered in the internodal shoot-root signaling process affectin plant development manifested highly increased nodulation (up to 40 times more nodules) after inoculation with *Bradyrhizobium japonicum*. The NTS-1 gene is significant not only because of its involvement in the shoot-root signaling circuit [8, 9], but also because supernodulation has several pleiotropic effects, such as nitrate tolerance and altered lateral root growth [10].

The cloning of chemically induced mutant alleles has shown that chemical mutagenesis generally results in single nucleotide changes or very small deletions [11-14]. In parallel, for several non-legume species mutants have been isolated after particle bombardment or ionizing mutagenesis, such as fast neutron and  $\gamma$ -rays [12, 15-17]. Ionizing mutagens are thought to produce double strand DNA breaks that are then repaired by mechanisms which are not clearly understood but could be

were self-pollinated and  $M_2$  seeds were harvested and analyzed in bulk. Briefly,  $M_2$  plants were inoculated three days after sowing with microsymbiont *Bradyrhizobium japonicum* strain USDA 110 (approximately  $10^{10}$  bacteria per plant) and were cultured in sand/vermiculite (1:2 ratio). The plants received 1.2 L of nitrogen-free ferruginous nutrient solution [24] three times a week. The nodulation phenotype of plants was scored 4 weeks after germination. Putative mutants were transferred to new pots and supplied with full-nutrient solution [24]. After screening about 5000  $M_2$  plants, one mutant with highly increased nodule number was found. This mutant was advanced through five generations of self-pollination and visual selection of progeny and showed a stable supernodulation phenotype. The mutant (designated FN37) was then subjected to genetic and molecular analysis (FN37 seeds are available from the corresponding author upon request).

## 2.2. Grafting Experiments

All four shoot/root, wild-type/mutant combinations were tested with at least four plants per graft. We have used a described protocol [8, 25] with some modifications. Plants were grafted 2 weeks after sowing by wedge-shaped grafts in the hypocotyls with the cotyledon left on the scion. Graft junctions were externally supported by short well-tinned nylon tubes. Plants were then covered with plastic bags or cut 2 L soft drink bottles and inoculated four days after grafting with either *B. japonicum* USDA 110 strain or Nodulaid 100 (Bio-Care Technology Pty Ltd., Somersby, NSW, Australia). The bottle lids were slowly opened and then discarded to permit venting and hardening off. Plastic covers were removed 1 week after and plants were harvested 9-10 weeks after germination and scored for nodulation phenotype.

## 2.3. BAC DNA Isolation, Fingerprinting, and End Sequencing

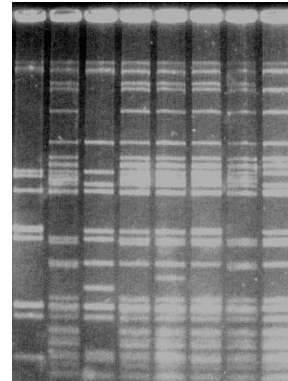
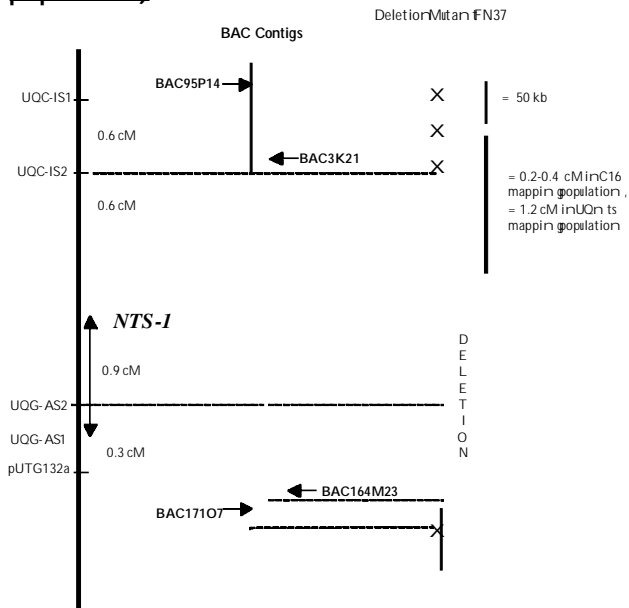
Soybean BAC libraries [26-28] arrayed as individual duplicated clones on 2205 × 2205 cm Nylon filters (4 × 4 grid) in 96-well plates (p3 919 Tm) (-24 (d) -2r 0 0 1 229 869 T (l) -24 (te) (an) --24 (es) r-24 (d) ) TJ1 0 0 1 443 72 (l) -24 (t24 (owly) 0 0 1 618 1293 68 (l) -2

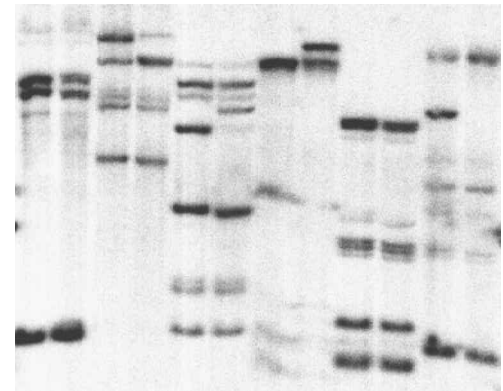
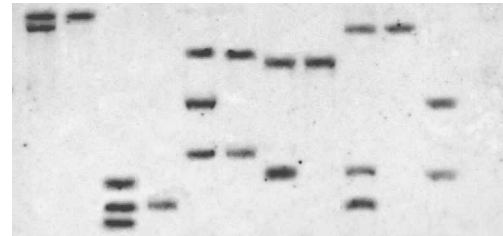
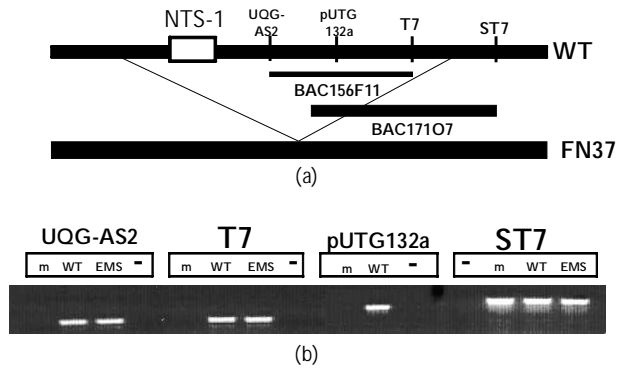
ATT C (annealing temperature 55 °C); for UQC-IS2 (T7 end of BAC95P14): 5'-AGC TTG TTA GAC CTG AAG GAT GTT C and 5'-TAT TAC TTA GTC AAC GTG AAA TCT CC (annealing temperature 60 °C); for



**Genetic Map  
(UQnts mapping  
population)**

**Physical Map**





**Figure 4.** PCR analysis of deletion mutant FN37 with probes mapped close to NTS-1. (a) Schematic representation of four PCR probes used for the analysis. The T7 probe was derived from the "south" end of the BAC156F11; ST7 is the "south" end of BAC171O7. The order of these probes relative to NTS-1 has been established by previous mapping [32]. (b) Agarose gel analysis of amplified probes. DNA lanes: m = FN37; WT = wild-type G. soja PI468.397; EMS = nts382 mutant; - = negative (no DNA) control.

In order to obtain more information on the genomic environment of NTS-1 and to determine physical/genetic distance relationships for this area of the soybean genome, we have sequenced and annotated the entire 35 kb long BAC171O7 (Men et al., in prep.). From the sequencing data, the physical distance between pUTG132a and UQG-AS1 is 83.7 kb. On the other hand, mapping of  $F_2$  soybean recombinants in the interval between pUTG132a and UQG-AS1 showed that the genetic distance between these two markers is 0.3 cM [34], giving a physical/genetic distance in this region of soybean genome as 279 kb/cM. Since the ST7 end of

“south” contig, the genetic/physical distance ratio in this region is 279 kb/cM. Genetic distance between pUTG132a and UQC-IS3 is 1.8 cM [32, 34], and physical distance between UQC-IS3 and UQC-IS4 is about 40 kb based on BAC129E8 analysis. Therefore the estimated size of the deletion in FN37 mutant is 460 kb, assuming physical collinearity between *G. max* and *G. soja* genomes.

#### 4. CONCLUSIONS

The chromosomal deletion approach is especially valuable for organisms that have repeated genomes as the deletion creates partial diploidy in defined chromosomal segments. Deletion mutants offer an additional advantage for gene analysis using reverse genetics. Using transgenesis, new genes can be added; interpretation of results will be aided by the complete absence of the resident copy for the reintroduced gene. Even a non-sense mutation, normally viewed as a “null,” still expresses the mRNA and would still be detected in genomic and gene expression analyses. Therefore large chromosomal deletion mutants remove all interfering functional and structural elements for a reverse genetics approach.

Here we report the application of fast neutron mutagenesis for the isolation of deletion mutations in soybean; one such mutation (FN37) altered the symbiotic phenotype as evidenced by a loss of autoregulation of nodulation. This mutant closely resembled degum mutants with the supernodulating phenotype [4–9] and provided additional value for map-based cloning and genome definition of the soybean NTS-1 locus [36]. By all developmental criteria, the FN37 mutant was similar to other supernodulation mutants, because (i) it lacked autoregulation of nodulation as manifested by a highly increased nodule number after inoculation with *B. japonicum* and (ii) demonstrated root control of the supernodulation phenotype. FN37 also had nodulation tolerance to inhibitory levels of nitrate (data not shown), reduced nodule size when supernodulated, and an altered root system (Fig. 1a).

Using molecular markers close to NTS-1 alleles derived from EMS mutants, we confirmed that FN37 was deleted in the same chromosomal region of soybean linkage group H. Most of the “south” BAC contig markers (bar the southern tip of the contig) were deleted, whereas all probes from the “north” contig (bar the UQC-IS4 marker) were present in FN37. It was therefore possible to define both deletion breakpoints located within the region of UQC-IS4 (“north” end) and within 40 kb of the fully sequenced soybean BAC171O7. Results based on BAC sizes and recombination distances suggested a large deletion. BAC156F11 and BAC164M23 (Fig. 2) were both 140 kb in size with approximately 125 kb overlap. Since both ends of these BACs as well as probes located between them (i.e., pUTG132a) were missing from FN37 DNA, we conclude that both the entire BAC156F11 and BAC164M23 were deleted. Based on the

absence of UQC-IS4 marker and the genetic/physical distance ratio in this region of soybean genome, the deletion in FN37 is about 460 kb.

FN mutagenesis recently has been effectively implemented in model species *Arabidopsis* and rice [23]. Li and co-authors used 60 Gy dosage to bombard *Arabidopsis* seeds (51,840 lines were analyzed). Neither large deletions nor visible *Arabidopsis* mutant phenotypes were reported for this screen [23]. In soybean, we used a lower dosage (8 Gy) to generate a deletion at least one order of magnitude larger than reported in *Arabidopsis*. This deletion resulted in a severe root phenotype, namely supernodulation. As revealed by BAC171O7 sequencing several soybean genes and ESTs are deleted in FN37. Since soybean is an ancestral tetraploid, duplication of many regions and genes is a common theme, as verified here with the UQC-IS4-based Southern blot (Fig. 5b). However, regional diploidy must exist as single recessive mutations such as *nts-1* can be found. Gene redundancy would explain the appearance of FN37 plants, which showed no growth abnormalities and were fertile even after losing about 460 kb of chromosomal DNA. For example, in *Arabidopsis* due to the small genome size, gene redundancy is probably much lower than in other plant species and gene density is higher. Hence, the presence of a large deletion could result in lethality of a plant with a small genome because of multiple null alleles for developmentally important genes or low transmission through the male gametophyte. Indeed, the deletion sizes reported in [23] were between 0.8 and 12 kb. In contrast, in polyploid species, such as hexaploid wheat, deletions can be as large as 70 Mb (for example, around the homeologous chromosome pairing gene *Ph1* [38, 39]). This supports the idea that deletion mutants in complex species still have redundant transcripts encoded by functional paralogs located elsewhere in the genome and therefore such mutants could be more easily recovered in mutagenesis programs.

Contiguous homozygous nonlethal deletions have been found in numerous organisms, including humans [40]. Alternatively, fast neutron bombardment may cause rearrangements more complicated than simple contiguous loss of chromosomal DNA. Cloning of transparent testa (•) avonoid pathway mutations in *Arabidopsis* generated by fast neutrons showed an inversion within the gene of interest combined with a translocation of another DNA fragment 38 centimorgans away on the same chromosome to one end of this inversion [17]. Interestingly, sequence analysis of the breakpoints in transparent testa DNA indicated that repair of radiation-induced damage involved mechanisms similar or identical to those that mediate the integration of foreign sequences into the plant genome [17]. Therefore, deletion mutagenesis of a given genome is most probably a combination of a random process of DNA breakage by particle bombardment followed by a nonrandom process that

function in a non-trophic specific double-strand breakage DNA repair mechanisms.

It is therefore possible that rearrangement other than the postulated contiguous deletion of the entire NTS-1 region could have occurred during our FN mutagenesis program. However, the results showing the absence of markers and BACs tightly linked to NTS-1 along with the observed shoot-controlled supernodulation phenotype of the FN37 mutant have led us to the hypothesis that FN37 is most probably null for NTS-1. Further proof of this hypothesis was recently achieved as the cloned NTS-1 gene was shown to be completely deleted from the FN37 genome based on Southern hybridization and expression studies [36].

**Acknowledgments:** We thank Dr. Helmut Brunner (IAEA, Seibersdorf, Austria) for fast neutron mutagenesis. Lisa Calfee-Richardson conducted the original M1 and M2 mutant isolation. Dr. A. Hussain aided mutant selection. We also thank Dr. Neal Quigley (University of Tennessee, Knoxville) and the Australian Genome Research Facility (Brisbane) for DNA sequencing. TSL is a recipient of a UQ postgraduate award. Research was supported by a UQ enabling grant to PMG, BJC, and AEM.

## References and Notes

- Endre G., Kereszt A., Kevei Z., Mihacea S., Kalo P., Kiss G. B. A receptor kinase regulates symbiotic nodule development *Nature* **417**, 962 (2002).
- Stracke S., Kistner C., Yoshida S., Mulder I., Sato S., Kaneko T., Tabata S., Sandlin N., Stougaard J., Szczyglowski K., Parniske M. A plant receptor-like kinase required for both bacterial and fungal symbiosis. *Nature* **417**, 959 (2002).
- Smil V. Biofixation and nitrogen in the biosphere and global food production. In Nitrogen fixation: a global perspective. Fillard T. M., O'Brian M. R., Layzell D. B., Vessey J. K., Newton W. Editors, CAB International Publishing, New York (2002), p. 7.
- Carroll B. J., McNeill D. L., Gresshoff P. M. Isolation and properties of soybean mutant which nodulate in the presence of high nitrate concentration. *Proc. Natl. Acad. Sci. USA* **82**, 4162 (1985).
- Akao S., Kouchi H. A supernodulation mutant isolated from soybean cultivar Enrei. *Soil Sci. Plant Nutr.* **38**, 183 (1992).
- Gremaud M. F., Harper J. E. Selection and initial characterization of partially nitrateresistant nodulation mutant of soybean. *Plant Physiol.* **89**, 169 (1989).
- Moran D., Sagan M., Prado-Vivan E., Duc G. Influence of genes determining supernodulation on root colonization by the mycorrhizal fungus *Glomus mosseae* in *Pisum sativum* and *Medicago truncatula* mutant. *Mycorrhiza* **10**, 37 (2000).
- Delves A. C., Mathews A., Day D. A., Carter A. S., Carroll B. J., Gresshoff P. M. Regulation of the soybean *Rhizobium* nodulesymbiosis by shoot and root factors. *Plant Physiol.* **82**, 588 (1986).
- Francisco P. B. J., Harper J. E. Translocation of leaf signals to regulate soybean nodulation. *Plant Sci.* **107**, 167 (1995).
- Wopereis J., Pajuelo E., Dazzo F. B., Jian G., Gresshoff P. M., De Bruijn F. J., Stougaard J., Szczyglowski K. Short root mutant of *Lotus japonicus* with a dramatically altered symbiotic phenotype. *Plant J.* **23**, 97 (2000).
- Buschges R., Hollricher K., Panstruga R., Simon G., Wolter M., Frijters A., van-Daalen R., van der Lee T., Diergaarde P., Groenen dijk Töpsch S., Vos P., Salamin F., Schulze-Lefert P. The barley Mlo gene: a novel control element of plant pathogen resistance. *Cell* **88**, 695 (1997).
- Christie J. M., Reymond P., Powell G. K., Bernasconi P., Raibekas A. A., Liscum E., Briggs W. R. Arabidopsis NPH1: a novel protein with the properties of a photoreceptor for phototropism. *Science* **282**, 1698 (1998).
- Mita S., Monnier J., Loeb L. A. Resistance of HeLa cell mitochondrial DNA to mutagenesis by chemical carcinogen. *Cancer Res.* **48**, 4578 (1988).
- Chan G. C., Kwok S. F., Bleeker A. B., Meyerowitz E. M. Arabidopsis ethylene response gene ETR1: similarity of product to two-component regulators. *Science* **262**, 539 (1993).
- Bruggeman R. P., Doan B., Han-Dwenger K., Storz G. Characterization of a novel allele of the Arabidopsis HY4 locus. *Genetics* **149**, 1575 (1998).
- Sun T. P., Goodman H. M., Ausubel F. M. Cloning in the Arabidopsis GA1 locus by genomic subtraction. *Plant Cell* **4**, 119 (1992).
- Shirley B. W., Hanley S., Goodman H. M. Effects of ionizing radiation on a plant genome analysis of two Arabidopsis transparent testate mutation. *Plant Cell* **4**, 333 (1992).
- Faris J. D., Gill B. S. Genomic targeting in high-resolution mapping of the domestication gene *g1* in wheat. *Genome* **45**, 706 (2002).
- Lisitsyn N. A. Representational difference analysis in detecting differences between genomes. *Trends Genet.* **11**, 303 (1995).
- Diatchenko L., Lau Y. F., Campbell A. P., Chen Chik A., Moqadam F., Huan G. B., Lukyanov S., Lukyanov K., Gurskaya N., Sverdlov E. D., Siebert P. D. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. USA* **93**, 6025 (1996).
- Simon G., van der Lee T., Diergaarde P., van Daalen R., Groenen dijk J., Frijters A., Buschges R., Hollricher K., Töpsch S., Schulze-Lefert P., Salamin F., Zabeau M., Vos P. AFLP-based mapping of the Mlo gene to a 30-kb DNA segment of the barley genome. *Genomics* **44**, 61 (1997).
- Michelmore R. W., Paran I., Kesseli R. V. Identification of markers linked to disease-resistance genes by bulked segregant analysis—a rapid method to detect markers in specific genomic regions by using segregating populations. *Proc. Natl. Acad. Sci. USA* **88**, 9828 (1991).
- Li X., Song Y., Century K., Straight S., Ronald P., Dong X., Lassner M., Zhang Y. A fast neutron deletion mutagenesis-based reverse genetics system for plants. *Plant J.* **27**, 235 (2001).
- Herridge D. F. Carbon and nitrogen nutrition of two annual legumes [dissertation Perth (WA): University of Western Australia (1977)].
- Delves A. C., Higgins A., Gresshoff P. M. Shoot apex removal does not alter autoregulation of nodulation in soybean. *Plant Cell Environ.* **15**, 249 (1992).
- Meksem K., Zobrist K., Ruben E., Hyten D., Qian Zhou T., Zhang H. B., Lightfoot D. A. Two large-insert soybean genomic libraries constructed as a bin array vector: application in chromosome walking and genome-wide physical mapping. *Theor. Appl. Genet.* **101**, 747 (2000).
- Tomkins J. P., Mahalingam R., Smith H., Goicoechea J. L., Knapp H. T., Wing R. A. A bacterial artificial chromosome library for soybean PI 437654 and identification of a clone associated with cyst nematode resistance. *Plant Mol. Biol.* **41**, 25 (1999).
- Daneshmandi, Penuela S., Mudge J., Denner J., Nordstrom H., Martin ez J. P., Young N. D. A bacterial artificial chromosome library for soybean and identification of a clone encoding a major cyst nematode resistance gene. *Theor. Appl. Genet.* **96**, 196 (1998).
- Sambrook J., Fritsch E. F., Maniatis T. *Molecular cloning: laboratory manual*, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY (1989).
- Carroll B. J., Klimyuk V., Thomas C. M., Bishop G. J., Harrison K., Scofield S. R., Jones J. G. D. Germinal transposition of the maize element Dissociation from T-DNA loci in tomato. *Genetics* **139**, 407 (1995).
- Kolchinsky A., Landau-Elis D., Gresshoff P. M. Map ordering and linkage of molecular markers close to the supernodulation (*nts-1*) locus of soybean. *Molec. Gen. Genetics* **254**, 29 (1997).

32. Men A. E., Gresshoff P. M. DAF yields a clonal marker in the soybean (*Glycine max*) supernodulation-1 locus. *Plant J. Physiol.* **158**, 999 (2001).
33. Vos P., Hogers R., Bleeker M., Reijman M., van de Lee T., Hornes M., Frijters A., Pot J., Peleman J., Kuiper M., Zabeau M. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* **23**, 4407 (1995).
34. Searle I. Cloning of nodulation genes from soybean [dissertation Brisbane (QLD): University of Queensland Australia (2002).
35. Salimath S. S., Bhattacharyya M. K. Generation of a soybean BAC library and identification of DNA sequences tightly linked to the Rps1-k disease resistance gene. *Theor. Appl. Genet.* **98**, 712 (1999).
36. Searle I. R., Men A. E., Lanjiva T. S., Buzas D. M., Iturbide-Ormaetxe, Carroll B. J., Gresshoff P. M. Long distance signaling in legume nodulation requires a CLAVATA1-like receptor kinase. *Science*, October 31 (10.1126/science.1077937) (2002).
37. Shoemaker R. C., Polzin K., Labate J., Specht J., Brummer E. C., Olson T., Young N., Conibidol V., Wilcox J., Tamulon J. P. Genome duplication in soybean (*Glycine subgenus soja*). *Genetics* **144**, 329 (1996).
38. Gill K. S., Gill B. S., Endo T. R., Mukai Y. Fine physical mapping of Ph1, a chromosome pairing regulator gene in polyploid wheat. *Genetics* **134**, 1231 (1993).
39. Roberts M. A., Reader S. M., Dalgliesh C., Miller T. E., Foote T. N., Fish L. J., Snape W., and Moore G. Induction and characterization of Ph1 wheat mutants. *Genetics* **153**, 1909 (1999).
40. Bitner-Glindzisz, Lindley K. J., Rutland P., Blyden D., Smith V. V., Milla P. J., Hussain K., Furth-Lavi J., Cosgrove K. E., Shepherd R. M., Barnes P. D., O'Brien R. E., Farnham A., Sowden J., Liu X. Z., Scanlon M. J., Malcolm S., Dunin M. J., Aynsley-Greer A., Glaser B. A recessive contiguous gene deletion causing fan leaf hyperinsulinism in teropathin deafness identifies the Usher type 1C gene. *Nature Genet.* **26**, 56 (2000).