High-Efficiency Stable Transformation of the Model Fern Species *Ceratopteris richardii* via Microparticle Bombardment[1][W][OPEN]

Andrew R.G. Plackett, Liandong Huang, Heather L. Sanders[2], and Jane A. Langdale*

Department of Plant Sciences, University of Oxford, Oxford OX1 3RB, United Kingdom

Ferns represent the most closely related extant lineage to seed plants. The aquatic fern *Ceratopteris richardii* has been subject to research for a considerable period of time, but analyses of the genetic programs underpinning developmental processes have been hampered by a large genome size, a lack of available mutants, and an inability to create stable transgenic lines. In this paper, we report a protocol for efficient stable genetic transformation of *C. richardii* and a closely related species *Ceratopteris thalictroides* using microparticle bombardment. Indeterminate callus was generated and maintained from the sporophytes of both species using cytokinin treatment. In proof-of-principle experiments, a *35S::β-glucuronidase* (*GUS*) expression cassette was introduced into callus cells via tungsten microparticles, and stable transformants were selected via a linked hygromycin B resistance marker. The presence of the transgene in regenerated plants and in subsequent generations was validated using DNA-blot analysis, reverse transcription-polymerase chain reaction, and GUS staining. GUS staining patterns in most vegetative tissues corresponded with constitutive gene expression. The protocol described in this paper yields transformation efficiencies far greater than those previously published and represents a significant step toward the establishment of a tractable fern genetic model.

Ferns represent an underinvestigated group compared with many other taxa of land plants. Ferns and horsetails together comprise the monilophytes, which diversified from the seed plant (spermatophyte) lineage approximately 400 million years ago (Pryer et al., 2001). As such, monilophytes represent the closest extant sister group to seed plants. Comparisons between ferns and seed plants should thus provide important insights into the developmental mechanisms present in the ancestral tracheophyte from which both taxa derive and also elucidate subsequent evolutionary trajectories.

The most extensively studied fern species is *Ceratopteris richardii*, a homosporous fern increasingly viewed as a viable experimental model (Hickok et al., 1995; Chatterjee and Roux, 2000; Leroux et al., 2013). The *C. richardii* lifecycle comprises gametophyte and sporophyte stages that are capable of growing independently of one another. Dispersal is via haploid spores, which germinate to form thalloid gametophytes. Gametophytes develop into either chordate hermaphrodites, characterized by the presence of a lateral meristem (Banks, 1999), or, in the presence of a hermaphrodite-secreted antheridiogen, males (Banks, 1997). Sexual reproduction in this species requires the presence of water and occurs through fusion of retained egg cells and motile sperm. The resultant diploid embryo develops within the gametophyte archegonium (Johnson and Renzaglia, 2008). Subsequent growth of the sporophyte occurs indeterminately through divisions of a tetrahedral shoot apical cell (Hou and Hill, 2002), the products of which establish both frond primordia and a shoot-derived root system, each with their own associated apical cells (Hou and Hill, 2004). Frond and root development are both heteroblastic in nature, in that the morphology of newly arising organs alters with the age of the sporophyte (Hou and Hill, 2002). Ultimately, haploid spores are generated on the lower lamina surface of reproductive fronds. The spore-to-spore lifecycle takes an average of 22 weeks.

The establishment of a fern genetic model has been hindered by a number of technical factors, not least of which are large haploid genomes (Bennett and Leitch, 2001) that in the absence of a pressing incentive remain uneconomical to sequence. For example, *C. richardii* is estimated to have a haploid genome size of approximately 11.3 Gbp (Nakazato et al., 2006). The greatest impediment to detailed genetic analysis, however, is an inability to efficiently transform ferns. Transient transformation of fern gametophyte prothallus cells, typically for RNA interference, has been previously demonstrated through either direct DNA uptake by germinating *C. richardii* spores (Stout et al., 2003) or direct microparticle bombardment in *Adiantum capillus-veneris* (Kawai-Toyooka et al., 2004), *C. richardii* (Rutherford et al., 2004), and *Pteris vittata* (Indriolo et al., 2010). Evidence of stable transmission to the subsequent sporophyte generation was reported, but where quantified (Rutherford et al., 2004), transmission through self-fertilization

[1] This work was supported by an European Research Council Advanced Investigator Grant "Evolution and Development in Plants."

[2] Present address: Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, UK.

* Address correspondence to jane.langdale@plants.ox.ac.uk.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Jane A. Langdale (jane.langdale@plants.ox.ac.uk).

[W] The online version of this article contains Web-only data.

[OPEN] Articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.113.231357

was very low (7%), and a significant proportion of transmitted events ultimately reverted to a nonsilenced phenotype (32%). Transmission to subsequent generations was not determined. A recently published protocol utilizing *Agrobacterium tumefaciens*-mediated transformation of spores reported stable transformation in two fern species, *P. vittata* and *Ceratopteris thalictroides* (Muthukumar et al., 2013), but the very low transformation efficiencies achieved (0.053% and 0.03%, respectively) preclude routine adoption of this approach.

In this paper, we demonstrate the genetic transformation of both *C. richardii* and *C. thalictroides* using microparticle bombardment of callus tissue and hygromycin selection of regenerating transformed plants. Transgenes were stably inherited in subsequent generations. With transformation efficiencies of 72% (*C. richardii*) and 86% (*C. thalictroides*), this technical advance positions *C. richardii* as a tractable genetic model for the analysis of gene function in ferns.

RESULTS

Induction of Callus Tissue from Fern Sporophytes with Cytokinin

Callus tissue has been generated from numerous angiosperm species and maintained in an undifferentiated state by treatment with the phytohormones auxin and cytokinin (CK; Ikeuchi et al., 2013). A similar approach was attempted in both *C. richardii* and *C. thalictroides*, first by treating gametophytes with either CK or auxin. Although gametophyte development was visibly altered, neither hormone treatment induced callus formation in either species (Supplemental Fig. S1). To determine the effect on sporophyte development, 11-d-old *C. richardii* sporophytes (Fig. 1A) were incubated on Murashige and Skoog (MS) media containing CK or auxin analogs. After 14 d, new fronds and roots had emerged on untreated sporophytes (Fig. 1B), whereas sporophytes treated with the auxin analog 1-napthaleneacetic acid (NAA) produced new fronds, but new roots were replaced by disorganized, callus-like tissue (Fig. 1C). Treatment with a second auxin analog, indole-3-butyric acid (IBA), did not dramatically perturb shoot or root development (Fig. 1D); however, the tip of the embryonic root remained green for longer than in control or other hormone-treated sporophytes. In contrast to the root-specific effects of auxin, treatment with two separate CKs, benzylaminopurine (BAP) and kinetin (KT), prevented the production of both fronds and roots on growing sporophytes. Instead, undifferentiated callus tissue was visible at the shoot apex (Fig. 1, E and F) and, occasionally, the root apex (Fig. 1E). CK treatment of *C. thalictroides* sporophytes also successfully induced callus at the shoot apex (Supplemental Fig. S2).

When grown on MS media without further hormone treatment, shoot-derived *C. richardii* callus completely differentiated into new shoots and roots within 4 weeks (Fig. 1, G and H). Callus treated with auxin analogs also

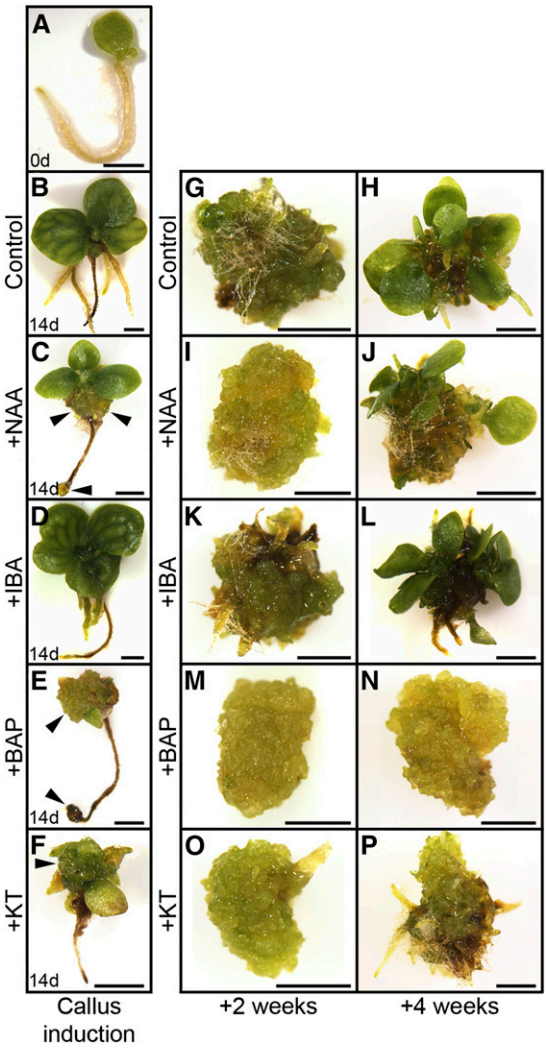

Figure 1. Induction and maintenance of *C. richardii* callus on CK. A to F, Eleven-day-old sporophytes (A) were grown for 14 d on media containing distilled water mock treatment (B), 5 μM NAA (C), 5 μM IBA (D), 5 μM BAP (E), or 5 μM KT (F). Callus was induced at the shoot apex by BAP and KT treatments (E and F; black arrowheads) and at the root apex by NAA and BAP treatments (C and E; black arrowheads). G and H, BAP-induced shoot callus entirely differentiates into shoots after 4 weeks without hormone treatment. I to L, Auxin treatment of callus tissue. Callus tissue differentiates into shoots when incubated with either 5 μM NAA (I and J) or 5 μM IBA (K and L). M to P, CK treatment of callus tissue. Incubation with 5 μM BAP prevents tissue differentiation (M and N), whereas incubation with 5 μM KT delays differentiation (O and P). Bars = 2 mm.

regenerated into new shoots over the same period (Fig. 1, I–L), with differentiation slightly delayed under NAA treatment (Fig. 1, I and J). By contrast, continued incubation on BAP maintained callus tissue in an apparently undifferentiated state (Fig. 1, M and N). Treatment with KT, although partially successful in preventing cell differentiation, was less effective (Fig. 1, O and P). Combined treatment with BAP plus NAA resulted in reduced callus growth, whereas BAP and BAP plus IBA treatments were essentially indistinguishable, both

yielding highly friable callus (Supplemental Fig. S3). Fern callus can thus be maintained on BAP or on a combination of BAP plus IBA. To determine callus longevity, both *C. richardii* and *C. thalictroides* calli were repeatedly subcultured on successive BAP plus IBA treatments at 14-d intervals. Indeterminate cell fate was successfully maintained in this manner for over a year.

High-Efficiency Transformation of *C. richardii* and *C. thalictroides* Using Microparticle Bombardment and Hygromycin Selection

Callus transformation was carried out using microparticle bombardment with a hygromycin-selectable *35S*::*GUS* construct (pCAMBIA1305.2; see "Materials and Methods"). To first determine the effectiveness of hygromycin as a selection agent, untransformed *C. richardii* callus was subjected to a range of antibiotic concentrations across different timeframes. A 2-week incubation period on 40 μg mL^{-1} hygromycin B was sufficient to prevent regeneration of shoots from untransformed callus (Supplemental Fig. S4) and was therefore used in all subsequent callus selection assays. Test bombardments with either the *35S*::*GUS* construct or with uncoated control microparticles were performed on both *C. richardii* and *C. thalictroides* callus. Callus was incubated on MS media containing CK (5 μM KT) during and following bombardment to prevent premature tissue differentiation. Callus was transferred to this media 2 d prior to bombardment and left on the same media after bombardment for a 3-d recovery period without antibiotic selection. After that time, GUS staining analysis was performed on samples of bombarded *C. richardii* callus tissue. Figure 2A shows that callus bombarded with the *35S*::*GUS* construct exhibited numerous spots of GUS staining, whereas callus bombarded with uncoated microparticles showed none (Fig. 2B). Multiple transformation events had thus taken place.

After the 3-d recovery period, the remaining callus was transferred to antibiotic selection media, and a week later, GUS assays were repeated. Once more, spots of GUS expression were visible within the population of *35S*::*GUS*-bombarded calli (Fig. 2C) but not on control calli (Fig. 2D). The frequency of spots visible on callus under selection was visibly reduced compared with callus stained immediately after bombardment (compare Fig. 2, A and C). This difference most likely reflects stable versus transient transformation events. Antibiotic selection was maintained for 14 d, after which time, callus was transferred to nonselective MS media to regenerate. At this stage, CK treatment was stopped. After a further 7 d, small protruding regions of green tissue were visible on *35S*::*GUS*-bombarded callus (Fig. 2E), whereas control callus had turned dark brown and stopped growing (Fig. 2F). Regenerating tissue subsequently went on to differentiate discrete organs, and continued GUS expression in these regenerating tissues was confirmed by GUS staining (Fig. 2G). Eight

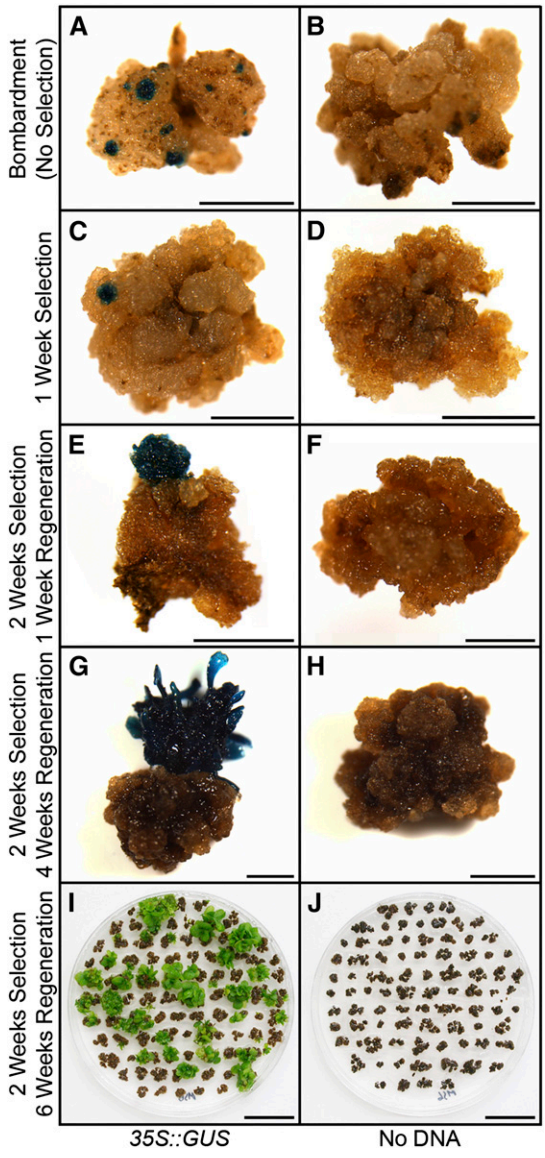

Figure 2. Transformation, selection, and regeneration of *C. richardii* callus. A to H, GUS analysis of regenerating callus tissue bombarded with *35S*::*GUS* (A, C, E, and G) or uncoated microparticles (B, D, F, and H) before (A and B), during (C and D), and after (E–H) antibiotic selection. Bars = 1 mm. I and J, Regenerating *C. richardii* T0 shoots 8 weeks after bombardment with *35S*::*GUS* (I) or uncoated microparticles (J). Bars = 20 mm.

weeks after bombardment, regenerated sporophyte shoots had successfully established an indeterminate growth pattern (Fig. 2I), with most individual calli regenerating more than one shoot.

To assess transformation and regeneration frequencies, 18 bombardments were performed on *C. richardii* callus over a period of 10 weeks, with each replicate comprising 45 to 60 calli. On average, 81.21% ± 2.45% of calli from each bombardment regenerated at least one shoot after selection, whereas corresponding control calli showed no regeneration (Table I). Of these regenerating calli, 88.06% ± 1.60% exhibited GUS expression when

stained, resulting in a final transformation efficiency of 71.58% ± 2.56%. A parallel experiment using *C. thalictroides* yielded efficiencies of 96.76% ± 0.82%, 88.58% ± 1.18%, and 85.79% ± 1.62%, respectively.

Once transplanted to soil, maturation of transgenic *C. richardii* sporophytes took 10 to 14 weeks (harvesting of first spores to harvesting of final spores), with a total minimum regeneration period of 18 weeks from bombardment to harvesting of earliest T1 spores (compared with 22 weeks spore to spore for untransformed plants). In comparison, maturation of *C. thalictroides* after tissue regeneration took 8 to 10 weeks, the total minimum regeneration period of 16 weeks being slightly longer than the lifecycle of untransformed plants (11 weeks).

Chimeric Transgene Expression in T0 Transformants

GUS staining of newly regenerated T0 *C. richardii* shoots revealed two types of expression pattern: expression in all shoot tissues (Fig. 3, A–C) or expression specifically in vasculature (Fig. 3, D and E). Across three independent bombardments, these occurred at mean frequencies of 32.60% ± 4.22% (all tissues) and 67.40% ± 4.22% (vasculature only) of GUS-expressing shoot clusters. There were no instances of these two expression patterns coexisting within the same shoot cluster. The same patterns of GUS expression were observed in regenerated T0 *C. thalictroides* shoots (Supplemental Fig. S5) at mean frequencies of 46.95% ± 7.95% and 53.05% ± 7.95%, respectively. Regenerated shoots were transplanted to soil and allowed to mature, with final T0 population sizes of 28 (*C. richardii*) and 43 (*C. thalictroides*) individuals. GUS staining of sporogenous frond tissue found the same two patterns in the sporophytes of both species (Fig. 3, F–L; Supplemental Fig. S5) at frequencies of 28.57% and 55.81% (whole-tissue staining, including staining of sporangia; Fig. 3, G, H, and K) and 42.86% and 18.60% (vasculature staining; Fig. 3L) of the *C. richardii* and *C. thalictroides* populations, respectively. The persistence of these two staining patterns suggests that expression patterns established in newly regenerated shoots are maintained through subsequent development.

Not all of the T0 shoots that regenerated after hygromycin selection displayed GUS staining. The extent of GUS expression in regenerating shoots also varied, from staining across entire shoot clusters originating from a single callus (Fig. 3A) to staining of individual shoots within a shoot cluster (Fig. 3B) and staining of sectors within a single shoot or frond (Fig. 3C). Staining across entire shoot clusters was observed at mean frequencies of 18.93% ± 2.92% (whole-tissue staining) and 45.54% ± 2.91% (vasculature only) of GUS-staining shoots in *C. richardii* and 25.20% ± 6.37% and 34.78% ± 6.02% in *C. thalictroides*, respectively. A proportion (11.94% ± 1.60% and 11.42% ± 1.18%) of the regenerated *C. richardii* and *C. thalictroides* populations displayed no GUS staining at all.

The occasional noncoincidence of GUS staining and hygromycin resistance was further observed in mature T0 sporophytes: 28.57% (*C. richardii*) and 25.58% (*C. thalictroides*) of mature individuals sampled within the regenerated T0 populations showed no GUS staining. Transfer DNA (T-DNA) expression was analyzed in greater detail in six T0 *C. richardii* transformants through reverse transcription (RT)-PCR (Fig. 3). Of these six, only two (plants 2 and 10) showed GUS staining (Fig. 3, G and H) in conjunction with amplification of both *35S::GUS* and hygromycin B resistance (Hyg^R) gene products (Fig. 3, M and N). Two further individuals (plants 16 and 17) showed no evidence of transgene expression except hygromycin resistance during tissue regeneration, and the remaining two (plants 18 and 23) each produced conflicting results: plant 18 was positive for GUS staining but negative for both *35S::GUS* and Hyg^R amplification, whereas plant 23 was positive for GUS staining and Hyg^R amplification but negative for GUS amplification. Different frond tissues were necessarily sampled for GUS and RT-PCR assays, which might explain these discrepancies (see "Discussion").

Transgene Inheritance and Stable Expression in T1 Transformants

To assess transgene inheritance, T1 spores that were harvested from 25 *C. richardii* T0 individuals were screened for hygromycin resistance (Fig. 4). The possibility that T0 transformants are chimeric necessarily creates the hypothesis that T1 progeny will comprise a mix of transformed and untransformed individuals, requiring an efficient method to identify transgenics. Empirical testing determined that 20 μg mL^{-1} hygromycin is sufficient to kill untransformed, germinating *C. richardii* spores (Fig. 4, A and B; Supplemental Fig. S4) and untransformed sporophytes (Supplemental Fig. S4).

Table I. *Estimated transformation efficiency after microparticle bombardment of C. richardii and C. thalictroides callus*

Values shown are the means of 18 independent bombardments of each species. All values are expressed as percentages of the population of callus bombarded ± SE. Regeneration efficiency refers to the number of separate calli bombarded that regenerated at least one shoot following hygromycin selection. Final transformation efficiency refers to the number of separate calli regenerating at least one shoot that also displayed GUS staining.

Species	Mean Regeneration Efficiency	Mean Final Transformation Efficiency
	% callus	
C. richardii	81.21 ± 2.45	71.58 ± 2.56
C. thalictroides	96.76 ± 0.82	85.79 ± 1.62

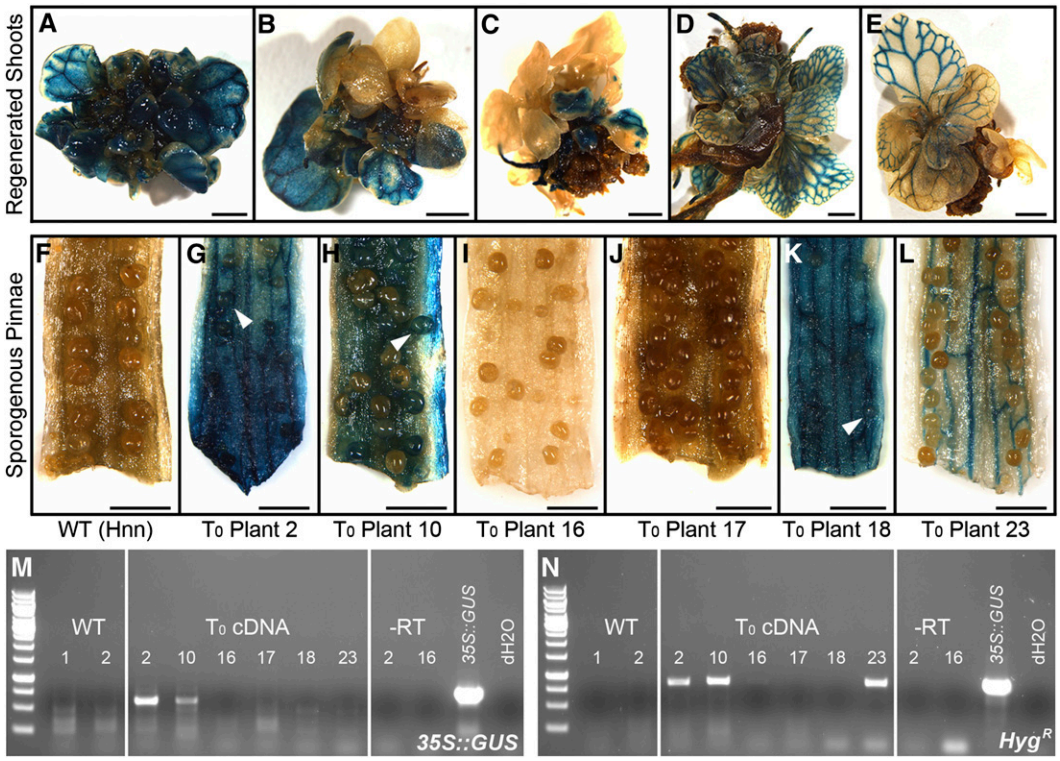

Figure 3. Chimeric transgene expression in *C. richardii* T0 transformants. A to E, Typical GUS staining patterns of T0 regenerated shoots 8 weeks after bombardment, staining either whole tissues (A–C) or restricted to the vasculature (D and E). Bars = 2 mm. F to L, GUS-stained sporogenous pinnae from untransformed (F) and mature regenerated T0 sporophytes (G–L). White arrowheads indicate GUS-stained sporangia containing T1 spores. Bars = 1 mm. M and N, RT-PCR analysis of *35S::GUS* (M) and Hyg^R (N) expression in T0 transformants depicted in G to L. RNA was extracted from sporogenous pinnae.

Under this selection regime, spores harvested from 18 T0 individuals (72%) produced hygromycin-resistant gametophytes. Approximately 500 to 1,000 spores from each line were screened, and compared with unselected controls, the estimated frequency of resistant individuals ranged from 1% (T1 line 2; Fig. 4, D and E) to up to 85% (T1 lines 10 and 17; Fig. 4, G, H, J, and K). In *C. thalictroides*, under similar screening conditions, 94% of lines produced resistant individuals, with frequencies within each line over a similar range (Supplemental Fig. S6). GUS staining of hygromycin-resistant T1 individuals revealed that gametophytes in 90% (*C. richardii*) and 68% (*C. thalictroides*) of transgenic lines also expressed *GUS*. Hygromycin selection of T1 germinating spores is thus a highly efficient method for identifying lines that carry intact transgenes.

Differences in *GUS* expression were occasionally found between the gametophyte and sporophyte stages of some T1 lines, in that constitutive expression was not seen in transgenic gametophytes but was seen in sporophytes. No variation in expression pattern was observed between individual resistant gametophytes within a line. Seventy percent of *C. richardii* T1 lines displayed GUS expression in all gametophyte tissues, including lines 2 (Fig. 4F), 10 (Fig. 4I), and 17 (Fig. 4L), the latter displaying no GUS expression in the T0 parent (Fig. 3, J and M). Two lines (16 and 18), which had low frequencies of resistant gametophytes (Fig. 4, M, N, P, and Q), showed GUS expression only in basal thallus tissues and rhizoids (Fig. 4, O and R), again inconsistent with the expression observed in the T0 parents (Fig. 3, I and K). A single T1 line (23) displayed no GUS expression within the gametophyte despite a high frequency of hygromycin resistance (Fig. 4, S–U). The absence of GUS expression from the gametophyte persisted in four out of five T_2 lines descended from this line (Supplemental Fig. S7), whereas sporophytic GUS expression was observed in all five. This absence of GUS expression in the gametophyte suggests that transgene expression might be influenced by surrounding genomic sequence, i.e. the transgene might be located near an element that represses expression in the gametophyte. The occurrence of GUS expression in the gametophyte stage of one descendent T_2 line (Supplemental Fig. S7) might therefore have occurred through genomic recombination. In *C. thalictroides* gametophytes, all GUS staining was constitutive where present, but nine lines (32.26%) lacked GUS staining despite being hygromycin resistant (Supplemental Fig. S6).

In contrast to the variable staining patterns observed in T1 gametophytes, GUS staining of *C. richardii* T1 sporophytes revealed constitutive expression in all lines tested (Fig. 5, A–E; Supplemental Fig. S8), with the exception of line 23 (Fig. 5F), where GUS expression (and hygromycin

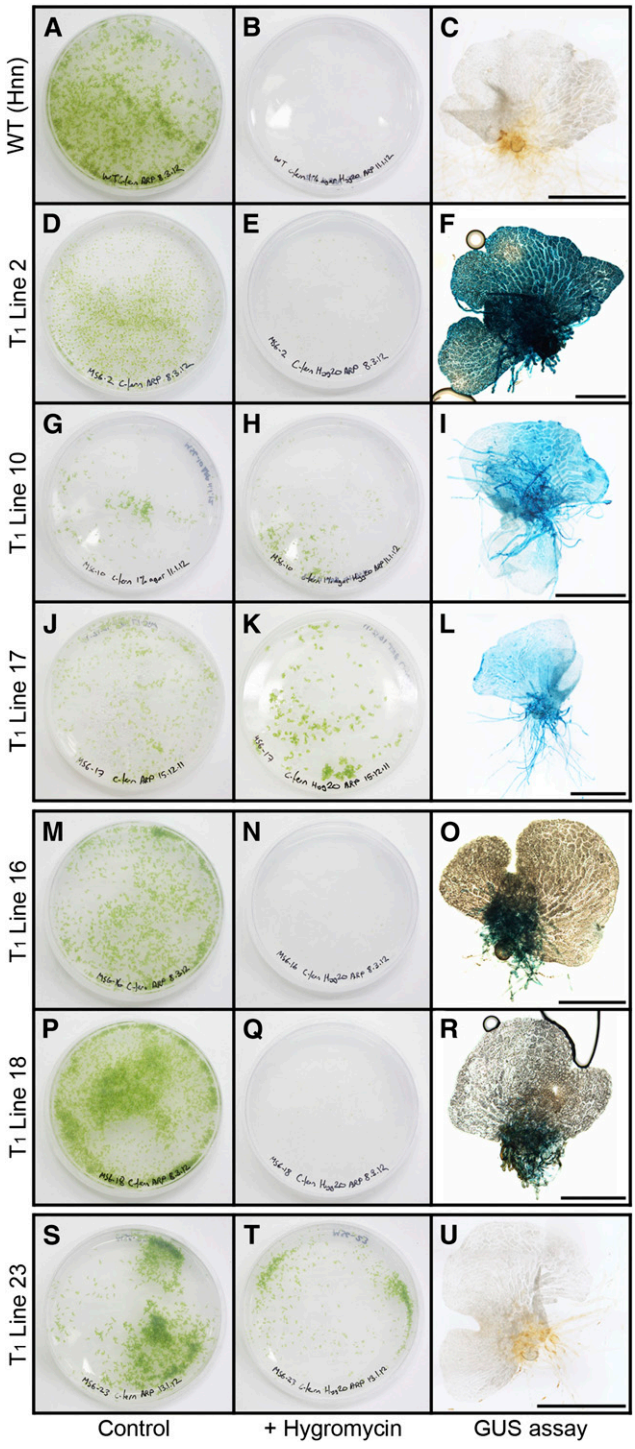

Figure 4. Hygromycin resistance and GUS expression are maintained in *C. richardii* T1 gametophytes. Antibiotic resistance screening and GUS analysis of untransformed gametophytes (A–C) and T1 progeny of T0 transformants (D–U). Growth was compared between control media (A, D, G, J, M, P, and S) and media containing 20 μg mL^{-1} hygromycin (B, E, H, K, N, Q, and T), sufficient to kill wild-type (WT) gametophytes at spore germination (B; Supplemental Fig. S4). GUS expression was compared between untransformed gametophytes (C) and T1 gametophytes resistant to hygromycin (F, I, L, O, R, and U). Bars = 200 μm.

resistance) was initially lower than in other lines. Notably, vasculature-specific GUS expression was not observed in the mature sporophyte tissues of any line, even when present in the T0 parent (Fig. 3E; Supplemental Fig. S8). RT-PCR analysis later in sporophyte development confirmed stable *35S*::*GUS* (Fig. 5G; Supplemental Fig. S8) and *Hyg*^{R} (Fig. 5H; Supplemental Fig. S8) expression in all individuals tested, including those in *C. richardii* line 23. Constitutive GUS expression in all sporophytes was subsequently confirmed by GUS staining of vegetative (Fig. 5, I–M; Supplemental Fig. S8) and reproductive fronds (Fig. 5, N–W; Supplemental Fig. S8).

DNA-Blot Analysis of Transformed *C. richardii* Lines

To determine transgene copy number in transformed *C. richardii* T0 plants, genomic DNA (gDNA) blots were hybridized to fragments of both the *Hyg*^{R} and *GUS* genes (Fig. 6A). Figure 6, B and C, show that the fragments hybridized to multiple copies of each transgene in all T0 transformants tested. The fewest insertions were found in T0 plant 2, with two *Hyg*^{R} and two *GUS* fragments hybridized. The remaining individuals all contained in excess of eight copies of each transgene. Hybridization patterns in individuals 10, 16, 17, and 18 were very similar, raising the possibility that these lines were derived from a single transformation event. Importantly, in each T0 individual, both *Hyg*^{R} and *GUS* probes hybridized to genomic fragments greater than the size of the introduced plasmid (11.9 kb; Fig. 6A), with at least one instance per individual of a hybridized fragment being shared between the two probes (Supplemental Fig. S9), supporting linked insertion of the *Hyg*^{R} and *GUS* genes.

To assess the inheritance of transgene insertions, DNA-blot analysis was performed on T1 progeny from four of the T0 transformants analyzed above. Similar numbers of hybridized fragments were identified in T1 individuals (Fig. 6, D and E) as in the T0 parents (Fig. 6, B and C). Of the two progeny tested from line 2, one (plant 2) demonstrated a hybridization pattern very similar to the T0 parent for both *Hyg*^{R} (Fig. 6, B and D) and GUS (Fig. 6, C and E) probes, but the second (plant 1) had apparently lost the insertion carrying the linked transgenes (Supplemental Fig. S9). The T1 individuals tested from other lines also demonstrated very similar hybridization patterns to their T0 parents, including the presence of linked *35S*::*GUS* and *Hyg*^{R} cassettes (Supplemental Fig. S9). Transgene insertions thus remained stably integrated within the *C. richardii* genome between the T0 and T1 generations, and linkage was maintained through meiosis.

DISCUSSION

Stable transformation of *C. richardii* and its sister species, *C. thalictroides*, has been achieved using a combination of tissue culture, microparticle bombardment, and antibiotic selection. Callus was initiated from the apical region of

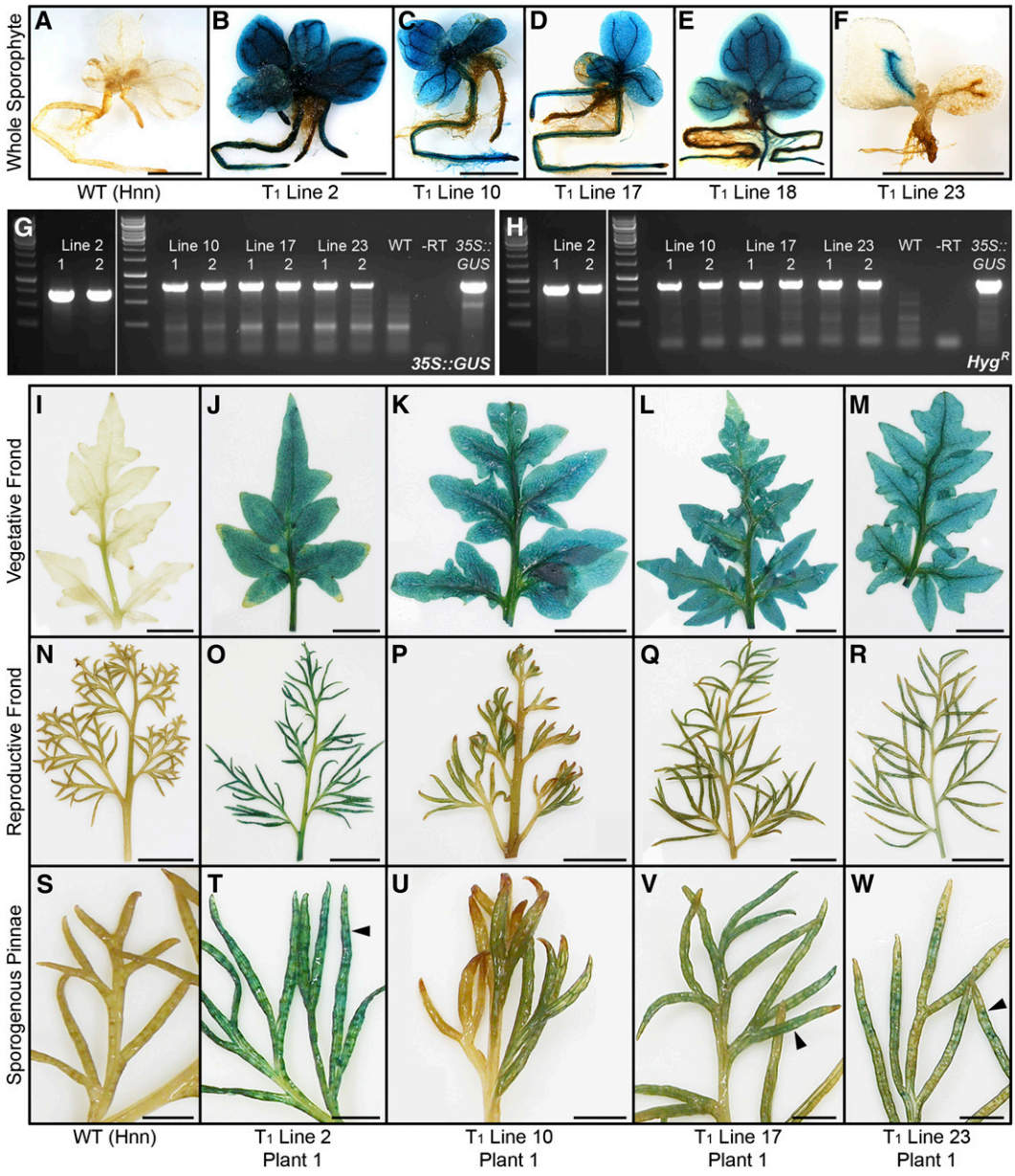

Figure 5. Constitutive transgene expression in *C. richardii* T1 sporophytes. A to F, GUS analysis of whole sporophytes 28 d after gametophyte fertilization, comparing untransformed (A) against individual transgenics from separate T1 lines (B–F). Bars = 2 mm. G and H, RT-PCR analysis of *35S*::*GUS* (G) and *Hyg*^{R} (H) expression in individual sporophytes from T1 lines depicted in B to F. RNA was extracted from vegetative frond tissue. I to W, GUS expression in vegetative (I–M) and reproductive fronds (N–W) of untransformed (I, N, and S) and individual sporophytes from T1 lines (J–M, O–R, and S–W). Black arrowheads indicate GUS-stained sporangia containing T_2 spores. Partial staining of regions within reproductive fronds probably reflect limitations in GUS substrate access or potentially regions of frond senescence prior to GUS staining. Bars = 20 mm (I–R) and 5 mm (S–W).

developing sporophytes of both species by application of the phytohormone CK and was maintained in an indeterminate state in vitro under the same treatment (Fig. 1; Supplemental Figs. S2 and S3). A *35S*::*GUS* cassette linked to a *Hyg*^{R} selectable marker cassette was introduced into callus cells by microparticle bombardment, and the regeneration of transformant sporophytes was successfully selected through hygromycin selection in tissue culture (Figs. 2 and 3). GUS expression was detected in bombarded *C. richardii* calli before (transient expression), during, and after (stable expression) antibiotic selection. The expression of the binary cassette in regenerated *C. richardii* T0 tissues was confirmed by RT-PCR and GUS staining (Figs. 3–5). *35S*::*GUS* was successfully transmitted through the gametophyte and sporophyte stages of the T1 generation of both species

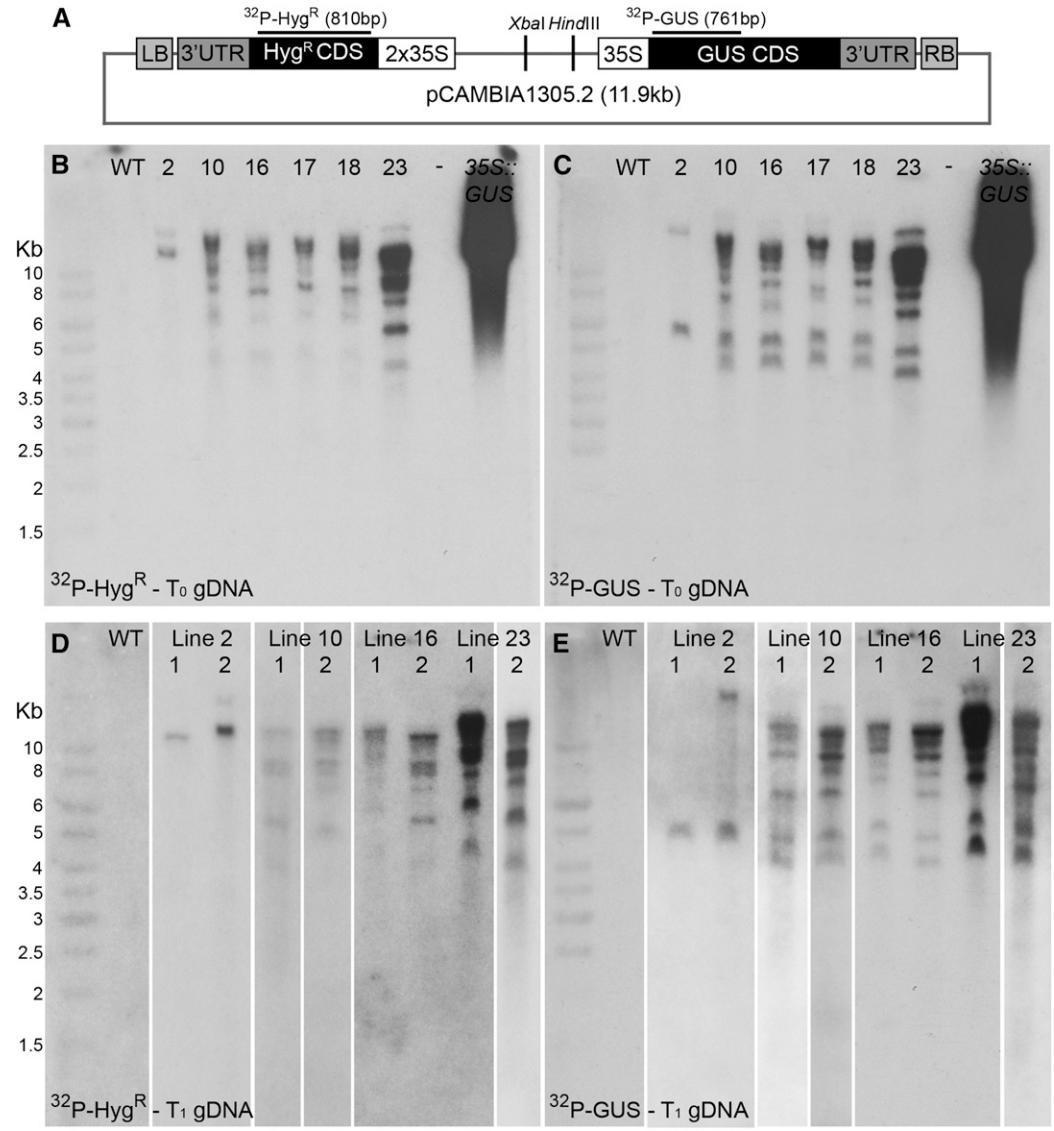

Figure 6. Transgene copy number in *C. richardii* transgenic lines. A, Map of pCAMBIA1305.2 (see "Materials and Methods"). Fragments used for hybridization to *Hyg*^{R} and *35S*::*GUS* are indicated, and relevant restriction enzyme sites are shown. B and C, DNA blots of T0 transformants, hybridized with *Hyg*^{R} (B) and *35S*::*GUS* (C) fragments. D and E, Composite DNA blots of individual T1 sporophytes, hybridized with *Hyg*^{R} (D) and *35S*::*GUS* (E) fragments. All lanes are scaled against the 1-kb ladder shown.

(Figs. 4 and 5), suggesting stable integration into the *Ceratopteris* spp. genomes. DNA-blot analysis of individual *C. richardii* T0 sporophytes and T1 progeny confirmed inheritance of the two cassettes, with multiple insertion events present in each of the lines examined (Fig. 6). This approach achieved transformation efficiencies of 72% for *C. richardii* and 86% for *C. thalictroides*.

Although past attempts to transform *C. richardii* via *A. tumefaciens* reportedly failed (Hickok et al., 1987), a protocol for successful *A. tumefaciens*-mediated transformation of *C. thalictroides* has recently been published (Muthukumar et al., 2013), with transformation achieved through infection of geminating spores. Interestingly, Muthukumar et al. (2013) report that their attempts to use hygromycin as a selectable marker for transformation were unsuccessful. This is potentially explained through our observation that hygromycin killed untransformed T1 gametophytes on germination (Supplemental Fig. S4). Transformed T0 spores may not have sufficient time to express the introduced *Hyg*^{R} gene at levels sufficient to confer resistance. By contrast, hygromycin selection was found to be a very efficient screening mechanism when identifying transformants regenerating from callus tissue. Regardless of the selection technique, *A. tumefaciens*-mediated transformation of both *C. thalictroides* and *P. vittata* spores occurred at very low frequencies (0.03% and 0.053%, respectively; Muthukumar et al., 2013). Attempts in the same report to directly

transform a population of *P. vittata* spores by microparticle bombardment similarly yielded a very low transformation efficiency of only 0.012%. The available data thus suggest that transformation of spores is an inherently less efficient method than transformation of callus tissue.

Using the protocol described in this paper, the time from callus transformation to recovery of T1 spores was 18 and 16 weeks for *C. richardii* and *C. thalictroides*, respectively, in contrast to an 11- to 13-week period for *C. thalictroides* via *A. tumefaciens*-mediated transformation (Muthukumar et al., 2013). Despite the longer time frame, transformation of the sporophyte generation confers the advantage of direct production of T1 spores without an intervening recombination/outcrossing event (sexual reproduction of T0 gametophytes). Outcrossing of T0 transformant gametophytes could conceivably reduce initial transgene copy number; however, cross fertilization of two independent transformants is also possible. Notably, Muthukumar et al. (2013) report occurrences of multiple transgene insertions, similar to those observed using microparticle bombardment. Overall, the initial high efficiency of transformation offered by microparticle bombardment and the concomitant ability to screen effectively using hygromycin offset the slightly longer T0 generation time required and provide significant benefits over the *A. tumefaciens*-mediated protocol.

Evidence for a Conserved Role of CK in the Shoot Meristem of Ferns and Angiosperms

Treatment with CK was sufficient to induce the formation of callus tissue in place of new fronds at the shoot apex of *C. richardii* and *C. thalictroides* sporophytes and to maintain the callus in an undifferentiated state (Fig. 1; Supplemental Fig. S2). Application of auxin failed to induce shoot callus but did perturb cellular activity specifically at the root apex. It was recently reported that auxin treatment of the lycophyte *Selaginella kraussiana* similarly disturbs root organization (Sanders and Langdale, 2013). Organogenesis in the *C. richardii* shoot arises from divisions of a single tetrahedral apical cell (Hou and Hill, 2002), instead of a multicellular meristem as found in angiosperms (Sussex, 1989). The observed formation of callus suggests that CK treatment can block the differentiation of cells derived from the apical initial cell. In Arabidopsis, CK acts downstream of *knotted1*-like homeobox genes to maintain undifferentiated cell fate in the shoot apex, in part through antagonism of the gibberellin signaling pathway (Jasinski et al., 2005; Bartrina et al., 2011). Given the results observed here, it is possible that CK acts in both ferns and angiosperms to promote indeterminate cell fate at the shoot apex. Interestingly, CK treatment did not induce callus formation from *C. richardii* or *C. thalictroides* gametophytes, although morphology at the notch meristem was slightly affected (Supplemental Fig. S1). This could indicate that the mechanisms regulating initial cell specification differ between the notch meristem and shoot apex and could reflect an important distinction between two-dimensional (gametophyte) and three-dimensional (sporophyte) growth and patterning.

Developmental Trajectories Explain Chimeric versus Stable Transgene Expression in T0 and T1 Generations

The transmission of transgenes from regenerated T0 plants to stable T1 lines was assessed in *C. richardii* by comparing the transgenic status of T0 parents (as assessed by GUS staining; Fig. 3; Supplemental Fig. S5) to their T1 progeny (as assessed by gametophyte hygromycin resistance; Fig. 4; Supplemental Fig. S6). Sixty percent of lines demonstrated consistent transgenic status between the T0 and T1 generations, i.e. both parent and offspring were transgenic (52%) or neither parent nor offspring were transgenic (8%). The remaining 40% of lines showed inconsistent inheritance patterns, with transgenic T1 individuals identified from apparently nontransgenic T0 parents (20%) or nontransgenic T1 progeny descending from apparently transgenic T0 parents (20%). In *C. thalictroides*, corresponding frequencies of 67% consistent (64% plus 3%) and 33% inconsistent (3% and 30%) lines were recorded. A distinction in GUS expression patterns between the T0 and T1 generations was also observed, with frequent examples of partial or tissue-specific (vasculature) GUS staining in T0 regenerated sporophytes (young and mature) but constitutive GUS expression in subsequent T1 progeny. A low incidence (11%–12%) of regenerated shoot clusters that lacked any GUS staining could reflect stable transformation with only the Hyg^R cassette or subsequent loss of the *35S*::*GUS* cassette after hygromycin selection. Noncongruence of GUS staining patterns between the T0 and T1 generations is unlikely to reflect technical issues of GUS substrate penetration given the examples of constitutive staining observed. Instead, these results are most simply explained through the regeneration of chimeric T0 transformants, as previously seen in transformed gametophytes (Rutherford et al., 2004). This interpretation would also explain the observed discrepancies between GUS analysis, antibiotic selection, and transgene expression data in T0 plants (Fig. 3), as different fronds, potentially not all of them transgenic, were sampled for each analysis.

The emergence of chimeric shoots does not correspond with our current understanding of fern shoot development from single apical initial cells (Hou and Hill, 2002; Sanders et al., 2011). However, in accordance with the observations made above regarding callus induction, we hypothesize that induction of callus through CK treatment prevents the mitotic derivatives of the apical initial from differentiating into frond and root initials and thus artificially expands the population of undifferentiated shoot apical initial cells. When this constraint is removed after bombardment, normal developmental patterning and gradients are presumably reimposed onto an abnormally large population of undifferentiated cells. This could theoretically result in the incorporation of multiple apical initials into single regenerating shoots, allowing

the formation of different tissue types from distinct subpopulations of initials. This scenario could theoretically explain our observations of vasculature-specific GUS staining patterns. However, given that regenerated plants appear morphologically similar to untreated controls, such a hypothesis would also imply that fern shoots and organs can successfully organize both from a founding population of multiple cells and from single initials.

In contrast to the scenario proposed above for T0 transformants, T1 individuals must necessarily arise from a single progenitor cell (spore and zygote); thus, all cells within the individual have a common genetic ancestry. This is evidenced by the stable and constitutive GUS expression observed in the T1 generation (Figs. 4 and 5; Supplemental Figs. S6 and S8). In the case of T1 gametophytes, variations in the frequency of transgenics within each line (Fig. 4) most likely reflect the frequency of transformed sporangia on chimeric fronds: each sporangium arises from a single separate initial cell (Hill, 2001). This conclusion is supported by observations of T0 pinnae containing both GUS-stained and unstained sporangia (Fig. 3H; Supplemental Fig. S5). Importantly, the recovery of constitutively expressing T1 progeny from apparently chimeric T0 transformants demonstrates that chimeric expression in the T0 generation does not represent a barrier to establishing stable and pure-breeding transgenic lines and argues that screening for stable transformants should occur in the T1 generation instead of in regenerated T0 shoots.

Transgene Copy Number

Microparticle bombardment resulted in the incorporation of multiple T-DNA fragments into the genomes of individual *C. richardii* T0 transformants, including fragmented copies of individual expression cassettes. The complex insertion of multiple transgene copies is a known factor in biolistic-based transformation techniques (Hansen and Wright, 1999) and can in part be mitigated through bombardment with linearized DNA (Lowe et al., 2009). In all of the T0 plants tested, the presence of linked Hyg^R and *35S::GUS* cassettes was revealed by shared hybridization to large gDNA fragments. Importantly, very little rearrangement of hybridization fragments was observed between the T0 and T1 generations, suggesting that transgene insertions remain essentially stable after initial integration. Within the relatively small sample of nine transgenic lines selected for DNA-blot analysis in this study, one (line 2) was found to carry an entire transgene containing both the Hyg^R and *35S::GUS* cassettes, plus a single unlinked copy of both the Hyg^R and *35S::GUS* cassettes. This observation suggests that the isolation of single insertion lines is feasible, especially if coupled with outcrossing to untransformed individuals. Although microparticle bombardment typically results in a higher T-DNA copy number in transgenics than *A. tumefaciens*-mediated transformation, as demonstrated by side-by-side transformations into barley (*Hordeum vulgare*; Travella et al., 2005), recent *A. tumefaciens*-mediated transformation of *C. thalictroides* resulted in similar copy numbers to those reported here (Muthukumar et al., 2013).

C. richardii as a Fern Genetic Model

The protocol described in this paper was able to successfully generate stable transformants in *C. richardii* and *C. thalictroides* at high efficiencies and may be more generally applicable to other fern species. *C. richardii* has previously been proposed as a candidate model fern; examples from a number of important classes of transcription factor have already been identified (Hasebe et al., 1998; Aso et al., 1999; Himi et al., 2001; Sano et al., 2005), and phylogenetic and cross-species complementation analyses have been published, for example with *CrLEAFY* (Maizel et al., 2005). With its smaller size, more rapid lifecycle, and smaller genome (3.7 Gbp; Bennett and Leitch, 2001), *C. thalictroides* must now also be seriously considered as a candidate model. Although the advantage of a smaller genome in this species is offset by polyploidy in relation to *C. richardii* (McGrath et al., 1994), the power of *C. richardii* as a genetic tool is most likely to be further enhanced by the availability of *C. thalictroides* as a closely related, comparable, genetically tractable species. The advent of a high-efficiency stable transformation system in *C. richardii* and *C. thalictroides* removes one of the final technical barriers for the adoption of ferns as genetic models.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All plant material was derived from *Ceratopteris richardii* strain Hnn (Hickok et al., 1995) and *Ceratopteris thalictroides* strain C-21 (Carolina Biological Supplies). Plants were grown in Sanyo MLR-350H incubators (Panasonic) at 28°C, 90% humidity, 16 h of light/8 h of dark, and fluence of 150 μmol m^{-2} s^{-1}. Gametophytes were grown and fertilized on 1% (w/v) agar media (pH 6.0) containing Ceratopteris (C-fern) nutrient mix (prepared following protocol in Hickok and Warne, 1998). Sporophytes were subsequently transplanted to Sinclair potting growing medium (William Sinclair Horticulture), typically when the third frond had expanded. Callus tissue was cultured on 0.7% (w/v) agar media (pH 5.8) containing 1× MS nutrients (Duchefa Biochemie) and 2% (w/v) Suc and supplemented with hormone (5 μM) and/or hygromycin B (40 μg mL^{-1}) treatments (Sigma Aldrich) as specified in the text. Attempts to grow callus on C-fern media were not successful (Supplemental Fig. S3). All hormone stocks were prepared to 1,000× working concentration in 1 N NaOH. Hygromycin B was prepared in distilled water as a 1,000× stock.

Spores were sterilized by incubating for 10 min at room temperature in sodium hypochlorite solution (2% [v/v] chlorine) and 0.1% (v/v) Tween, which was subsequently removed by six sequential rinses in sterile distilled water. Sterile spores were imbibed in distilled water and incubated for 48 h at room temperature in darkness before sowing. Gametophytes were fertilized between 9 and 11 d after germination by the application of sterile distilled water. Fertilization of transgenic T1 gametophytes was not successful if grown under hygromycin selection. T1 sporophytes were recovered through fertilization within T1 gametophyte populations grown without hygromycin selection and transgenic individuals subsequently identified though hygromycin selection on C-fern media. Twenty micrograms per milliliter hygromycin is sufficient to kill untransformed young sporophytes within 7 d (Supplemental Fig. S4). Transgenic sporophytes were removed from selection after 14 d and transplanted to soil.

Ceratopteris spp. Microparticle Bombardment

All callus utilized in bombardments was derived from sporophyte shoot tissues under BAP treatment; root-derived callus excised from NAA-treated *C. richardii* sporophytes differentiated solely into roots within 14 d, irrespective of hormone treatment (Supplemental Fig. S3). Bombardments utilized the *35S*::*GUS* plasmid pCAMBIA1305.2 (Cambia). Bombardment was performed using a PDS-1000/He biolistic delivery system (Bio-Rad). Tungsten M-20 microparticles (diameter approximately 1.3 μm; Bio-Rad) were prepared for bombardment according to Sanford et al. (1993). Bombardments were performed according to the manufacturer's instructions. Transgenic plants were regenerated from bombardment of 2-week-old callus tissue at 900 pounds per square inch under vacuum conditions of 28 pounds per square inch, with callus tissue placed at a distance of 6 cm from the firing disc. Bombarded callus tissue was allowed to recover for 3 d before transfer to hygromycin selection. All mean efficiency values are expressed as a percentage of the bombarded population ± SE.

Analysis of Transgenic Lines

gDNA was extracted from sporophyte frond tissues using a hexadecyltrimethylammonium bromide based protocol modified from Porebski et al. (1997). Hexadecyltrimethylammonium bromide (2%) extraction buffer also contained 2% (w/v) polyvinylpyrrolidone-40 (PVP-40), 0.3% (v/v) β-mercaptoethanol, and 50 μg mL^{-1} ribonuclease A (Sigma Aldrich), extracting gDNA from ≤1 g of tissue in 10 mL of buffer. Chloroform:isoamyl alcohol (24:1) extraction was performed three times, and NaCl/ethanol precipitation was performed twice. gDNA was resuspended in 1× Tris-EDTA buffer (pH 8.0) at 4°C overnight.

RNA was extracted from ≤100 mg of sporophyte frond tissues using the RNeasy RNA extraction kit (Qiagen). Complementary DNA was synthesized from 250 ng of RNA template using Superscript III Reverse Transcriptase (Life Technologies). Genotyping PCR and RT-PCR were performed using the following primer pairs: HygF2 (CTTCTACACAGCCATCGGTC) and HygR (CCGATGGTTTCTACAAAGATCG) and GUSF (CTTGCCATCCTTGTCCTCC) and GUSR4 (CGAAGTTCGGCTTGTTACG).

C. richardii gDNA (10 μg per sample) was prepared for DNA blotting by digestion with *Hin*dIII and *Xba*I (New England Biolabs) and separated by gel electrophoresis (25 V, 16 h). Blots were prepared and hybridized to ^{32}P-dCTP-labeled DNA probes as described by Langdale et al. (1988). Probes against the *Hyg*^{R} and *35S*::*GUS* cassettes were synthesized from 810- and 761-bp fragments (Fig. 6A), using the Redprime II DNA labeling kit (G.E. Healthcare). Primers used to synthesize probe templates are as described above for PCR.

Histochemical GUS staining was performed on gametophyte and sporophyte tissues using 0.5 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Melford) at 37°C for 16 h, following 20-min pretreatment in 90% (v/v) acetone at 4°C. GUS-stained tissue was cleared by incubation in 70% (v/v) ethanol.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. CK and auxin treatments do not induce callus formation in gametophytes of *C. richardii* or *C. thalictroides*.

Supplemental Figure S2. CK treatment induces shoot apical callus formation in *C. thalictroides* sporophytes.

Supplemental Figure S3. Maintenance of undifferentiated *C. richardii* callus on CK-MS media.

Supplemental Figure S4. Hygromycin sensitivity of *C. richardii* callus, gametophytes, and sporophytes.

Supplemental Figure S5. Chimeric GUS expression in *C. thalictroides* T0 transformants.

Supplemental Figure S6. Stable GUS expression in *C. thalictroides* T1 transgenic lines.

Supplemental Figure S7. Absence of GUS expression during gametophyte development of *C. richardii* transgenic line 23 persists in the T_2 generation.

Supplemental Figure S8. Transgene expression patterns change from chimeric to stable between the T0 and T1 generations of *C. richardii* transgenic lines 24, 25, and 28.

Supplemental Figure S9. *Hyg*^{R} and *35S*::*GUS* remain linked between the T0 and T1 generations of *C. richardii* transgenic lines.

ACKNOWLEDGMENTS

We thank Julie Bull for help with plant maintenance and Laura Moody and Mara Schuler for useful comments on the manuscript.

Received October 28, 2013; accepted March 11, 2014; published March 12, 2014.

LITERATURE CITED

Aso K, Kato M, Banks JA, Hasebe M (1999) Characterization of homeodomain-leucine zipper genes in the fern *Ceratopteris richardii* and the evolution of the homeodomain-leucine zipper gene family in vascular plants. Mol Biol Evol **16:** 544–552

Banks JA (1997) Sex determination in the fern *Ceratopteris*. Trends Plant Sci **2:** 175–180

Banks JA (1999) Gametophyte development in ferns. Annu Rev Plant Physiol Plant Mol Biol **50:** 163–186

Bartrina I, Otto E, Strnad M, Werner T, Schmülling T (2011) Cytokinin regulates the activity of reproductive meristems, flower organ size, ovule formation, and thus seed yield in *Arabidopsis thaliana*. Plant Cell **23:** 69–80

Bennett MD, Leitch IJ (2001) Nuclear DNA amounts in Pteridophytes. Ann Bot (Lond) **87:** 335–345

Chatterjee A, Roux SJ (2000) *Ceratopteris richardii*: a productive model for revealing secrets of signaling and development. J Plant Growth Regul **19:** 284–289

Hansen G, Wright MS (1999) Recent advances in the transformation of plants. Trends Plant Sci **4:** 226–231

Hasebe M, Wen CK, Kato M, Banks JA (1998) Characterization of MADS homeotic genes in the fern *Ceratopteris richardii*. Proc Natl Acad Sci USA **95:** 6222–6227

Hickok LG, Warne TR (1998) C-Fern Manual. Carolina Biological Supply Company, Burlington, VT

Hickok LG, Warne TR, Fribourg RS (1995) The biology of the fern *Ceratopteris* and its use as a model system. Int J Plant Sci **156:** 332–345

Hickok LG, Warne TR, Slocum MK (1987) *Ceratopteris richardii*: applications for experimental plant biology. Am J Bot **74:** 1304–1316

Hill JP (2001) Meristem development at the sporophyll pinna apex in *Ceratopteris richardii*. Int J Plant Sci **162:** 235–247

Himi S, Sano R, Nishiyama T, Tanahashi T, Kato M, Ueda K, Hasebe M (2001) Evolution of MADS-box gene induction by *FLO/LFY* genes. J Mol Evol **53:** 387–393

Hou GC, Hill JP (2002) Heteroblastic root development in *Ceratopteris richardii* (Parkeriaceae). Int J Plant Sci **163:** 341–351

Hou GC, Hill JP (2004) Developmental anatomy of the fifth shoot-borne root in young sporophytes of *Ceratopteris richardii*. Planta **219:** 212–220

Ikeuchi M, Sugimoto K, Iwase A (2013) Plant callus: mechanisms of induction and repression. Plant Cell **25:** 3159–3173

Indriolo E, Na G, Ellis D, Salt DE, Banks JA (2010) A vacuolar arsenite transporter necessary for arsenic tolerance in the arsenic hyperaccumulating fern *Pteris vittata* is missing in flowering plants. Plant Cell **22:** 2045–2057

Jasinski S, Piazza P, Craft J, Hay A, Woolley L, Rieu I, Phillips A, Hedden P, Tsiantis M (2005) KNOX action in Arabidopsis is mediated by coordinate regulation of cytokinin and gibberellin activities. Curr Biol **15:** 1560–1565

Johnson GP, Renzaglia KS (2008) Embryology of *Ceratopteris richardii* (Pteridaceae, tribe Ceratopterideae), with emphasis on placental development. J Plant Res **121:** 581–592

Kawai-Toyooka H, Kuramoto C, Orui K, Motoyama K, Kikuchi K, Kanegae T, Wada M (2004) DNA interference: a simple and efficient gene-silencing system for high-throughput functional analysis in the fern adiantum. Plant Cell Physiol **45:** 1648–1657

Langdale JA, Rothermel BA, Nelson T (1988) Cellular pattern of photosynthetic gene expression in developing maize leaves. Genes Dev **2:** 106–115

Leroux O, Eeckhout S, Viane RL, Popper ZA (2013) *Ceratopteris richardii* (C-fern): a model for investigating adaptive modification of vascular plant cell walls. Front Plant Sci **4:** 367

Lowe BA, Shiva Prakash N, Way M, Mann MT, Spencer TM, Boddupalli RS (2009) Enhanced single copy integration events in corn via particle bombardment using low quantities of DNA. Transgenic Res **18:** 831–840

Maizel A, Busch MA, Tanahashi T, Perkovic J, Kato M, Hasebe M, Weigel D (2005) The floral regulator LEAFY evolves by substitutions in the DNA binding domain. Science **308:** 260–263

McGrath JM, Hickok LG, Pichersky E (1994) Assessment of gene copy number in the homosporous ferns *Ceratopteris thalictroides* and *C. richardii* (Parkeriaceae) by restriction fragment length polymorphisms. Plant Syst Evol **189:** 203–210

Muthukumar B, Joyce BL, Elless MP, Stewart N (2013) Stable transformation of ferns using spores as targets: *Pteris vittata* (Chinese brake fern) and *Ceratopteris thalictroides* (C-fern 'Express'). Plant Physiol **163:** 648–658

Nakazato T, Jung MK, Housworth EA, Rieseberg LH, Gastony GJ (2006) Genetic map-based analysis of genome structure in the homosporous fern *Ceratopteris richardii*. Genetics **173:** 1585–1597

Porebski S, Bailey LG, Baum BR (1997) Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Mol Biol Rep **15:** 8–15

Pryer KM, Schneider H, Smith AR, Cranfill R, Wolf PG, Hunt JS, Sipes SD (2001) Horsetails and ferns are a monophyletic group and the closest living relatives to seed plants. Nature **409:** 618–622

Rutherford G, Tanurdzic M, Hasebe M, Banks JA (2004) A systemic gene silencing method suitable for high throughput, reverse genetic analyses of gene function in fern gametophytes. BMC Plant Biol **4:** 6

Sanders HL, Darrah PR, Langdale JA (2011) Sector analysis and predictive modelling reveal iterative shoot-like development in fern fronds. Development **138:** 2925–2934

Sanders HL, Langdale JA (2013) Conserved transport mechanisms but distinct auxin responses govern shoot patterning in *Selaginella kraussiana*. New Phytol **198:** 419–428

Sanford JC, Smith FD, Russell JA (1993) Optimizing the biolistic process for different biological applications. Methods Enzymol **217:** 483–509

Sano R, Juárez CM, Hass B, Sakakibara K, Ito M, Banks JA, Hasebe M (2005) KNOX homeobox genes potentially have similar function in both diploid unicellular and multicellular meristems, but not in haploid meristems. Evol Dev **7:** 69–78

Stout SC, Clark GB, Archer-Evans S, Roux SJ (2003) Rapid and efficient suppression of gene expression in a single-cell model system, *Ceratopteris richardii*. Plant Physiol **131:** 1165–1168

Sussex IM (1989) Developmental programming of the shoot meristem. Cell **56:** 225–229

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