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SID 5 Research Project Final Report

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Project identification

1. Defra Project code: AR1005

2. Project title

   Biotechnology Resources for Arable Crop Transformation (BRACT)

3. Contractor organisation(s)

Prof. John Snape, Department of Crop Genetics, John Innes Centre, Colney, Norwich, NR4 7UH

Dr Huw Jones, Rothamsted Research West Common, Harpenden, Herts, AL5 2JQ

4. Total Defra project costs: £ 898,468

5. Project: start date .... 01 June 2003

   end date .... 28 Feb 2007

6. It is Defra's intention to publish this form.
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   In all cases, reasons for withholding information must be fully in line with exemptions under the Environmental Information Regulations or the Freedom of Information Act 2000.

   (b) If you have answered NO, please explain why the Final report should not be released into public domain

Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

Biotechnology Resources for Arable Crop Transformation – (BRACT)

Executive Summary

Introduction

An efficient transformation capability is of major importance, both as an analytical tool, and for crop improvement. To facilitate the use of transformation technology within the UK a need was identified for a centre of expertise to develop and provide efficient transformation technology to scientific community. The main objective of the BRACT project was therefore to provide a highly efficient, robust transformation capability for the main UK arable crops while also facilitating further improvements in transformation technology for the UK research community. The BRACT project, a collaborative project between JIC and R.Res, has achieved this aim and now provides a range of transformation facilities in major UK crops. During the project significant improvements to the transformation technology have been made and a new set of Gateway® compatible constructs developed to facilitate a range of transformation requirements. BRACT has provided training in transformation technology to many laboratories both in the UK and worldwide as well as undertaking the transformation component of a range of collaborative projects and providing a transformation service facility. The key achievements of the project are summarised below.

BRACT Objectives

Main objective:
To provide a highly efficient, robust transformation capability for the main UK arable crops while facilitating further improvements in transformation technology and delivery to the UK research community.

Technical and scientific objectives:
Objective 1 – Providing transformation resources to the UK research community (JIC & R Res)
Objective 2 – Vector construction. (JIC)
Objective 3 - Vector testing. (JIC & R.Res)
Objective 4 – Develop an efficient *Agrobacterium*-mediated transformation system for wheat. (R.Res)
Objective 5 – *Agrobacterium*-mediated transformation of barley. (JIC)
Objective 6 – Identify and test high throughput genotypes of oilseed rape. (JIC)

Objective 7 – Production of marker free transgenic plants. (JIC & R.Res)
Objective 8 – Stabilisation of transgene expression. (JIC)
Objective 9 - Website development. (JIC)
Objective 10 – Planning future transformation resources. (JIC & R.Res)

Methods and results of the research

During the early phase of the BRACT project, a consultation exercise was carried out to determine the demand for transformation facilities and the needs of the research community. A BRACT advisory group was established to provide advice and expertise in the establishment of the transformation facility and the BRACT website (www.bract.org) was developed as the main route to inform users of the resources available.

One of the key aims of the project was to develop a set of Gateway® compatible vectors based on the pGreen/pSoup system (Hellens et al, 2000) to enable easy building of constructs for a range of transformation applications. During the project the pBract series of vectors was developed specifically for wheat, barley and Brassicas, but with vectors also available for pea and *Arabidopsis* transformation. These vectors are all detailed on the website and allow basic transformation, over-expression and antisense studies, silencing using RNAi and production of clean-gene transformants. All of the pBract series vectors have been tested in the appropriate crop or are currently undergoing testing and will be made available to the research community.

Wheat was one of the key crops considered in the BRACT project and the basic protocol for *Agrobacterium*-mediated wheat transformation was re-established and improved. Over the three years of the project the method has been used to transform three bread wheat varieties (the winter wheat cultivar Florida, and two spring wheat varieties Cadenza and Fielder), and one durum wheat variety Ofanto. The targeted transformation efficiency for wheat of 5% (independent transgenic lines/total numbers of immature embryos inoculated) has been achieved in some experiments in all varieties with a range of 0.4-9.7%. Wheat transformation is now available as a service facility at 'full economic cost'. During 2005 we made over 500 transgenic wheat lines, either by biolistics or *Agrobacterium*, with approximately forty constructs both for in-house projects and for external customers. As knowledge of biological systems gained in model species is transferred to crops and as more candidate genes for crop traits are identified, we believe the demand for this service will increase.

Barley was another of the main BRACT crops. Routine transformation of the spring cultivar Golden Promise has been undertaken throughout the life time of this project. A number of interested parties from both the UK and Overseas have attended the lab for training purposes and the BRACT barley protocol has been shown to be highly portable and readily reproducible. A number of factors affecting transformation efficiency have recently been evaluated resulting in a significant increase in transformation efficiency from around 2% to an average of 10%. An extensive survey of 11 winter lines from the current recommend list was carried out and although several cultivars (Pearl, Haka, Jewel & Heligan) were identified as possible transformation targets, only transformed callus lines could be generated. JIC now offers a very efficient barley transformation service based on cost recovery for the spring genotype Golden Promise.

During the 3 years BRACT has been funded by Defra, the Brassica transformation group has made considerable progress and successfully made the transition from R&D to an established transformation facility. Demand for Brassica transformation within the UK has been high. For *Brassica oleracea* the high throughput (HTP) genotype AGDH1012 is used as a 'model' genotype for the routine testing of gene function and for training in Brassica transformation. Based on the work in *B. oleracea,* the same screening method was used within the BRACT programme to identify HTP *B. napus* genotypes for routine use. Currently transformation efficiencies of 12% and 17% for the spring (Q6) and winter (Q31) genotype respectively are being achieved. Screening of *B. carinata* and *B. rapa* has also been undertaken as these species would add to the BRACT portfolio and would be of significant interest to the research community. BRACT has established itself as a centre of excellence for Brassica transformation, not only within the UK but also internationally.

Discussion and future work

The BRACT project has addressed the need for focussed provision of transformation resources within key UK crops and has provided a transformation facility for the UK research community. By consulting with the research community, BRACT has ensured that the resources available closely match the needs of the community. During the course of the project, significant improvements have been made to the protocols for *Agrobacterium*-mediated transformation of wheat, barley and oilseed rape. Transformation efficiencies in some crops are approaching the level where the technology could be used to provide a range of functional genomics tools to further aid the determination of gene function. In addition to the improvements

in transformation efficiencies, BRACT has provided a set of easy to use constructs that should reduce the time involved in preparation of final constructs containing genes or sequences of interest. BRACT can help with all stages of crop transformation through planning, preparation of constructs, production of transgenic plants to analysis of T_0 and T_1 populations. The BRACT group is already involved in a number of collaborative projects and is providing transformation services on a cost recovery basis. Requirements for crop transformation as an essential tool for determination of gene function are growing and BRACT should be in a strong position to meet this need and therefore to ensure the continuation and further growth of the transformation facility.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the scientific objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Transfer).

(Please see attached project report.)

SID 5 Research Project Final Report - AR1005
Section 8 – Project Report to Defra

Biotechnology Resources for Arable Crop Transformation (BRACT)

Introduction

An efficient transformation capability is of major importance both as an analytical tool and for crop improvement. To facilitate the use of transformation technology within the UK a need was identified for a centre of expertise to develop and distribute best practice among the scientific community. The main objective of the BRACT project was therefore to provide a highly efficient, robust transformation capability for the main UK arable crops while also facilitating further improvements in transformation technology for the UK research community. The BRACT project, a collaborative project between JIC and R.Res, has achieved this aim and now provides a range of transformation facilities in major UK crops. During the project significant improvements to the transformation technology have been made and a new set of Gateway® compatible constructs developed to facilitate a range of transformation requirements. BRACT has provided training in transformation technology to many laboratories both in the UK and worldwide as well as undertaking the transformation component of a range of collaborative projects and providing a transformation service facility. The key achievements of the project are summarised below under the individual objective headings.

Objectives

Main objective:

To provide a highly efficient, robust transformation capability for the main UK arable crops while facilitating further improvements in transformation technology and delivery to the UK research community.

Technical and scientific objectives:

Objective 1 – Providing transformation resources to the UK research community (JIC & R Res)
Objective 2 – Vector construction. (JIC)
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Objective 8 – Stabilisation of transgene expression. (JIC)
Objective 9 - Website development. (JIC)
Objective 10 – Planning future transformation resources. (JIC & R.Res)

Methods and Results

Objective 1 – Providing transformation resources to the UK research community.

At the start of the BRACT project the existing most efficient and robust transformation methods for the main UK arable crops were brought together so that some transformation

resources could offered early in the project while improved methodology and vectors were being developed. During this early phase of the project a consultation exercise was carried out to determine the demand for transformation facilities and a BRACT advisory group was established to provide advice and expertise in the establishment of transformation facilities to meet the needs of the UK research community. The BRACT website was also developed as the main route to inform users of the resources available.

Of the researchers who responded to the consultation exercise, 68% could see an immediate use for a transformation facility, while 32% envisage they may have a need for such a facility in the future. A high demand for transformation technology as a research tool for testing gene function was identified with 64% of researchers needing to study gene function in plants under controlled environmental (glass-house) conditions with a further 14% expressing an interest in testing gene expression of plants *in vitro*. Clean gene technology (the delivery of transgenic plants free from selectable markers) was considered to be important by only 16% of researchers. This was surprisingly low, but may reflect that only 12% of researchers expressed an interest in testing gene function of transgenic plants grown under field conditions, with just 7% looking at commercial application. The availability of a range of ready made constructs, with a choice of selectable marker and reporter genes was of interest to 83% of researchers.

In terms of the facilities offered by BRACT, there was greatest interest in opportunities to develop projects on a collaborative basis (71%). Training facilities were of interest to 36% of researchers, a hotel facility to 22% and a full transformation service to 42% of those who responded.

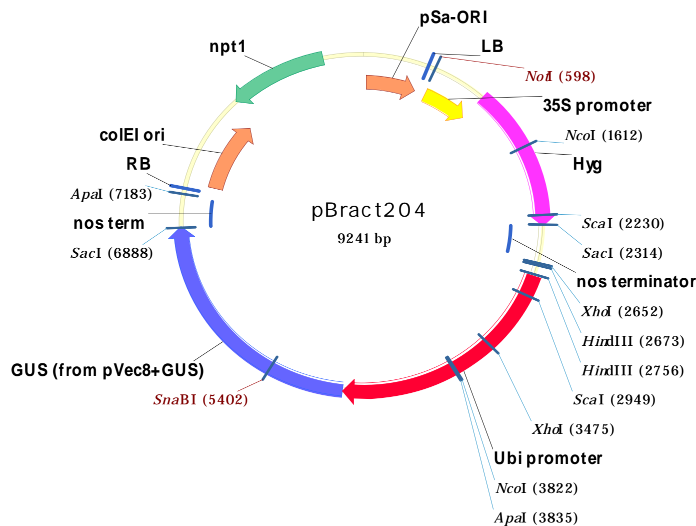
Objective 2 – Vector construction.

Objective 2 was to construct a set of Gateway® compatible vectors based on the pGreen/pSoup system (Hellens et al, 2000) to enable crop transformation. The vectors were designed specifically for wheat, barley and Brassica, but vectors are also available for pea/*Arabidopsis* transformation. These have been called pBract vectors.

Basic Vectors

A set of basic vectors were designed to enable control experiments to be carried out in wheat (pBract304), barley (pBract204; figure 1) and Brassica (pBract104) with *gus* (Ohta et al, 1990) as the reporter gene. The selection cassettes for each crop were chosen based on the best protocols used at the beginning of the project. These were for wheat; UbiBar, barley; 35SHyg and for Brassica; 35SKan. These basic vectors will also be useful when new transformation protocols are being developed.

Figure 1. pBract204 vector containing 35SHyg and UbiGUSint.



Barley and wheat vectors are also available with the *luc* reporter gene which can be used as an alternative to *gus* (pBract210 and pBract310). The UbiGUS and UbiLUC promoter-gene-terminator fragments are available as Gateway Entry vectors (pBEnt011 and pBEnt041), and therefore can be readily integrated into any destination vector.

pBract vectors are also available with the selection cassettes 35SKan, nosKan, 35SHyg or UbiBar present at the LB and an empty Gateway cassette at the RB. Thus the vector with the correct selection for the crop of interest can be chosen and the desired promoter-gene-terminator combination introduced using the Gateway LR reaction.

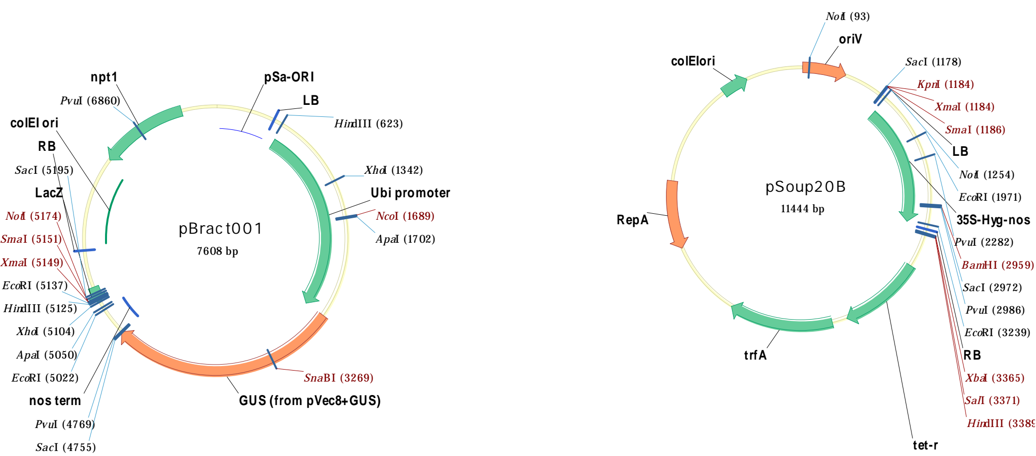
Clean Gene Vectors

To carry out clean gene work, selectable marker genes need to be easily removed from the plant following transformation. Constructs have been designed so that the selectable marker gene and gene of interest are present within different T-DNAs. Once the two T-DNAs have integrated within the plant genome the selectable marker gene may segregate away from the gene of interest within one generation, and plants containing only the gene of interest isolated.

The pGreen/pSoup system is particularly amenable to clean gene work as the two T-DNAs can be present on individual vectors. For each crop a basic set of clean gene vectors were constructed with the selectable marker gene cloned into pSoup and the *gus* reporter gene into pGreen (figure 2). To enable introduction of genes of interest, a pBract vector (pBract805) containing only a Gateway cassette is available for use in the clean-gene system.

The pSoup clean gene vectors contain the same selection cassettes as in the basic set of vectors for each of the three crops. To indicate that pSoup has been manipulated it has been given a number followed by 'B' to indicate it is part of the BRACT set of vectors.

Figure 2. The clean gene vectors available for barley transformation are pBract001 and pSoup20B.

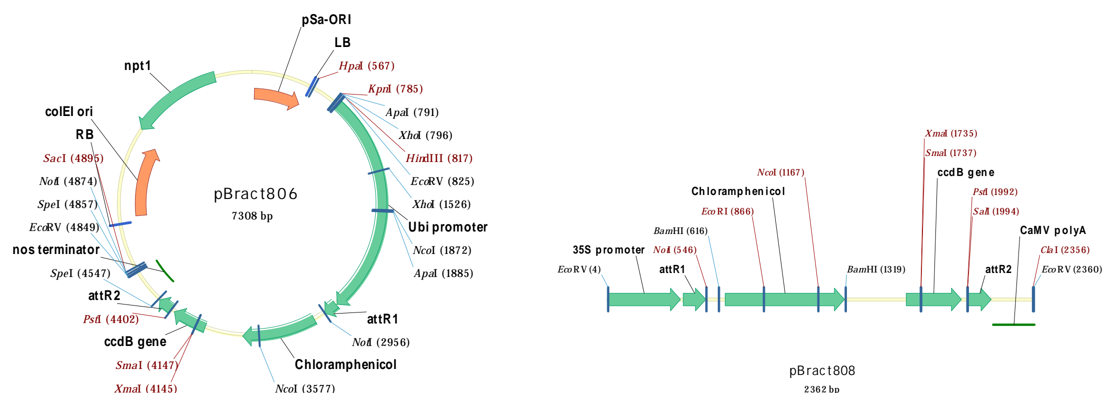


Over-expression and Antisense vectors

Over-expression and antisense studies can be very informative when trying to understand gene function. Novel genes can be expressed using over-expression vectors to determine the effects they have across species.

To date, over-expression cassettes with Ubi promoter-Gatecassf-nos terminator (pBract806) and 35S promoter-Gatecassf-CaMV (pBract808) have been constructed within a pGreen and pJIT61 backbone, respectively (figure 3). These over-expression cassettes have been designed so they can be easily digested and eluted as EcoRV fragments and cloned into the pBract vectors containing selection as required.

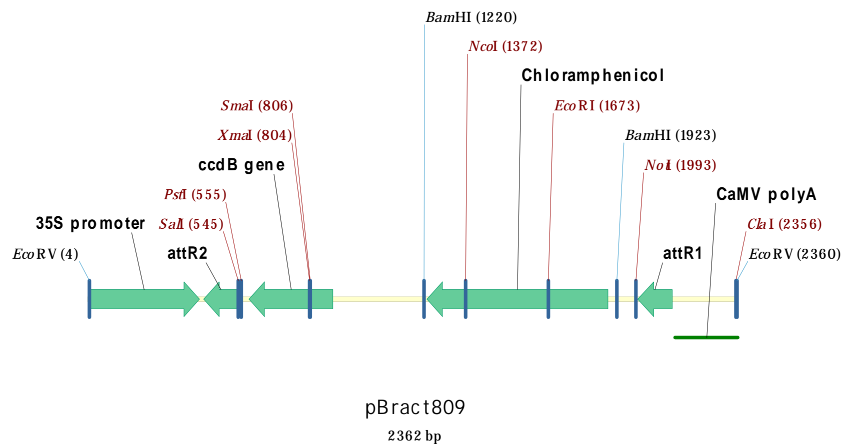
Figure 3. The pBract806 and pBract808 overexpression vectors contain Gateway cassettes in the forward direction driven by Ubi and 35S promoters, respectively.



The Ubi over-expression cassette has been cloned into the pBract202 vector containing 35SHyg ready for barley transformation (pBract 214).

An antisense Gateway cassette is available within the 35S promoter-CaMV terminator (pBract809; figure 4). As for the over-expression vectors, the antisense cassette can be eluted as an EcoRV fragment and cloned into a pBract vector containing the desired selection.

Figure 4. pBract809 antisense Gateway cassette available.



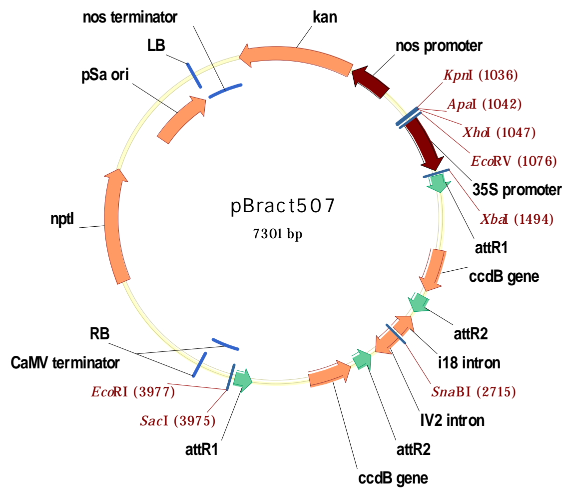
RNAi vectors

RNA interference (RNAi) is a reliable method of producing gene silencing. Antisense constructs can produce a range of gene silencing efficiencies, but RNAi constructs can reproducibly produce complete, or almost complete silencing.

The RNAi pBract vectors have been designed using inspiration from the constructs produced by Helliwell and Waterhouse, 2003. For constructs to initiate gene silencing they need to enable a hairpin structure to be formed *in vivo*, giving rise to double stranded RNA. The pBract RNAi cassette therefore consists of two Gateway cassettes cloned in opposing directions. A fragment of the gene to be silenced can be introduced via an Entry vector using the LR recombination reaction. From work carried out by Smith et al, 2000 it is known that splicing of an intron from the hairpin loop structure improves gene silencing efficiency, and therefore it was important that a functional intron was present in our construct. Observations have shown that with only one intron present this can rotate in direction during the LR reaction as the two Gateway cassettes undergo recombination with the Entry vector (Helliwell and Waterhouse, 2003). Thus, in some of the resulting constructs the intron will be in the reverse direction and the splice sites not recognised. The use of two introns cloned in opposing directions resolves this problem. Even if the section between the Gateway cassettes rotates, one intron will be in the correct orientation for splicing.

The RNAi cassette consisting of two Gateway cassettes and introns was constructed within a 35S promoter and CaMV terminator (35SRNAi). Two EcoRV sites straddle 35SRNAi so that this fragment can be eluted and cloned into a vector containing the desired selection. This has been done to form pBract507 containing nosKan (figure 5). The RNAi cassette alone can also be eluted as a HindIII/SacI fragment and placed within alternative promoter/terminator combinations. A vector containing the Ubi promoter driving the RNAi cassette with a nos terminator has been constructed with 35SHyg selection.

Figure 5. Diagram of pBract207 containing nosKan selection and 35SRNAi.



Sequencing and VectorNTI

All of the pBract constructs have been sequenced. The sequence and maps for each of the vectors are available as VectorNTI files at www.bract.org along with associated Word files and details for ordering. Although the vectors described above have been completed, they will only be available for ordering once testing in the individual crops in complete. Forty-four individual constructs are now detailed on the BRACT website.

Further work

The 35SGUS reporter gene cassette does not seem to be expressing in stably transformed Brassica lines, even though biolistic experiments showed detectable levels of GUS. Further testing of this cassette is being carried out. The wheat silencing construct containing UbiBar and UbiRNAi is still in progress and should be completed shortly.

Key

UbiBar = Ubiquitin promoter – bar – nos terminator
35SHyg = 35S promoter – hyg with intron – nos terminator
35SKan = 35S promoter – kan – CaMV terminator
UbiGUS = Ubiquitin promoter – gus with intron – nos terminator
UbiLUC = Ubiquitin promoter – luc with intron – nos terminator
Gatecassf = Gateway cassette in the forward direction
Gatecassr = Gateway cassette in the reverse direction

References

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

Ohta S, Mita S, Hattori T and Nakamura K (1990) Construction and Expression in Tobacco of a b-Glucuronidas (GUS) Reporter Gene Containing an Intron within the Coding Sequence. Plant Cell Physiol. **31(6)**: 805-813.

Helliwell C and Waterhouse P (2003) Constructs and methods for high-throughput gene silencing in plants. Methods **30(4)**; 289-295

Smith N A, Singh S P, Wang M-B, Stoutjesdijk P A, Green A G and Waterhouse P M (2000) Total silencing by intron-spliced hairpin RNAs. Nature **407**, 319-320.

Objective 3 - Vector testing.

Vector testing

As mentioned above, all vectors have been checked by complete sequencing. The basic vectors have all been tested in stable transformation experiments. pBract204 containing the Hygromycin selectable marker and the GUS reporter genes has been successfully transformed into barley on numerous occasions. Furthermore, several labs have already requested the vector for use in their own studies. pBract 304 has been used successfully for wheat transformation and pBract 204 also successfully used for barley at R. Res. pBract 104 has been tested in the Brassica transformation systems at JIC.

The clean gene vector combination pSoup20b (Hygromycin) and pBract001 (GUS) has also been successfully introduced into barley. This series of experiments is presently on-going and is covered by the time-only extension to the project.

RNAi vectors are currently being tested in both transient and stable transformation experiments. The results will be reported in the supplementary report to be produced in February 2007.

Objective 4 – Develop an efficient *Agrobacterium*-mediated transformation system for wheat.

04a. *Re-establish a basic protocol previously developed under MAFF funding AR1002 and demonstrate stable transformations using pAL154/pAL156 in wheat varieties already shown to be amenable (Florida and Cadenza).*

The basic protocol developed under MAFF AR1002 has been re-developed and optimised. Over the last three years this method has been applied, with minor variations, to transform three bread wheat varieties (the winter wheat cultivar Florida, and two spring wheat varieties Cadenza and Fielder), and one durum wheat variety Ofanto. To the best of our knowledge, this is the first report of durum wheat transformation by *Agrobacterium*. It has also been effective in transforming barley. The targeted transformation efficiency for wheat of 5% (independent transgenic lines/total numbers of immature embryos inoculated) has been achieved in some experiments in all varieties with a range of 0.4-9.7% (see Table 1). We believe that our current protocol which is unique in using fresh (less that 1 hr after isolation) immature embryos could be extended to different varieties and maybe different species with minor modifications (see Table 2).

Since the beginning of the BRACT project, 204 independent transgenic wheat plants have been produced by this protocol. Some parameters have been found to be important in achieving efficient transformation mediated by *Agrobacterium* in wheat, such as additional Komari fragment in the helper plasmids, the concentration of acetosyringone, appropriate density of *Agrobacterium*, pH of the inoculation medium etc (see Figure 6).

04b. *Assess highly regenerable Bobwhite lines with the aim of providing a higher efficiency Agrobacterium-mediated transformation system for wheat.*

Two Bobwhite accessions reported to be highly regenerable were tested for their transformability. In our hands these wheat types were recalcitrant and no transformed lines could be generated. In consultation with the project officer, alternative spring types Cadenza and more recently, Fielder were incorporated as alternatives. We have generated transgenic

lines with both these varieties and our latest data indicate that response of Fielder (kindly provided by Dr. Dong Fang Chen at Syngenta), is better than Cadenza. (Table 1).

Knowledge transfer and training

Training workshop for wheat *Agrobacterium* transformation.

A hands-on workshop to teach the wheat transformation protocol took place at Rothamsted over three days in March-April 2005. Seven scientists from leading transformation labs in six different EU countries were given intensive training in the wheat transformation method developed at RRes (see below).

Visiting workers trained in wheat transformation at RRes in last three years

<table>
<tr><th>Name</th><th>Organisation</th><th>Fellowship and length of visit</th></tr>
<tr><td>Michela Janni</td><td>University of Tuscia, Italy</td><td rowspan="6">RRes training workshop, 3 days.</td></tr>
<tr><td>Silvia Travella</td><td>Institute of Plant Biology, University of Zürich, Switzerland</td></tr>
<tr><td>Klara Meszaros</td><td>Agricultural Research Institute of the Hungarian Academy of Scieneses Martonvásár, Hungary</td></tr>
<tr><td>Istvan Csoti</td><td>Faculty of Science, Eotvos Lorand University, Budapest, Hungary</td></tr>
<tr><td>Pierre Barret</td><td>INRA, Clermont-Ferrand, France</td></tr>
<tr><td>Khaled Masmoudi</td><td>Centre of Biotechnology of Sfax, Tunisia.</td></tr>
<tr><td></td><td></td><td></td></tr>
<tr><td>D. P. Biradar</td><td>Department of Agronomy, University of Agricultural Sciences, Dharwad, India</td><td>Commonwealth Universities Commission Fellowship. Nov 2004-April 2005</td></tr>
<tr><td>Mariann Rakszegi,</td><td>Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvasar, Hungary.</td><td>Visiting worker
March 2005-Aug 2005.</td></tr>
<tr><td>Sylvie Bernard,
Isabelle Nadaud and
Andre Madeore,</td><td>INRA Clermont-Ferrand, France.</td><td>Invited training visit
1st- 2nd March 2004.</td></tr>
<tr><td>Silvia Rubio Diaz,</td><td>University of Alcala, Madrid, Spain.</td><td>Short Term Training Fellowship, Ministry of Science and Technology Spain. Sept 2002-Jan 2003.</td></tr>
<tr><td>M.S. Patil,</td><td>University of Agricultural Sciences, Dharwad, India.</td><td>Commonwealth Universities Commission Fellowship. Oct 2002-April 2003.</td></tr>
<tr><td>Teun van Herpen,</td><td>Plant Research International, Wargeningen, The Netherlands.</td><td>PhD seconded training. Feb 2004-July 2005.</td></tr>
</table>

Katumasa Niwa,	Assistant Professor of Plant Breeding Department of Agriculture, Tokyo University of Agriculture, Japan	Visiting worker Sept 2005 – Aug 2006
Lanqin Xia,	National Wheat Improvement Centre, Institute of Crop Science, Chinese Academy of Agricultural Sciences (CAAS), Beijing, China	Visiting worker June 2005 – Dec 2005

Provision of wheat transformation as a service

Wheat transformation is now available as a service facility at 'full economic cost'. During 2005 we made over 500 transgenic wheat lines, either by biolistics or *Agrobacterium*, with approximately forty constructs both for in-house projects and for external customers. As knowledge of biological systems gained in model species is transferred to crops and as more candidate genes for crop traits are identified, we believe the demand for this service will increase.

Table 1. Summary of numbers of transgenic plants made and transformation efficiencies for three bread wheat and one durum variety

	Variety	No. of immature explants used	No. of transgenic plants	Transformation efficiency (ave %)	Transformation efficiency (max %)
Bread wheat	Florida (W)	5611	88	1.6	7.1
	Cadenza (S)	1421	28	2.0	4.5
	Fielder (S)	2587	61	2.4	6.3
Durum wheat	Ofanto	1161	27	2.3	9.7

Table 2. Susceptibility of different wheat varieties and species to infection by AGL1 pAL154/pAL156.

Variety	Type	No. of IE	% of IE with GUS	Average GUS spots/explant
Bobwhite	S	37	86.5	53.51
Canon	S	92	82.6	28.92
Baldus	S	49	89.7	36.73
Imp	S	36	61.1	9.27
L88-31	S	61	86.9	43.28
Riband	W	88	29.5	7.96
Buster	W	39	74.4	20.71
Tm23	Monococcum	82	89.0	40.12

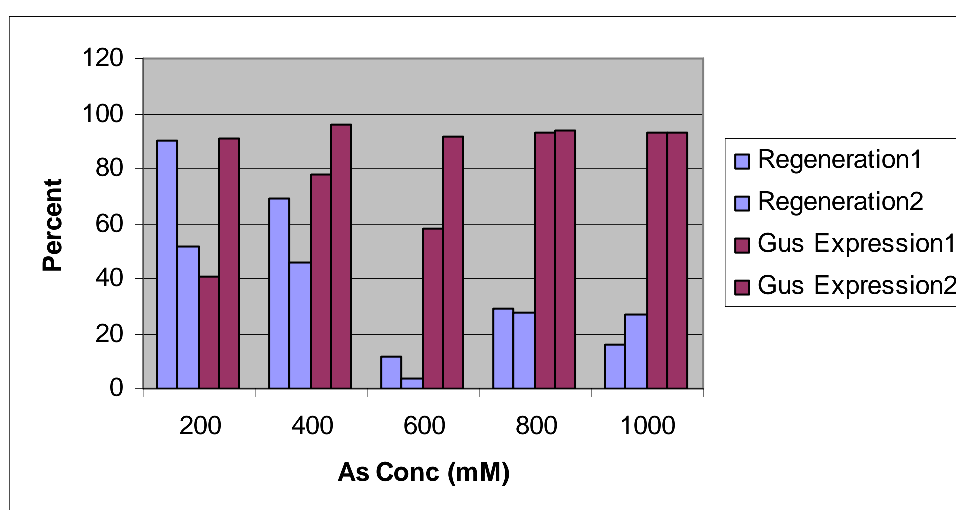


Figure 6. T-DNA transfer (measured as GUS expression) and wheat plant regeneration with increasing levels of the phenolic Vir-gene inducer Acetosyringone in the medium. Bars show mean results of at least 50 embryos and for each Acetosyringone concentration the experiment was repeated twice. An acetosyringone concentration of 400 mM was chosen as a compromise between T-DNA delivery and the capacity for regeneration.

Objective 5 – *Agrobacterium*-mediated transformation of barley.

Routine transformation

Routine transformation of the spring cultivar Golden Promise has been undertaken throughout the life time of this project. A number of interested parties from both the UK and Overseas have attended the lab for training purposes. Barley transformation is now successfully established at Institutes such as the Scottish Crop Research Institute, Institute of Arable Crop Research and MTT Agrifood Research, Finland as a direct result of the BRACT project. Excellent feedback has been received and this has been incorporated into regularly updated protocols, all of which are available on the website. The BRACT barley protocol has been shown to be highly portable and readily reproducible. In work linked to the BRACT project, a number of factors affecting transformation efficiency have recently been evaluated resulting in a very significant increase in transformation efficiency from around 2% to an average of

10% with some experiments much higher. Some of the methods used to achieve this improvement are described below.

Copper as a media additive

Preliminary results have shown that raising the level of copper in the barley callus induction medium triggers an earlier production of embryogenic callus and a more rapid shift into the regeneration phase. The existing barley transformation protocol already uses increased copper in the later stages of the culture process. However, adjusting the timing of the additional copper appears to have a significant effect on transformation efficiency. The full results of these experiments will be reported in the supplement to the report in February 2007.

Other improvements to transformation efficiency

Additional improvements to the efficiency of barley transformation have been made by treating immature embryos with *Agrobacterium* on the same day as they are isolated rather than 1 day after isolation. Filter sterilising the callus induction medium has been shown to give improved results compared to the use of autoclaved medium. Also one of the most important factors in the barley transformation process is the quality of the donor plants providing the immature embryos. By paying particular attention to the plant growth conditions further improvements to transformation efficiencies have been made.

Subculture technique

Most tissue culture methods, when selecting barley transgenics, rely on a regular subdivision of the callus, to promote regeneration of transformed material. However, three small independent experiments (50 - 100 embryos each) have demonstrated no significant reduction in transformation efficiency if the callus is only broken up in the latter stages of the experiment, when transferred to regeneration medium. This substantially reduces the time spent subculturing material and reduces the quantity of media required. These both represent significant efficiency savings with the only reduction observed being in the number of clones per line, not the number of independent lines.

Winter cultivars

As previously reported, an extensive survey encompassing 11 winter lines (including two six row barleys) from the current recommend list, identified several cultivars (Pearl, Haka, Jewel & Heligan) as suitable targets for transformation. However, none were as prolific as Golden Promise *in vitro*. Furthermore, the winter cultivars exhibited a more severe hypersensitive reaction in response to the addition of the *Agrobacterium*, resulting in phenolic browning of the tissue and loss of regeneration potential. As a consequence, only transgenic callus lines were recovered.

In an attempt to mitigate the hypersensitive response, a number of strategies were investigated.

- An initial pre-culture period of up to 14 days, prior to addition of the *Agrobacterium*, enabled callus formation to commence and the tissue to stabilise *in vitro*. A visual reduction in phenolic browning was observed, but at an increased risk of subsequent overgrowth of the explant by the *Agrobacterium.* An initial pre-culture period of 7 days was deemed optimal and it was from these experiments that the transformed calli were generated.
- The addition of cysteine to culture media has previously been shown to have a positive effect in barley transformation systems (Hensel & Kumlehn 2005). However, at a concentration of 400 mg l^{-1} the effect was detrimental, resulting in a severe reduction in callus formation, no regeneration and an apparent desiccation of the tissue.
- Patent WO 00/63398 describes a method of producing transgenic wheat by the injection of *Agrobacterium* and subsequent co-cultivation *in planta*. A series of five experiments, attempted to replicate this in barley, in the anticipation that the *in planta*

co-cultivation may overcome the loss of regeneration potential. However, early results were disappointing with little callus production observed from the subsequently isolated embryos.

- pBract204 was transformed into three different *Agrobacterium* host strains; AGL1, EHA105 and GV3101. AGL1 is used for routine Golden Promise transformation work, however the additional hosts were assessed for any possible reduction in the hypersensitive response of Haka, Heligan and Pearl. In transient assays on 500 embryos, AGL1 consistently produced superior results with a mean of 1.57 transformation events per embryo, with no significant difference between the winter lines, or the Golden promise control. EHA105 resulted in 0.08 transformation events in Haka and GV3101 did not produce any.
- As described above experiments to determine the effect of additional copper on the transformation of the winter lines are currently in progress.

Mature seeds

A major expense and potential delay in the production of transgenic barley is the requirement for a regular supply of high quality (CER grown) immature embryos. This is particularly problematic when studying winter cultivars which require an additional eight to ten weeks vernalisation period. Sharma et al. (2005) published a protocol describing plant regeneration from mature embryos. This method was examined to determine whether it could be used to produce target tissue for transformation of Golden Promise. However, the method did not produce regenerable material of sufficient quality for transformation.

Provision of barley transformation as a service

JIC now offers a very efficient barley transformation service based on cost recovery. The BRACT group have assisted collaborating groups with grant proposals containing barley transformation. In addition to providing training for visitors to the lab, the BRACT group is also hosting students wishing to use the methodology in their research projects. Training and collaborations in barley transformation are summarised below.

Training and collaborations

Training provided to:

Scottish Crop Research Institute
Rothamsted Research
MTT Agrifood Research Finland
Institute of Genetics and Cytology, Belarus

Collaborations involving barley transformation:

University of Southampton
University of Liverpool
University of Nottingham
University of Birmingham
and four JIC groups.

References

Hensel G & Kumlehn J (2004) Genetic transformation of barley (*Hordeum vulgare* L.) by co-cultivation of immature embryos with *Agrobacterium.* In: Curtis I S (Ed) Transgenic Crops of the World- Essential protocols, Kluwer Academic Publishers. pp 35-44.

Sharma V K, Hansch R, Mendel R R & Schulze J (2005) Mature embryo axis-based high frequency somatic embryogenesis and plant regeneration from multiple cultivars of barley (*Hordeum vulgare* L.) J Exp Bot. 56: 1913-1922.

Objective 6 – Identify and test high throughput genotypes of oilseed rape.

Brassica transformation

During the 3 years BRACT has been funded by Defra, the Brassica transformation group has made considerable progress and successfully made the transition from R&D to an established transformation facility. Demand for Brassica transformation within the UK has been high. BRACT has already completed a number of programmes, both as 'service provider' where T_0 transgenics have been supplied to researchers, and as part of collaborative programmes, which commonly results in production of T_1 progeny. Demand for phenotypic progeny testing and evaluation, has also been high. In the last 18 months, collaborations have already brought in revenue of over £80K.

The UK brassica research community is well established, with a good network of scientists who meet regularly. Through this BRACT is being promoted as the UK centre of excellence for Brassica transformation. The use of BRACT for transformation work is now being costed into a number of future and proposed research grants (approximately £250K in grants currently submitted). This is an encouraging position to be in at the end of this Defra funded project.

BRACT now offers a range of resources and facilities for Brassica transformation, focusing on *B. oler*acea and *B. napus*. The following report summaries the outputs from this study, and highlights the resources now available to the scientific community.

Brassica oleracea transformation

The routine transformation of *B. oleracea* was already established at JIC as part of a previous Defra funded programme of research (HH0909SFV). As part of this study a high throughput (HTP) *B. oleracea* genotype (AGDH1012, Sparrow et al. 2004) was identified and proposed as a 'model' genotype for the routine testing of gene function.

AGDH1012 is now being offered as a resource to the Brassica scientific community, as an easy-to-transform model genotype, through BRACT. The transformation protocol has been simplified and full protocols including a photo board of the transformation protocol are now available via the BRACT website www.bract.org . Due to the robustness and efficacy of this genotype it has been used for all training work, both hands-on training at JIC, and remote training using the BRACT website and email correspondence as a freely available resource. We currently have a 100% success rate with technology transfer of this protocol. This genotype is also being used by BRACT for all routine *B. oleracea* transformation work. Currently we are able to go from transformation initiation, to PCR tested T_0 plantlets *in vitro* within 8-10 weeks; and to T_1 seed production in as little as 40 weeks.

Brassica napus (Oilseed rape OSR) transformation

Based on a strong understanding of the genetic basis of transformation in *B. oleracea* (Sparrow et al., 2004), the same screening method was used within the BRACT programme to identify HTP *B. napus* genotypes for routine use. Winter OSR is the favoured crop in the UK, and it was therefore desirable for BRACT to identify high throughput winter varieties for testing gene function. However, for research purposes spring OSR is often desirable as this does not require a vernalisation (cold) period before the plants will flower, thus shortening the time required for the production of subsequent generations. Our objective was to identify both winter and spring genotypes with transformation efficiencies of greater than 10%. This was above the current rate of our in-house best at the time (genotype W10, transformation efficiency of 5-8%). During the reporting research phase of BRACT, 81 *B. napus* genotypes

were screened for ease of transformation. This included genotypes from the HGCA recommended list, breeding material and developmental mapping populations.

Of the 81 genotypes screened, 20 had the desired tissue culture shoot regeneration potential (>50% of explants producing shoots) and mode of regeneration (shooting via a slight callus phase, and critically in the absence of tissue culture blackening), to be taken through to test for susceptibility to *Agrobacterium*. This screen identified 9 of the 81 original genotypes, as potential candidates for transformation studies, based on a high shoot regeneration potential, an absence of tissue culture blackening, and a high susceptibility to *Agrobacterium*.

All 9 genotypes were trialled for transformation potential, and transgenic shoots were successfully recovered from all genotypes. Two genotypes (a winter and spring genotype) were identified as strong candidates for use in routine transformation work. These lines come from a doubled haploid population developed at JIC and are currently being mapped, by Ian Bancroft's group (we acknowledge Ian Bancroft, Colin Morgan and Rachel Wells for supplying material of the DH 'Q' population). Transformation rates for the two selected lines were above the target objective of >10%. These lines also benefit from being highly self-compatible, which will enable an efficient turn around of generations, without the need for lengthy hand-pollination, and thus share some of the desirable traits that make AGDH1012 such a good resource. Currently transformation efficiencies of 12% and 17% for the spring (Q6) and winter (Q31) genotype respectively are being achieved.

Demand has been higher for spring *B. napus* transformation, and Q6 is now being used for all our routine *B. napus* transformation work. Technology transfer with this genotype has also been successful.

Additional work

Investigation of alternative promoters

A high proportion of researchers wishing to use the BRACT facility, have approached us with constructs containing a whole array of different promoters and selectable markers. This is often due to researches wishing to express genes in Brassica using constructs that have been used for testing in Arabidopsis. As the BRACT facility further develops, it would be useful to make the system as user friendly as possible. BRACT has therefore encouraged the scientific community to update us on their requirements.

In response to requests from the scientific community, we are currently investigating the NOS promoter, as an alternative to the 35S promoter to drive the kanamycin selectable marker gene. The rational for this is two fold, firstly NOS Kan is often used in Arabidopsis transformation, and secondly with interest in RNAi increasing it would be desirable to have an alternative to 35S driving the selectable marker (as 35S is often the promoter of choice for driving the gene of interest).

Using constructs developed by BRACT members, pBRACT102 (35S Kan) and pGreen0029 (NOS Kan) we are currently testing the viability of NOS as an alternative promoter. The Brassica transformation protocol was developed using 35S driven kanamycin, and AG DH1012 is sensitive to kanamycin. NOS is a much weaker promoter, and under the current selection levels (15mg/l kanamycin in *B. oleracea* AG DH1012), fails to regenerate transgenic shoot at an acceptable level (if at all). A range of selection levels are currently being investigated.

Screening of *B. carinata* and *B. rapa*

Two MSc students from the MSc in Plant breeding and Biotechnology at the University of East Anglia in conjunction with JIC conducted research programmes to screen *B. rapa* and *B. carinata* genotypes for transformation competent genotypes.

The screening methods employed were as described for *B. oleracea* and *B. napus* (Sparrow et al., 2004). *B. carinata* lines were identified with shoot regeneration potentials of > 80%, that also exhibited high levels of susceptibility to *Agrobacterium* (>70%). In preliminary studies 2 genotypes were identified as potential candidates for further investigation having demonstrated the ability to produce transgenic shoots. In *B. rapa*, which remains a highly recalcitrant species, tissue culture competent genotypes (with shoot regeneration levels of >50%) were identified. However, the average regeneration response in this population (30 genotypes screened) was just 12 %. All genotypes were screened for susceptibility to *Agrobacterium,* overall susceptibility was high (average 60 %). From the initial screen 3 genotypes were identified with reasonable shoot regeneration potential, and susceptibility to *Agrobacterium*. These genotypes were taken through for transformation trials, using a construct containing the GUS reporter gene, and early GUS expressing shoots were successfully obtained in all 3 genotypes. This demonstrated that the screening method optimised during the BRACT programme, could be applied to other brassica species. The best combination of shoot regeneration potential and *Agrobacterium* susceptibility was 60% and 48% respectively, for genotype R018. Although transgenic shoots were produced in this genotype, overall efficiency was low, and whole plant recovery has yet to be demonstrated. However, the ability to produce transgenic *B. rapa* shoots was a significant advance, and has provided a good starting point for further research with this genotype. R018 is currently being used to produce a TILLING population at JIC, and the *B. rapa* genome is currently being sequenced (Multinational Genome Project http://www.brassica.info/). The ability to transform *B. rapa,* at a level that would allow routine use would be a major scientific achievement. The ability to offer transformation in both of these species would add to the BRACT portfolio and would be of significant interest to the research community.

Conclusions

BRACT has established itself as a centre of excellence for Brassica transformation, not only within the UK but also internationally. BRACT resources (protocols and germplasm) are now being used by the scientific community in laboratories in Europe, China, New Zealand and the US.

Easy to transform genotypes of *B. oleracea* and *B. napus* have been identified, and simplified protocols disseminated to the scientific community.

Training and collaborations:

Training given to:
Birmingham University
Warwick HRI
Sainsbury Laboratories

Protocols and Germplasm sent out to:
INRA-France
Warwick –HRI (AGDH1012 and also developmental *B. napus* material)
Hunan Agricultural University-China
Huazhong Agricultural University, Wuhan, China
Berkley University, San Francisco, USA
Crop and Food Research, New Zealand
Planet Biotechnology, San Francisco, USA
National University of Ireland, Maynooth, Co. Kildare, Ireland
Lund University, Lund, Sweden

Institute of Plant Breeding, Christian-Albrechts-University, Germany

Reference

Sparrow PAC, Dale PJ and Irwin JA (2004). The use of phenotypic markers to identify *Brassica oleracea* genotypes for routine high-throughput *Agrobacterium*-mediated transformation. Plant Cell Reports. 23:64-70

Pea Transformation

Transformation of *Pisum sativum* (pea) has been established in very few laboratories throughout the world. BRACT has overcome the transformation difficulties of this important European legume using the methodology first described by Bean *et al*., 1997, re-establishing pea transformation at JIC. BRACT is providing pea transformation as part of the EU's Grain Legumes Integrated Project (GLIP). The pea transformants produced by BRACT, provide an essential component for the study of gene function in comparative genetic experiments within GLIP. BRACT collaborates with groups across Europe as part of GLIP.

Bean, S. J, Gooding, P. S, Mullineaux, P. M. and Davies, D. R. (1997). A simple system for pea transformation. *Plant Cell Rep.* **16:** 513-519.

Objective 7 – Production of marker free transgenic plants.

Due to technical problems experienced during the production of the constructs, testing of the BRACT clean gene vectors in Brassica was not possible during the course of this programme. However, we can report that the production of marker free plants in AG DH1012 (*B. oleracea*) and W10 (*B. napus*) has already been demonstrated within the group using existing pGreen/pSoup constructs. Figure 1, shows segregation patterns for the *gus* and *nptII* genes in a population of *B. napus* T_1. These findings were repeated in a number of progeny of *B. napus* and *B. oleracea*, containing single and multiple loci inserts (Bean et al., in preparation) full data not presented.

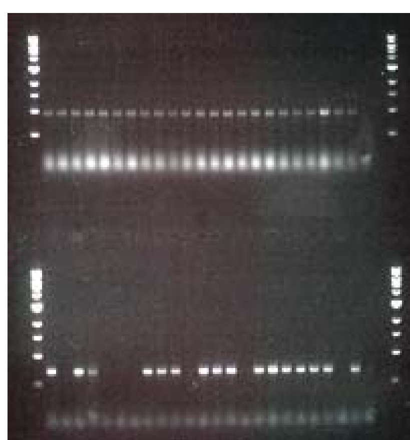


Figure 7. PCR segregation patterns in T_1 *B. napus* lines. Lanes 1-22 represent 22 T_1 *B. napus* progeny from a T_0 plant containing a single loci insertion of the *gus* and *nptII* genes; lane 23 contained a positive control, and lane 24 a negative control. The upper row shows PCR data for presence of the *gus* gene, and the lower row corresponded to data for presence of the *nptII* gene. Lanes 2,5,6,7,11,15 and 22 show loss of the *nptII* gene in the resulting T_1.

Experiments using BRACT constructs pSoup20b and pBract001 are underway in barley. Transgenic plants have been regenerated from a number of individual experiments by selecting for hygromycin resistance. Plants are being tested for the presence of the *Gus* gene and seed collected so that segregation analysis in the T1 generation can be undertaken. These results will be reported in the supplementary report to be produced in February 2007.

Objective 8 – Stabilisation of transgene expression.

Previous work in wheat showed that additional introns inserted within the coding region of a gene help to stabilise transgene expression between the T_1 and T_2 generations (Bourdon et al. 2004). Following on from this work, both the T_3 and T_4 generations of the wheat lines transformed with constructs with or without additional introns have now been analysed for luciferase expression levels to see if the stabilising effect was still apparent in later generations. Analysis of the T_3 and T_4 luciferase expression data did not show any further stabilisation of luciferase expression in the lines containing the additional introns. Constructs suitable for barley transformation containing the luciferase gene either with or without introns within the coding sequence have now been prepared and transformed into barley. Analysis of the resulting lines should establish whether the stabilising effect between the T_1 and T_2 generations can be demonstrated in barley as well as wheat.

Reference

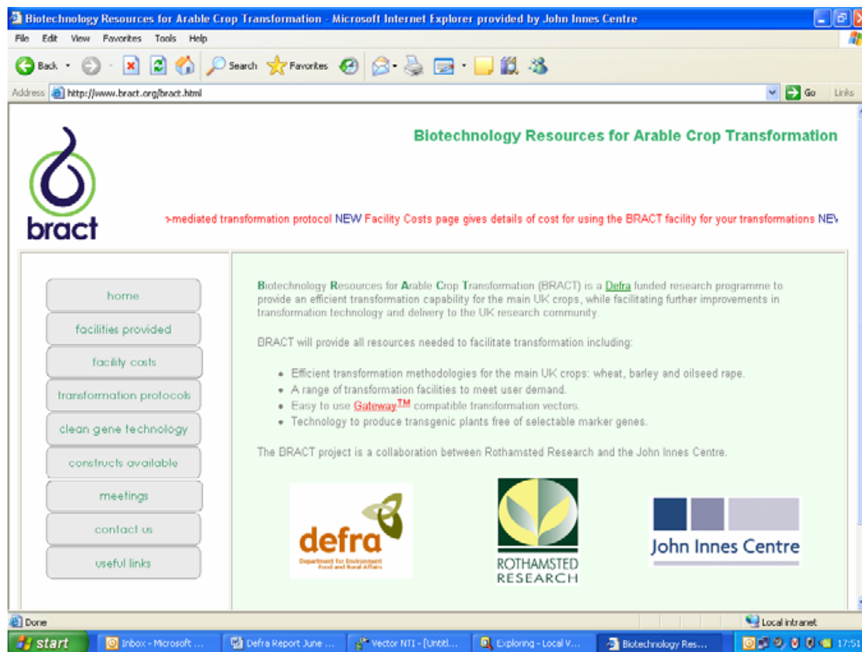
Bourdon V., Wickham A., Lonsdale D., Harwood W A. (2004) Additional introns inserted within the luciferase reporter gene stabilise transgene expression in wheat. Plant Science **167**: 1143-1149

Objective 9 - Website development.

BRACT website (www.bract.org)

The BRACT website was designed to inform the UK research community about the facilities that BRACT provides. Over the course of the three year project it has been updated a number of times to include details of new protocols, transformation picture boards, costs for using our facilities and details of the BRACT workshop.

The numbers of unique visitors to www.bract.org has been monitored since 20th January 2005, and during the last 17 months a total of 2298 unique visitors have been recorded. This equates to an average of 30 visitors per week, or 127 per month. Of the total unique visitors to www.bract.org 46.4% have been from the UK. This shows that the BRACT facilities available to the UK research community are reaching our target audience. One area that has been very important for promoting the work of BRACT has been through weblinks on related websites, such as pGreen, Brassica Info and Grain Genes. 58% of visitors to www.bract.org come via links on other websites and search engines. Google is the most popular search engine, and when used to search for the terms ‘bract’ and ‘crop transformation’ retrieves the BRACT link as the top hit.



The website also includes a facility for interested parties to sign up to updates from the BRACT group and we currently have 67 on our email distribution list, 21 of whom are from the UK. Recently details of all of the new BRACT constructs have been added to the website together with complete construct maps.

Objective 10 – Planning future transformation resources.

One of the key components of objective 10 was to establish a self-supporting transformation facility to meet the future needs of the UK research community. This has been achieved for all three major BRACT crops, wheat, barley and oilseed rape where we are able to offer a range of transformation facilities meeting the communities needs. During the BRACT project various advertising material has been produced to inform the research community of the transformation resources available. At the beginning of the project a logo was designed, and this has been used on our website, business cards and promotional posters. A second part of objective 10 was to organise a workshop on crop transformation. A very successful workshop was held at JIC on April 5th 2006 that brought together many UK researchers interested in crop transformation. Both UK and international speakers allowed the workshop to consider the latest developments in crop transformation technology. All details of the BRACT workshop were advertised at www.bract.org and also through posters which were sent to all UK universities and relevant institutions. A booklet was produced for the workshop containing details of the facilities that BRACT has to offer, together with details of the pBract constructs. The presentations given at the workshop were recorded onto DVD, and copies of these can be found in the John Innes Centre library for loan.

A BRACT database has been developed and this is being used to record all transformation experiments undertaken at JIC. Following some further small modifications, the database will be made available to other research groups producing transgenic plants.

The BRACT logo.

Discussion and Future Work

The BRACT project has addressed the need for focussed provision of transformation resources within key UK crops and has provided a transformation facility for the UK research community. By consulting with the research community, BRACT has ensured that the resources available closely match the needs of the community.

During the course of the project, significant improvements have been made to the protocols for *Agrobacterium*-mediated transformation of wheat, barley and oilseed rape. These improvements mean that the effort needed to produce the required number of transgenic lines containing a construct of interest has been very much reduced making the technology more readily available to the research community. Transformation efficiencies in some crops are approaching the level where the technology could be used to provide a range of functional genomics tools to further aid the determination of gene function. In addition to the improvements in transformation efficiencies, BRACT has provided a set of easy to use constructs that should reduce the time involved in preparation of final constructs containing genes or sequences of interest. BRACT can help with all stages of crop transformation through planning, preparation of constructs, production of transgenic plants to analysis of T_0 and T_1 populations.

During the time-only extension to the BRACT project, the analysis of the experiments to produce clean-gene transformants in barley will be completed. The final construct for silencing experiments in wheat will be completed and where testing of constructs is still in progress, this will also be concluded.

BRACT is already involved in a number of collaborative projects and is providing transformation services on a cost recovery basis. The challenge for BRACT is firstly to retain key staff skilled in crop transformation. This could be done by individuals carrying out BRACT work together with work on other funded projects. The second challenge for BRACT is to remain at the forefront of crop transformation technology and to continue to improve and update the transformation resources offered to respond to changing needs of the research community. BRACT has already established strong links with the research community and is a partner in a number of grant proposals utilising crop transformation. Requirements for crop transformation as an essential tool for determination of gene function are growing and BRACT should be in a strong position to meet this need and therefore to ensure the continuation and further growth of the transformation facility.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

Please see attached list of published material.

SID 5 Section 9

References to published material

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