Inheritance and expression of transgenes in white clover

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Abstract

White clover plants from a range of cultivars can now be routinely transformed with cloned foreign genes. However, before transgenic white clover cultivars can be developed, these inserted genes must be stably inherited and expressed at appropriate levels in progeny. Primary transgenic white clover plants containing a *uidA* (GUS) reporter transgene were outcrossed and the inheritance and expression of the *uidA* gene was examined over two generations, in several different cultivar backgrounds. Both Mendelian inheritance and consistent expression in different genetic backgrounds were obtained from strongly expressing primary transgenic plants. However, primary transgenic plants with weak or variable expression gave non-Mendelian inheritance and inconsistent expression of the transgene in progeny plants. Transgenic BC_1 plants were also intercrossed to produce a segregating F_2 population containing individuals heterozygous or homozygous for the transgene. In these populations heterozygous and homozygous plants had similar levels of *uidA* gene expression. These results indicate that F_2 plants, homozygous for a transgene, might be used to develop a transgenic cultivar. However, both selection of a primary transgenic plant with stable, high level expression of the introduced gene and progeny testing, to determine the influence of genetic background, are prerequisites to such a development.

Keywords: gene expression, inheritance, transgene, *uidA* reporter, white clover

Introduction

Recombinant DNA techniques can now introduce cloned foreign genes into white clover (Voisey et al. 1994). This incorporation of novel genes is being used to develop transgenic white clover cultivars with traits not found within the natural genetic variation of this species.

Ideally an introduced gene should be expressed in every genotype of a transgenic cultivar. This can be achieved by having the transgene in a homozygous state, i.e. where both chromosomes of a homologous pair contain the transgene. This ensures all progeny of the next generation will inherit the transgenic trait. However, when a plant is initially transformed, the transgene is present in a heterozygous form. Since white clover is outcrossing and predominantly self incompatible it is necessary to cross primary transgenic plants to produce an BC_1 population, then intercross BC_1 transgenic plants to produce an F_2 population. The F_2 population will have a highly heterogeneous genetic background and a variable allelic copy number (0 to 2) for the transgene. Therefore, it is essential to establish the effects of both allelic composition at individual loci and differing genetic backgrounds on transgene expression.

Multiple copies of a transgene within a plant can result in increased levels of expression, however, there are also examples where expression is reduced or silenced (Hobbs et al. 1993). It is therefore necessary to check the expression level of each transgene before they are incorporated into a breeding programme.

This paper reports an evaluation of the inheritance and expression of an introduced *uidA* reporter gene in transgenic white clover plants.

Materials and methods

Primary transgenic plants

Primary transgenic white clover plants were as described in Voisey et al. (1994). The transferred DNA (T-DNA) in these plants has a β-glucuronidase *uidA* (GUS) reporter gene and a neomycin phosphotransferase II (*nptII*) gene. Expression of the *nptII* gene confers kanamycin antibiotic resistance and allows preferential selection for transgenic plants. Both genes are driven by a Cauliflower mosaic virus 35S promoter. Antibiotic resistant plantlets obtained were screened for GUS activity and placed in a containment glasshouse. The presence of the *nptII* and *uidA* transgenes was confirmed by Southern analysis (Voisey et al. 1994). A histochemical GUS assay was used to determine the presence of an active *uidA* gene whilst a fluorometric assay was used to quantify its expression (Voisey et al. 1994).

BC_1 population

Five primary transgenic plants (GH7, GH8, GH129, GH177, G195) expressing the *uidA* gene were reciprocally crossed to a non-transgenic control. The BC_1 seed was germinated *in vitro* (Voisey et al. 1994)

and plantlets established in a containment glasshouse. Histochemical and fluorometric assay for GUS activity were performed on fully developed glasshouse grown plants.

BC_2 population

Five BC_1 plants derived from a primary transgenic plant (GH7), with a high level of GUS activity and a single transgene (Voisey et al. 1994), were outcrossed to a second non-transgenic control plant to produce a BC_2 population. The level of GUS activity in both the BC_1 and BC_2 populations was compared.

F_2 population

The primary transgenic plant GH7, was also used to generate a population of individuals (heterozygous or homozygous) for the *uidA* transgene.

As the primary transgenic plant was self-incompatibile, it was crossed with a non-transgenic control. Five of the resulting BC_1 progeny that exhibited high GUS activity were intercrossed to produce a segregating F_2 population. Individual F_2 plants were then screened for the presence of the *uidA* gene using a histochemical assay. The level of GUS activity in *uidA* positive plants was determined using a fluorometric assay.

GUS positive F_2 plants were test crossed with a non-transgenic control plant to determine whether the plant was heterozygous or homozygous for the transgene. Twenty seeds from each cross were germinated *in vitro* on 0.8% agar. After 1 week the root tip of each seedling was used to determine *uidA* gene segregation ratios using the histochemical assay. The shoot tips were placed on CR medium without hormones (White & Voisey, 1994) for subsequent GUS testing after 3–4 weeks in culture.

Statistical analysis

Chi-square tests were carried out to test whether segregation ratios deviated from those expected for Mendelian inheritance of a single dominant gene. Analysis of variances was used to determine statistical differences ($P \leq 0.05$) for *uidA* expression levels in the BC_1, BC_2 and F_2 generations respectively.

Results

BC_1 population inheritance

No differences were found between the reciprocal crosses of transgenic plants and the non-transgenic plants. Hence data from the reciprocal crosses were pooled for further analysis. All 5 primary transgenic plants contained 1 copy of the *uidA* transgene (Southern analysis, data not presented). With Mendelian inheritance, we expected that progeny from the primary transgenics would segregate in a 1:1 ratio for the *uidA* transgene. Results of the segregation analysis using the GUS histochemical assay are presented in Table 1. Only progeny from primary transgenic plants GH195 and GH7 exhibited the expected 1:1 segregation ratio for GUS activity. Progeny from 2 other primary transgenics (GH8 and GH129) contained less than the expected number of GUS positive plants (χ^2 ldf $P<0.05$). Primary transgenic GH177 was unusual in that no GUS positive progeny could be obtained from this plant.

Table 1: Histochemical staining was used to assess segregation of the *uidA* transgene in BC_1 progeny.

Primary transgenic	Progeny positive	Negative	Deviation from 1:1 ratio χ^2 ldf
GH177	0	85	P<0.001
GH8	20	50	P<0.001
GH195	28	23	NS
GH7	28	38	NS
GH129	6	59	P<0.001

A correlation between the level and pattern of GUS activity in the primary transgenic and segregation of 1:1 GUS activity in the progeny was observed. GH7 and GH195 both exhibited strong uniform GUS activity throughout the plant. GUS expression, although strong in some instances, was found to be a lot more variable in GH8 and GH129, while only weak GUS activity was observed in GH177.

Quantification of GUS activity

GUS activity was quantified in the BC_1 populations of 3 primary transgenics, GH7, GH8, GH177 (Figure 1). The GUS activity observed in the BC_1 population reflected that found in the original primary transgenic with the exception of GH177 where no GUS activity was found in any of the progeny. Most of the BC_1 progeny from GH7 exhibited high levels of GUS activity whereas the progeny for GH8 exhibited low activity.

The median values obtained for the BC_1 populations were lower than those of the original primary transgenic, reflecting the considerable variation in GUS activity found within the BC_1 populations. Though individuals were identified with the same mean GUS activity as that of the primary transgenic, others were found with significantly lower GUS activity (Figure 2).

No difference was found in GUS activity (Figure 2) amongst the BC_1, BC_2 and F_2 populations derived from GH7 for *uidA* positive individuals.

F_2 population

The primary transgenic plant used to generate the F_2 population contained 1 copy of the *uidA* transgene.

Figure 1: Quantification of GUS activity in BC, populations of *uidA* positive plants from 3 primary transgenic plants (GHl77, GH8, GH7). The mean value for the primary transgenic parent is marked with a black circle.

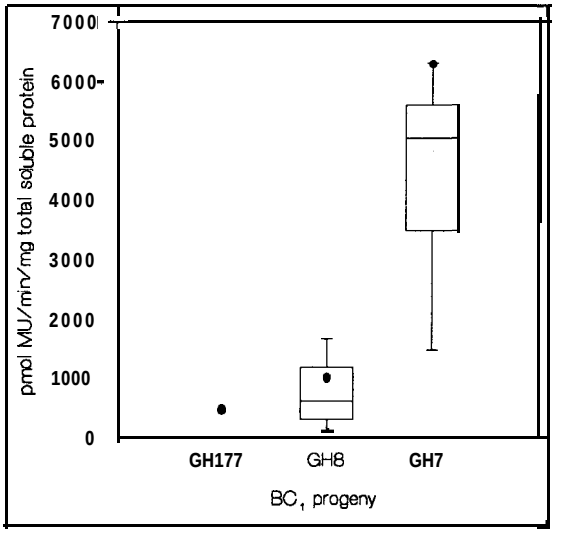
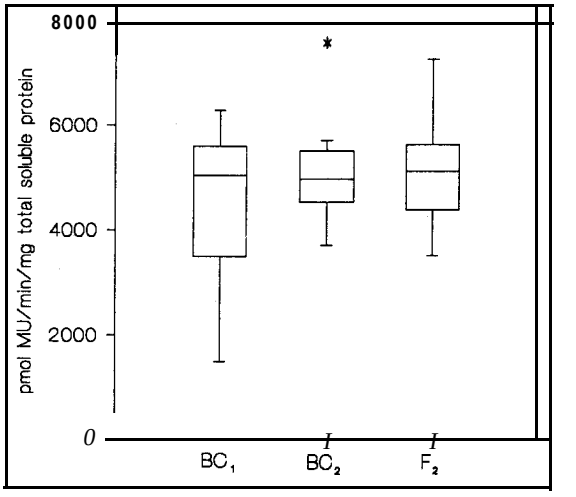


Figure 2: Quantification of GUS activity in 3 populations of *uidA* positive progeny derived from primary transgenic plant GH7.
BC_1 : primary transgenic x non-transgenic plant
BC_2 : BC, x non-transgenic plant
F_2 : BC, x BC,



Therefore we expected that if the gene was inherited in a classical Mendelian manner, ¾ of the progeny in the F_2 population would be either homozygous or heterozygous for the *uidA* transgene.

Individual plants within the F_2 populations were screened for GUS expression (Table 2) and were found to deviate from the expected 3:1 ratio (χ^2 I df $P<0.05$) in that there were significantly less than the expected number of GUS positive plants.

Table 2: Segregation of the *uidA* transgene in a F_2 population derived from GH7, based upon a histochemical assay.

	GUS positive	GUS negative
Observed	48	28
Expected (3:1 ratio)	57	19

Since heterozygous and homozygous plants in the F_2 progeny both exhibit GUS activity, it was necessary to test cross individual plants to a non-transgenic control to identify the two groups. In the heterozygous group, the transgene occurs in only 1 of the 2 chromosomes in a homologous pair. Therefore the *uidA* gene is transmitted to only half of the progeny whereas in a homozygous plant all the progeny would inherit the transgenic trait.

Thirty-one GUS positive F_2 plants were progeny tested. F_2 plants were classed as homozygous for the *uidA* transgene if all 20 progeny exhibited GUS activity in a histochemical assay. Those F_2 plants with some GUS negative progeny were classified as heterozygotes. Results of the segregation analysis for the 31 GUS positive F_2 plants tested are illustrated in Table 3. Six homozygous plants were identified. The ratio of homozygote to heterozygote plants (1:4) was less than the expected 1:2 (χ^2 0.10 > P > 0.05), especially for crosses dlxd2 and cl xc3.

Table 3: Segregation of homozygous and heterozygous plants in a F_2 population derived from GH7, based upon a histochemical assay.

Cross	Homozygous plants	Heterozygous plants	Total
d1 x d2	2	10	12
cl xc3	1	11	12
al x dl	3	4	7
Total observed	6	25	31
Expected (1:2 ratio)	10.3	20.7	31

GUS activity within the F_2 population

GUS expression for known heterozygous and homozygous individuals was quantified using a fluorometric assay. The mean GUS activity for the homozygous population though slightly lower than that of' the heterozygous population, was not significantly different (Figure 3).

Discussion

We have demonstrated that the *uidA* transgene can be stably transmitted to white clover progeny in a Mendelian manner. However, non-Mendelian inheritance was also observed in the BC, progeny of some primary transgenics. This is not unusual and has been observed by others working with transgenic plants (Deroles & Gardner 1988; Peng et al. 1995).

Variation was also noted in expression of the *uidA* gene within BC, populations. This variation and some cases of non Mendelian inheritance, emphasise the need to select high expressing individuals before proceeding to a breeding programme.

The level of GUS expression in the BC, and BC, populations was similar to that of the primary transgenic parent and Mendelian inheritance was observed in progeny from primary transgenic plants with strong uniform GUS activity. Thus it is possible to identify primary transgenic plants whose progeny will also inherit and exhibit good expression of a transgene. Deroles and Gardner (1988) have also noted in petunia plants that a strongly expressed kanamycin gene was usually transmitted in a Mendklian fashion to progeny, whereas poorly expressing transgenic plants tended to exhibit reduced transmission ratios.

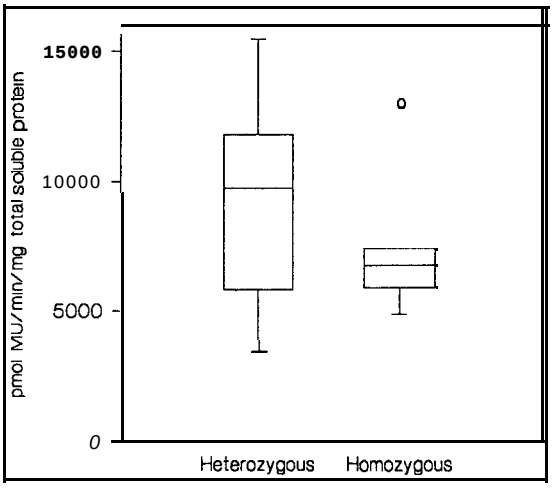
In the F_2 population the number of homozygous plants expressing the *uidA* gene was under-represented. This is not uncommon since many workers have demonstrated that although a plant may inherit a transgene it does not necessarily mean that the gene will be expressed (Lambe et al. 199.5).

In this study the heterozygous plants contain 2 copies of the CaMV 35S promoter; 1 to drive the *uidA* transgene and the other to drive the antibiotic resistance gene. In creating a homozygous transgenic plant the number of transgenes is doubled, so that a homozygous plant contains 4 copies of the CaMV 35S promoter. Because multiple copies of a gene are more prone to genetic inactivation than single copies (Hobbs et al. 1993), the apparent lack of transgenic plants in the homozygous class may be due to transgene silencing.

Despite the presence of 4 copies of the CaMV 35S promoter, we have demonstrated it is still possible to obtain homozygous GUS positive plants that express at a similar level to that of heterozygous plants. Peng et al. (1995) also found no difference in GUS activity between transgenic homozygous and heterozygous rice plants, whereas others (Hobbs et al. 1993; Last & Gray, 1990; Dean et al. 1988) have found increased activity of a single copy transgene in a homozygous state.

The ability to select high expressing transgenic plants homozygous for an introduced gene is a considerable advantage in breeding programmes. In a homozygous plant the transgene is fixed so that all progeny from this plant inherit the transgene (Woodfield et al. 1996).

Figure 3: Comparison of GUS activity in heterozygous vs. homozygous plants expressing the *uidA* transgene within the GH7 derived F_2 population.



It is therefore possible to intercross a primary transgenic plant to the parent plants or a range of plants of an existing white clover cultivar and select for high expressing homozygous transgenic plants from each line. These individuals can then be intercrossed so that the transgenic trait can be fixed within the cultivar. The new cultivar would still retain the high degree of genetic diversity present in the original cultivar.

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