Title
Improving nitrogen use efficiency in barley (Hordeum vulgare L.) through the cisgenic approach

Authors
Kichey, Thomas
Holme, Inger
Møller, Inge Skrumsager
et al.

Publication Date
2009-04-29

Peer reviewed

Introduction

Barley (*Hordeum vulgare* L.) belongs in terms of yield and cultivated area to the four most important crops produced in the world (http://apps.fao.org). Since this crop is used both for human and animal consumption, an increase in grain production will be required to meet the food demand of the growing world population. Historically, the yield potential of cereals per unit of land area has been increased by repeated selection and cross-breeding of the most productive strains. Nevertheless, a powerful tool to increase grain yield has been the use of mineral fertilizers, chemical herbicides and fungicides.

Among all the fertilizers applied in the field, nitrogen (N) is the most important for plant productivity and grain quality (Frink et al. 1999). However, in developed countries, N use efficiency (NUE; defined as grain dry matter per unit of N available from the soil, fertilizer included) is very low (Raun and Johnson, 1999). Thus, intensive agricultural practices held in Europe and Northern America have led to both higher production costs and a greater risk from environmental hazards such as ground and surface water pollution by nitrate leaching (Mariotti, 1996).

Due to the current economical and ecological pressure, more sustainable agricultural practices are being developed using lower levels of N fertilization. Reducing the amount of N fertilizers applied to the field while at the same time maintaining yields is therefore the main challenge faced by breeders in selecting for cereal cultivars that absorb and/or metabolize N more efficiently.

Plant varieties can be improved through classical plant breeding (crossing and mutation) or genetic modification. However, the improvement of productivity and quality traits require high genetic variation and numerous crosses/backcrosses with wild species. Genetic modification of plants carries the promise of developing new cultivars with improved productivity, NUE and grain quality for food and feed. However, in Europe, genetic modification faces a substantial skepticism among the opinion and public authorities. With respect to public concerns and the promises of generating genetically modified plants with positive impact on productivity and resource use efficiency, the cisgenesis concept gradually made its appearance (Rommens et al. 2004). In contrast to transgenesis where genes and DNA sequences are moved across the species barriers, cisgenesis is characterized by the introduction of genes (including introns) with their native promoters from crossable species or from the crop plant itself (Schouten et al. 2006). Furthermore, all “helper” genes and gene sequences of foreign origin must be removed from the transformed plant lines. This technology has been made possible in crops by the development of new transformation protocols and the isolation of genes of interest.

In crops, numerous studies haves been undertaken in order to identify the limiting steps in the control of N uptake, N assimilation and N recycling during growth and development (Hirel *et al.* 2001; Kichey et al. 2006; Habash et al. 2007). A number of putative candidate genes involved in yield and in control of nutrient use efficiency were identified. Recent studies in maize and rice have provided strong evidence that the cytosolic isozyme of glutamine synthetase (GS1), a central enzyme in N assimilation and transport, is involved in growth and grain filling (Martin et al. 2006; Tabuchi et al. 2005). Other studies also suggest that the tonoplast intrinsic protein (TIP), an aquaporin, would

be involve in N storage (Bertl and Kaldenhoff, 2007) and thus may play a role in N management.

We have undertaken the development of new lines of genetically modified barley using the cisgenesis concept with the aim to improve NUE. Here we describe gene isolation and cloning, plant transformation and regeneration.

Results and discussion

Preparation of plasmid vector

Agrobacterium-mediated transformation was chosen since it has been shown in barley to be twice as efficient as particle bombardment (Travella et al. 2005). Since the cisgenesis concept requires the transfer of large pieces of DNA into the plant and an easy removal of selection markers, the binary Ti vector pGreenII was selected (Hellens et al. 2000). The pGreen vector series are small plasmids with high efficiencies in routine *in vitro* recombination procedures in *Escherichia coli*. In *Agrobacterium*, pGreen can replicate only if another plasmid, pSoup, is co-resident in the same strain. pSoup provides replication functions *in trans* for pGreen. The plant selection gene is carried on pSoup allowing for the separate transfer and integration of the two genes into the host genome.

pGreenII has been engineered to enable the size of the vector to be kept to a minimum and to both allow easy cloning and guaranty that gene transfer occurs without transfer of bacterial sequences. The inner part between the left border and the right border of the vector was removed and replaced by a cloning cassette so that the subsequent cloning can be done with the USER™ system (Geu-Flores et al. 2007).

Cloning of genes

The genomic sequence of TIP2 was available in the DNA DataBase (http://www.ncbi.nlm.nih.gov/). Primers have been designed to amplify the gene, its promoter and its terminal sequence. A 3532bp fragment, containing 1999bp upstream the gene and 564bp downstream, was obtained and cloned into the modified pGreenII vector. The sequence was checked by DNA sequencing.

For the GS1 gene, a probe of about 1kb was synthesized by PCR in order to screen a lambda phage library. The phage clone containing the GS1a isoform has been detected and sequenced. A 5.2kb gene fragment, including 1.5kb promoter and 491bp terminator was cloned into pGreenII by PCR.

Plant transformation and regeneration

Although barley has proved to be one of the most difficult crops to transform, numerous reports of gene transfer into barley cells and tissues have appeared during the past decade. The transfer of the genomic TIP2 and GS1a sequences into barley has been undertaken according to the method developed by Holme et al. (2006). Ovules of the

cultivar Golden Promise were isolated a few hours after pollination and infected with *Agrobacterium tumefaciens* strain carrying the binary vector pGreenII and its helper plasmid pSoup. About 250 embryos were transformed for gTIP2 and 500 embryos for gGS1a. According to Afolabi et al. (2004), out of 100 transformants between 2.7 and 5.4 plants would be suitable with the cisgenesis concept. This calculation includes marker and vector-backbone free transformants. Plants containing the resistance gene transformed with gTIP2 or gGS1a are growing in the greenhouse.

Conclusions and perspectives

We are developing a new generation of genetically modified barley based on the concept of cisgenesis in order to improve the NUE. Transgenic regenerants (T_0) transformed with the genomic TIP2 or GS1a have been obtained and are growing in greenhouse. They are in process to be tested for the presence and the number of cisgenes. The T_0 containing only one cisgene will be allowed to self pollinate and the T_1 generation will be tested for transcript levels, protein production and the presence of vector backbone and selection gene. Plants with simple integration pattern of expressed cisgenes and without vector backbone and selection gene sequences will be grown to maturity. The T_2 generation will subsequently be subjected to physiological analysis and biochemical characterization.

This study is currently completed by another cisgenic approach with the aim to increase the availability C-skeletons, a limiting factor for glutamate synthesis. Genomic gene of phosphoenolpyruvate will be transfer into barley using the same method and the transformants will then be crossed with the previously described cisgenic plants.

This new generation of genetically modified plants based on the cisgenesis concept could be a good alternative to conventional strategies having a positive impact on the environment and meeting the future demands and challenges associated with plant production while decreasing the potential risk linked to transgenic crops.

References

Afolabi A.S., Worland B., Snape J.W., Vain P. (2004) A large-scale study of rice plants transformed with different T-DNAs provides new insights into locus composition and T-DNA linkage configurations. Theor Appl Genet. 109: 815-826

Bertl A., Kaldenhoff R. (2007) Function of a separate NH3-pore in Aquaporin TIP2;2 from wheat. FEBS Lett. 581: 5413-5417

Frink C.R., Waggoner P.E., Ausubel J.H. (1999) Nitrogen fertilizer: retrospect and prospect. Proc. Natl. Acad. Sci. USA. 96: 1175-1180.

Geu-Flores F., Nour-Eldin H.H., Nielsen M.T., Halkier B.A. (2007) USER fusion: a rapid and efficient method for simultaneous fusion and cloning of multiple PCR products. Nucl. Acids Res.35: 7 e55

Habash D.Z., Bernard S., Schondelmaier J., Weyen J., Quarrie S.A. (2007) The genetics of nitrogen use in hexaploid wheat: N utilisation, development and yield. Theor Appl Genet. 114: 403-419

Hellens R.P., Edwards E.A., Leyland N.R., Bean S., Mullineaux P.M. (2000) pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. Plant Mol. Biol. 42: 819-832

Hirel B., Bertin P., Quilleré I., Bourdoncle W., Attagnant C., Dellay C., Gouy A., Cadiou S., Retailliau C., Falque M., Gallais A. (2001) Towards a better understanding of the genetic and physiological basis for nitrogen use efficiency in maize. Plant Physiol. 125:1258-1270

Holme I.B., Brinch-Pedersen H., Lange M., Holm P.B. (2006) Transformation of barley (*Hordeum vulgare* L.) by *Agrobacterium tumefaciens* infection of *in vitro* cultured ovules. Plant Cell Rep. 25: 1325-1335

Kichey T., Heumez E., Pocholle D., Pageau K., Vanacker H., Dubois F., Le Gouis J., Hirel B. (2006) Combined agronomic and physiological aspects of nitrogen management in wheat highlight a central role for glutamine synthetase. New Phytol. 169: 265-278

Mariotti A. (1996) Quelques réflexions sur le cycle biogéochimique de l'azote dans les agrosystèmes. In: Lemaire, G., Nicolardot, B. Eds. Maîtrise de l'azote dans les agrosystèmes. pp. 9-24. INRA Editions, Paris.

Martin A., Lee J., Kichey T., Gerentes D., Zivy M., Tatout C., Dubois F., Balliau T., Valot B., Davanture M., Tercé-Laforgue T., Quilleré I., Coque M., Gallais A., Gonzalez-Moro M.B., Bethencourt L., Habash D.Z., Lea P.J., Charcosset A., Perez P., Murigneux A., Sakakibara H., Edwards K.J., Hirel B. (2006) Two cytosolic glutamine synthetase isoforms of maize are specifically involved in the control of grain production. Plant Cell. 18: 3252-3274.

Raun W.R., Johnson G.V. (1999) Improving nitrogen use efficiency for cereal production. Agron. J. 91: 357-363.

Rommens C.M., Humara J.M., Ye J., Yan H., Richael C., Zhang L., Perry R., Swords K. (2004) Crop improvement through modification of the plant's own genome. Plant Physiol. 135: 421-431

Schouten H.J., Krens F.A., Jacobsen E. (2006) Cisgenic plants are similar to raditionally bred plants: international regulations for genetically modified organisms should be altered to exempt cisgenesis. EMBO Rep. 7: 750-753

Tabuchi M., Sugiyama T., Ishiyama K., Inoue E., Sato T., Takahashi H., Yamaya T. (2005) Severe reduction in growth and grain filling of rice mutants lacking OsGS1;1, a cytosolic glutamine synthetase 1;1. Plant J. 42: 641-655

Travella S., Ross S.M., Harden J., Everett C., Snape J.W., Harwood W.A. (2005) A comparison of transgenic barley lines produced by particle bombardment and *Agrobacterium*-mediated techniques. Plant Cell Rep. 23: 780-789