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Development of a multiplex amplicon-sequencing assay to detect low-frequency mutations in poinsettia (*Euphorbia pulcherrima*) breeding programmes

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Abstract

Poinsettia is an economically important ornamental potted plant in which certain bract colour variants are often obtained by mutation breeding. Previously, in poinsettia, we identified *Bract1*, a *GST* gene involved in the sequestration and transport of anthocyanins to the vacuole. This gene carries a short, highly mutable 4-bp repeat in its coding region. Loss of one repeat unit leads to a loss of function for *Bract1*, and in homozygous mutants, anthocyanin-based coloration is absent, resulting in white or cream-coloured bracts. Although mutation induction through ionizing radiation leads to a high frequency of mutations in *Bract1*, mutants are difficult to obtain from homozygous red genotypes. In this study, we used *Bract1*-specific amplicon sequencing as a tool to identify mutations in pools of tissues, which enabled the detection of mutations in dilutions of up to one mutant in 50 nonmutated samples. This approach enabled efficient screening of recalcitrant homozygous genotypes for mutated alleles and the reduction of the mutation load in the application of ionizing radiation in mutation breeding programmes.

KEYWORDS

amplicon-seq, *Bract1*, heterozygous mutations, irradiation mutagenesis, mutation breeding, poinsettia breeding

1 | INTRODUCTION

Mutation breeding is an established and successful method for generating variation in ornamental plants, because most economically important traits, for example, flower characteristics or growth habits, are easily monitored after mutagenic treatment (Ibrahim et al., 2018; Schum & Preil, 1998). Induced mutations in ornamental plants focus on economically valuable aesthetic values, such as flower and leaf characteristics (colour, size, morphology and fragrance), growth habits (compact, climbing and branching), physiological traits (photoperiodic response, early flowering, free flowering and flower keeping quality), and biotic and abiotic stress responses (Schum & Preil, 1998). The applicability of induced mutagenesis has been exploited in several ornamental species (Datta, 2020), with over 700 mutant varieties being obtained for more than 80 different species (FAO & IAEA, 2019). Among all ornamental-related traits, the development of new colours has strong appeal among consumers; therefore, it is one of the most exploited traits in mutagenesis breeding (reviewed by Datta, 2020; Ibrahim et al., 2018).

Poinsettia, also known as nochebuena, Weihnachtsstern or Christmas star (Trejo et al., 2012), is considered an important ornamental plant, especially due to its attractive, mostly red-coloured bracts, and it is primarily consumed during Christmas season in North America, Europe and Asia (Vilperte et al., 2019). The total consumption of ornamental poinsettias in Germany alone was estimated to be ~32 million potted plants in 2018, with ~90% of the sales comprising red- and white-coloured varieties (van der Ploeg, 2018). Nonetheless, the introduction of varieties with colour variations, such as pink, yellow and even bicoloured, scattered and marbled colourations, adds considerable ornamental value for product development. Ionizing radiation is an important tool in mutation breeding for obtaining such a range of colours, with nearly all pink and white varieties being obtained after γ- or X-ray mutagenesis of red varieties. Poinsettia mutation breeding is usually performed on cuttings that are irradiated with moderate dosages (~20–30 Gy), and mutants are selected on side shoots of the originally irradiated plants.

Anthocyanins have been identified as the main pigments in poinsettia bracts (Moustaka et al., 2018; Nitarska et al., 2018; Slatnar et al., 2013; Stewart et al., 1980), but extensive molecular studies of colour formation and accumulation remain lacking for this species. Dihydroflavonol-4-reductase (DFR) was suggested to promote the conversion of green leaves into red bracts (Gu et al., 2018). The orange colour of the poinsettia cultivar 'Harvest Orange' was related to a nonsense mutation in a flavonoid 3′-hydroxylase (*F3′H*) allele, thereby leading to a higher accumulation of pelargonidin (Nitarska et al., 2018). The pink colouration in pink and marble bracts can be attributed to periclinal chimeric structures (Nielsen et al., 2003). Previous unpublished studies, as well as the poinsettia transcriptome (Vilperte et al., 2019), have demonstrated the expression of most structural genes and the related enzyme activities involved in the formation of red anthocyanin pigments. Thus, the appearance of acyanic (uncoloured) varieties in poinsettias, despite gene expression being present, is referred to as the '*white paradox*'.

A glutathione *S*-transferase (GST) gene (*Bract1*) was recently determined to be involved in the transport and accumulation of anthocyanins in poinsettia bracts (Vilperte et al., 2019, 2020). A 4-bp deletion in a short repeat within the coding region is responsible for the lack of function of *Bract1* and thus explains the '*white paradox*' in poinsettia, that is, the appearance of white phenotypes upon irradiation treatment. The identified repeat seems to be highly unstable, because mutated plants can be easily detected among fewer than 200 shoots derived from an original set of only 10 mutated heterozygous plants (Vilperte et al.,2020). In contrast, de novo mutations derived from homozygous plants cannot be identified phenotypically due to the complete dominance of *Bract1* but only by molecular analysis.

In this study, we present a strategy for a multiplex amplicon-sequencing (hereafter named amplicon-seq) assay to detect newly generated heterozygotes among pooled progeny from irradiated poinsettia homozygotes and thus significantly increase the efficiency of the detection of heterozygous mutants. Employing different approaches, this study demonstrates the applicability of using an amplicon-seq strategy to detect low-frequency mutations in a heterozygous state, thereby enabling the detection of mutant alleles in larger pools of tissue samples. Most importantly, we developed a marker assay linked to colour variation that could be directly implemented in poinsettia breeding programmes, thereby contributing to the genetic resources by obtaining mutants from unexploited homozygous genotypes and improving the selection of prominent breeding material.

2 | MATERIALS AND METHODS

2.1 | Plant material

Three poinsettia (*Euphorbia pulcherrima* Willd. Ex Klotsch) varieties were utilized in this study: the red varieties 'Vintage' and 'Christmas Feelings' and the white variety 'Christmas Feelings Pearl'. The white variety 'Christmas Feelings Pearl' was obtained through γ-ray mutagenesis of the red variety 'Christmas Feelings' followed by shoot development and trait selection at the company Selecta One (Stuttgart, Germany; https://www.selecta-one.com/). Therefore, red- and white-bracted poinsettias from the same variety are referred to as 'pairs' due to their highly similar genetic backgrounds.

Rooted cuttings from all varieties were obtained from the company Selecta One and grown in a greenhouse at the Institute for Plant Genetics of the Leibniz Universität Hannover under short-day conditions to induce flower formation and to stimulate the development of coloured bracts. The plants were grown in 5-L pots containing Einheitserde P substrate (Hermann Meyer KG) with an average temperature of 22°C and 9 hr of daylight (15 hr of darkness).

2.2 | Plasmid controls

Plasmids obtained from Vilperte et al. (2020) were used as controls for amplicon sequencing. Briefly, the coding sequence of *Bract1* (GenBank—MW718861) was amplified from the poinsettia varieties 'Vintage' and 'Christmas Feelings Pearl' (GenBank—MW718847–MW718860) to capture both wild-type and mutated alleles (645/641 bp plus 50 bp of partial 5′ and 3′ flanking sequences) and inserted into the C757pGFPU10-35s-ocs-LH binary vector (DNA Cloning Service, Hamburg, Germany). The fragment was amplified using the primer pair EpGST_F1 (TCCGATCTAAGAAATCAAGGCTA) and EpGST_R1 (CAGTCGGCCGCTACATAGA; Vilperte et al., 2020). The vector contains a GFP gene under the control of the *Arabidopsis* ubiquitin promoter (*ProAtUbi::GFP*) and a 35S promoter upstream of the multiple cloning site into which inserts were cloned. The final expression vectors contained either the wild-type allele (*35S::Bract1*—695 bp) or the mutated allele (*35S::Bract1_mut*—691 bp).

2.3 | DNA isolation

DNA was isolated from ~100 mg of poinsettia leaf tissue using the NucleoSpin® Plant II Kit (Macherey–Nagel GmbH & Co.) according to the manufacturer's instructions. DNA concentrations and quality were assessed by photometry using a NanoDrop™ 2000 (Thermo Fisher Scientific) and by gel electrophoresis.

2.4 | Sample preparation

Plasmids containing the wild-type and mutated copies of the *Bract1* alleles were used as controls for the amplicon-seq analysis. Moreover, the poinsettia varieties were also used as controls: (a) 'Vintage' is homozygous dominant for the *Bract1* locus (designated RR), containing two copies of the wild-type allele; (b) 'Christmas Feelings' is heterozygous for the same locus (Rr) and contains both wild-type and mutated versions of the allele; and (c) 'Christmas Feelings Pearl' is homozygous recessive (rr) for the *Bract1* locus and contains two copies of the mutated allele. DNA samples from the varieties 'Vintage' and 'Christmas Feelings Pearl' were diluted to the same concentration (10 ng/μl) and used to create serial dilutions of wild-type (R) and mutated alleles (r). The following dilutions (R:r) were used: 1:1, 5:1, 10:1, 20:1, 50:1, 100:1 and 500:1.

2.5 | Genotyping

To detect changes in the repeat structure of the *Bract1* gene, a genotyping approach based on the fluorescent labelling of polymerase chain reaction (PCR) fragments (Schuelke, 2000) was employed. DNA samples were PCR-amplified in a 20-μl reaction containing 50 ng of DNA template, 1× Williams buffer, 0.15-mM each dNTP, 0.0125-μM forward primer, 0.07-μM universal IRD_{700}-labelled M13 primer, 0.25-μM reverse primer and 1 U of DCSPol DNA Polymerase (DNA Cloning Service, Hamburg, Germany). The cycling conditions were 94°C for 3 min; 24 cycles of 94°C for 45 s, 59°C for 1 min and 72°C for 1 min; six cycles of 94°C for 30 s, 52°C for 45 s and 72°C for 1 min; and a final extension of 10 min at 72°C. Fifty microlitres of formamide loading dye (98% formamide, 10-mM EDTA and 0.05% pararosaniline) was added to each reaction, which was subsequently incubated at 95°C for 5 min. The PCR products were resolved in a 6% (w/v) acrylamide gel via vertical electrophoresis using a LI-COR 4,200 DNA Analyser (LI-COR Biosciences). Primer sequences are available in Table 1.

2.6 | Amplicon sequencing

Control plasmids, poinsettia varieties 'Vintage', 'Christmas Feelingsa and 'Christmas Feelings Pearl', and serial dilutions of genomic DNA samples (1:1, 5:1, 10:1, 20:1, 50:1, 100:1 and 500:1) were submitted to amplicon sequencing. Briefly, *Bract1*-specific primers were tagged with an M13 tail and used in the first round of PCR (Table 2). Complementary M13 tags were subsequently used in a second PCR to differentiate PCR fragments arising from each variety. A schematic overview of the amplicon-seq approach is shown in Figure 1.

Both rounds of PCRs were performed in a 50-μl reaction containing 30 ng of DNA, 1× PrimeSTAR® Buffer (Mg^{2+} plus), 0.2 mM of each dNTP, 0.25 μM of forward and reverse primers and 1.25 U of PrimeSTAR® HS DNA Polymerase (Takara). The cycling conditions were 95°C for 3 min; 10 (for the second PCR) and 20 (for first PCR) cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 45 s; and a final extension of 10 min at 72°C. PCR products were pooled and sent to Eurofins Genomics Germany GmbH (Ebersberg) for Illumina sequencing. Primer sequences are available in Table 1.

Sequences were analysed using CLC Genomics Workbench (Qiagen). The Kruskal–Wallis test for non-parametric data ($p \leq .05$) was applied for pairwise comparisons of allele frequency. Statistical analysis and data plotting were performed by in-house scripts using the language and statistical environment R.

3 | RESULTS

3.1 | Detection of *Bract1* mutation by PCR fluorescent labelling

In a previous study (Vilperte et al., 2020), we used a genotyping approach based on the fluorescent labelling of PCR fragments to identify wild-type and mutated alleles in a range of poinsettia varieties. This approach produced satisfactory results to differentiate varieties containing a 4-bp *indel* in the *Bract1* locus in homozygous (dominant and recessive) and heterozygous states. However, heterozygous mutations arising from irradiation mutagenesis of homozygous dominant varieties have been shown to occur at low frequencies, and screening individual mutants by phenotype is impossible due to the dominance of *Bract1* (Vilperte et al., 2020). Therefore, we created serial dilutions of wild-type (R) and mutated (r) *Bract1* alleles to simulate mutation frequencies and evaluate the potential for detecting mutations at such dilutions through PCR-based techniques.

The amplification of the poinsettia varieties 'Vintage', 'Christmas Feelings' and 'Christmas Feelings Pearl' showed the expected allele configuration: homozygous wild-type, heterozygous and homozygous mutations, respectively (Figure 2). The mutated allele was detected with high accuracy at dilutions of 1:1, 5:1 and 10:1 in wild-type DNA, whereas the approach was not sensitive for detecting the mutations at dilutions of 100:1 and 500:1 (Figure 2). Although IRD labelling was highly sensitive with low background noise, only faint amplification could be observed for the 20:1 and 50:1 dilutions of mutated alleles in wild-type DNA, but the same faint signal could be detected in DNA of the 'Vintage' variety (RR) indicating background noise due to slippage of the DNA polymerase. Therefore, a reliable result could not be drawn for those samples. Nonetheless, the fluorescent labelling of PCR fragments approach followed by

TABLE 1 List of primers used in this study

Primer name	Sequence	Analysis
*Bract1*_F	GTAAAACGACGGCCAGTTGGCCTGCCTTTTAGAGAAA	Genotyping in acrylamide gel
*Bract1*_R	ACAAGTTCAGGGGGCTGAG	
IRD_{700}-labelled M13	FAM-GTAAAACGACGGCCAGT	
*Bract1*_F	GTAAAACGACGGCCAGTTGGCCTGCCTTTTAGAGAAA	First PCR round amplicon sequencing *Poinsettia samples*
*Bract1*_R	CAGGAAACAGCTATGACACAAGTTCAGGGGGCTGAG	
*Bract1*_F	GTAAAACGACGGCCAGTTGGCCTGCCTTTTAGAGAAA	First PCR round amplicon sequencing *Plasmid samples*
*Bract1*_plasmid_R	CAGGAAACAGCTATGACGGCCCTCTTTCTTCATATTTTG	
Tag1_F	AAGTCTTCGTGTAAAACGACGGCCAGT	Second PCR round for amplicon sequencing
Tag1_R	AATGCGCTATCAGGAAACAGCTATGAC	
Tag2_F	ACCTCATCTTGTAAAACGACGGCCAGT	
Tag2_R	ACGTGTTACTCAGGAAACAGCTATGAC	
Tag3_F	AACAACTGCTGTAAAACGACGGCCAGT	
Tag3_R	AAGCTCACTTCAGGAAACAGCTATGAC	
Tag4_F	ACAGTTAGCTGTAAAACGACGGCCAGT	
Tag4_R	ACGATACGTTCAGGAAACAGCTATGAC	
Tag5_F	ACAACCAGTTGTAAAACGACGGCCAGT	
Tag5_R	ACATGAGGTTCAGGAAACAGCTATGAC	
Tag6_F	AATCCTGGATGTAAAACGACGGCCAGT	
Tag6_R	AGAGTTGCTTCAGGAAACAGCTATGAC	
Tag7_F	ATGTTAGGATGTAAAACGACGGCCAGT	
Tag7_R	AAGACCGAATCAGGAAACAGCTATGAC	
Tag8_F	AGTATTCGATGTAAAACGACGGCCAGT	
Tag8_R	ATCGGACCATCAGGAAACAGCTATGAC	
Tag9_F	AAGCTACCATGTAAAACGACGGCCAGT	
Tag9_R	AAGGTCCTATCAGGAAACAGCTATGAC	
Tag10_F	ACTTGACGATGTAAAACGACGGCCAGT	
Tag10_R	ATCATTCGATCAGGAAACAGCTATGAC	

Abbreviation: PCR, polymerase chain reaction.

electrophoresis resolution could reliably detect heterozygous mutations in poinsettia DNA samples up to a 10:1 dilution.

3.2 | Amplicon-seq approach to identify heterozygous mutations

Our amplicon-seq analysis was performed in two experiments: (i) plasmid sequencing, where we used the plasmids *35S::Bract1* and *35S::Bract1_mut* to first evaluate if the approach would be suitable to detect the *Bract1* mutation and to determine the intrinsic error rates, and (ii) poinsettia sequencing, where we sequenced the *Bract1* locus from PCR products amplified from genomic DNA obtained from the varieties Vintage, Christmas Feelings and Christmas Feelings Pearl, and the serial dilutions 1:1, 5:1, 10:1, 20:1, 50:1, 100:1 and 500:1 were employed. The sequencing strategy consisted of a first round of PCR with *Bract1*-specific primers tagged with M13 tails followed by a second PCR with complementary M13 primers including tags to differentiate PCR fragments arising from each variety and PCR replicates (Figure 1).

Three independent PCRs were performed for each plasmid for the first amplicon-seq experiment, whereas for the second experiment (the poinsettia sample and serial dilutions), five independent PCRs were performed for each sample. The tags used for each sample are available in Table 1. The amplicon-seq experiments showed an average sequencing coverage of 135,890 reads for the plasmids and an average coverage of sequencing of 62,995 for the second experiment (Figure 3). The mutated *Bract1* allele (containing a 4-bp deletion) was detected in all analysed samples, including the *35S::Bract1* plasmid and the homozygous dominant variety Vintage (Data S1). On the other hand, the wild-type *Bract1* allele was also detected in the *35S::Bract1_mut* plasmid and the homozygous recessive variety Christmas Feelings Pearl. Figure 4 shows the percentage of the *Bract1*-mutated allele detected in each of the varieties and dilutions. The heterozygous variety Christmas Feelings showed a ratio of 1:1 from both alleles. The serial dilutions showed a serial

TABLE 2 Tag distribution for the amplicon-sequencing experiments

Experiment	Sample	Forward tag	Reverse tag (one tag per PCR replicate)
Plasmid sequencing	*35S::Bract1*	Tag_F1	Tag_R1-R3
	35S::Bract1_mut	Tag_F2	Tag_R4-R6
Poinsettia sequencing	Vintage	Tag_F1	Tag_R1-R5
	Chr. Feelings	Tag_F2	Tag_R1-R5
	Chr. Feelings Pearl	Tag_F3	Tag_R1-R5
	1:1	Tag_F4	Tag_R1-R5
	5:1	Tag_F5	Tag_R1-R5
	10:1	Tag_F6	Tag_R6-R10
	20:1	Tag_F7	Tag_R6-R10
	50:1	Tag_F8	Tag_R6-R10
	100:1	Tag_F9	Tag_R6-R10
	500:1	Tag_F10	Tag_R6-R10

Note: Sequence information for each of the tags is available in Table 1.

Abbreviation: PCR, polymerase chain reaction.

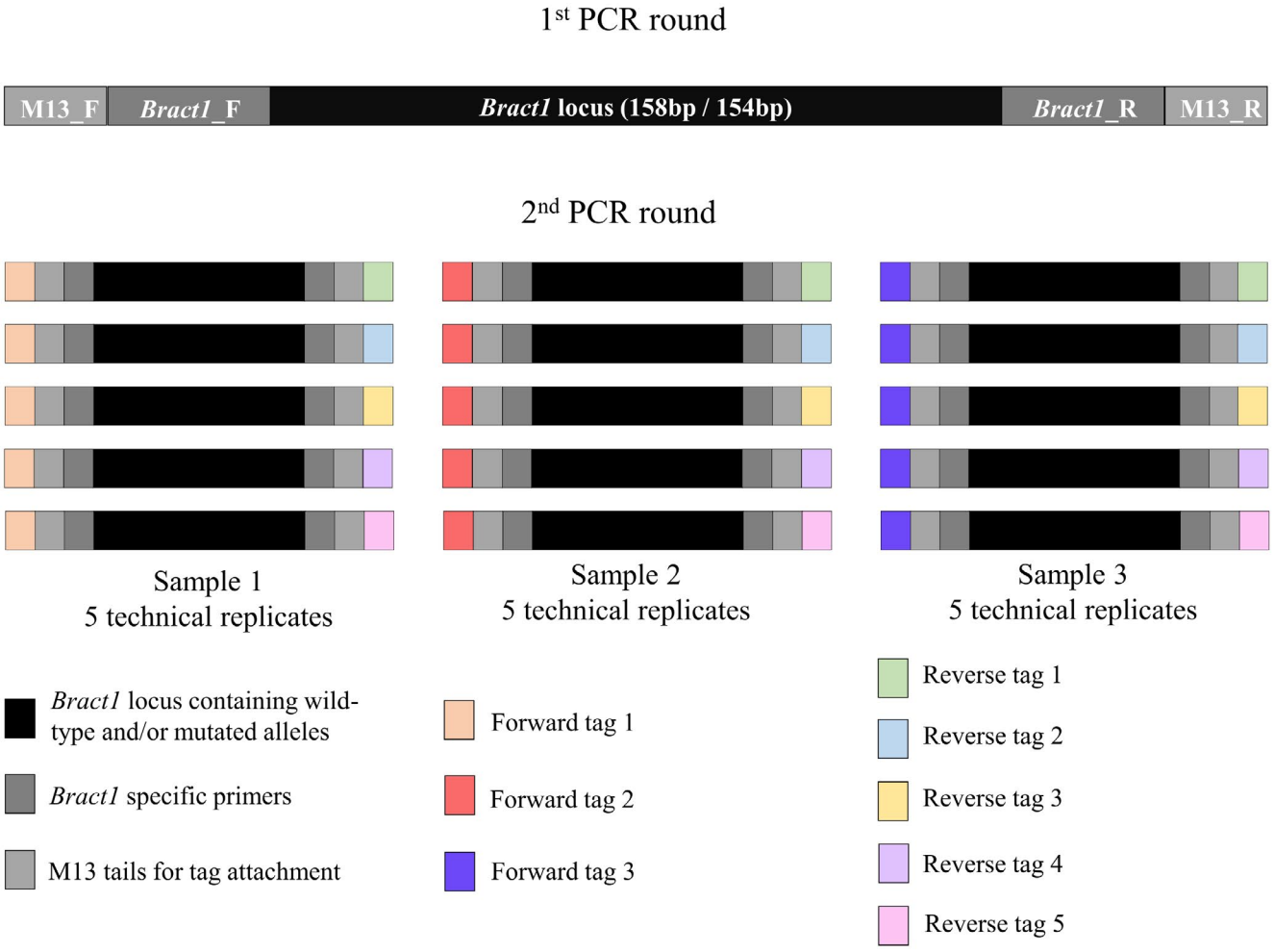

FIGURE 1 Schematic overview of the amplicon-sequencing (amplicon-seq) strategy for the *Bract1* locus. The first polymerase chain reaction (PCR) round amplifies the *Bract1* locus containing the 4-bp indel. The second PCR round introduces tags for each sample and PCR replicate prior to sequencing. The picture depicts an example for three hypothetical samples. The tag distribution for each sample can be seen in Table 2 [Colour figure can be viewed at wileyonlinelibrary.com]

reduction in the detection of the mutated allele, ranging from 36.1% (1:1) to 0.3% (500:1).

3.3 | Detection accuracy of heterozygous mutations

To address the detection accuracy of the amplicon-seq assay for the detection of heterozygous mutations in the poinsettia breeding pipeline, we compared the serial dilutions containing the lower concentrations of mutated alleles (20:1, 50:1, 100:1 and 500:1) to the control samples (*35S::Bract1* and Vintage). As previously mentioned, the mutated allele was detected in the control samples, which may be explained by the fact that library construction for sequencing relies on PCR, which is prone to polymerase slippage at repeat motifs, thereby leading to minor amplicon products that differ by multiples of the length of the repeat unit (also known as stutters; Ellegren, 2004). Therefore, the control samples used a threshold for the limit of detection of mutated alleles in our assay.

When compared with the *35S::Bract1* plasmid control, the dilutions 20:1 and 50:1 showed significant differences (Kruskal–Wallis,

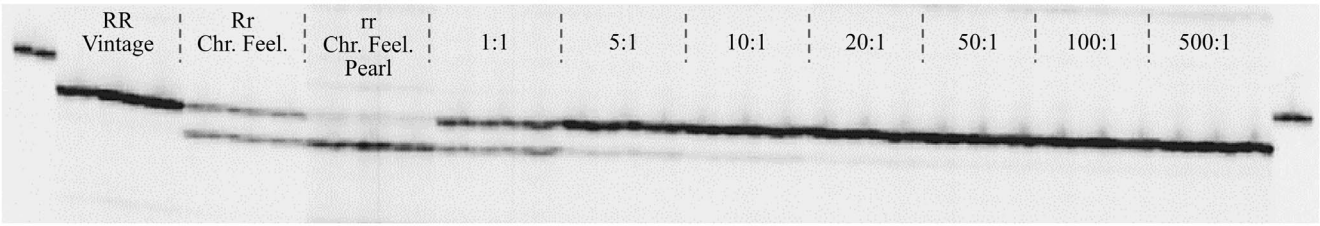

FIGURE 2 Detection of *Bract1* alleles in poinsettia genotypes and in serial dilutions of wild-type (R) and mutated (r) *Bract1* alleles using fluorescent labelling with polymerase chain reaction (PCR) fragments. For each poinsettia variety and dilution, three independent PCR analyses were performed. A size marker (~200 bp) is shown on both sides of the gel image. Chr. Feel., 'Christmas Feelings' variety; Chr. Feel. Pearl, 'Christmas Feelings Pearl' variety

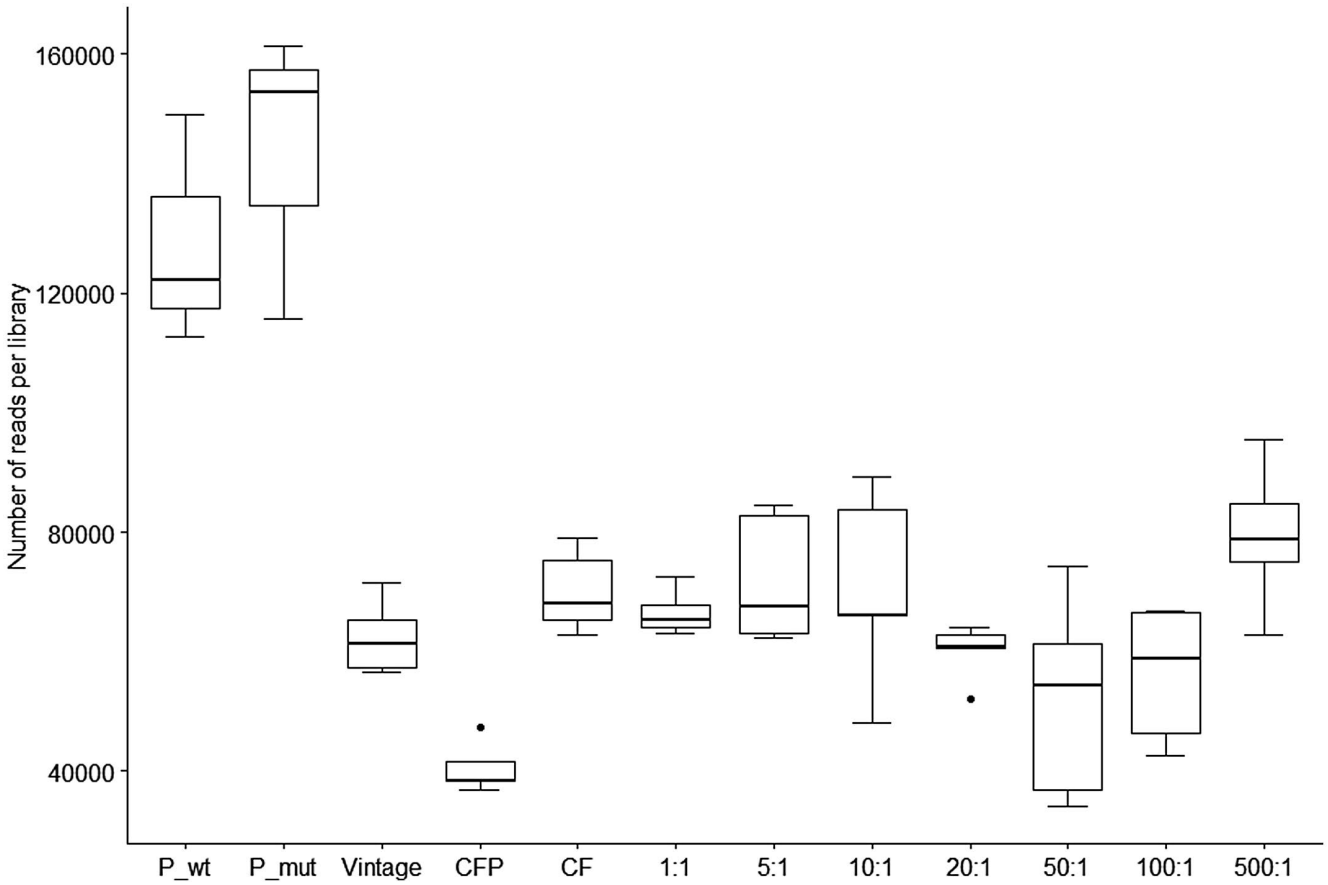

FIGURE 3 Sequencing coverage for two amplicon-sequencing (amplicon-seq) experiments. Boxplots for the plasmids *35S::Bract1* (P_wt) and *35S::Bract1_mut* (P_mut) were calculated with three independent replicates. Boxplots for the poinsettia samples and serial dilutions were calculated with five independent replicates. CF, 'Christmas Feelings' variety; CFP, 'Christmas Feelings Pearl' variety

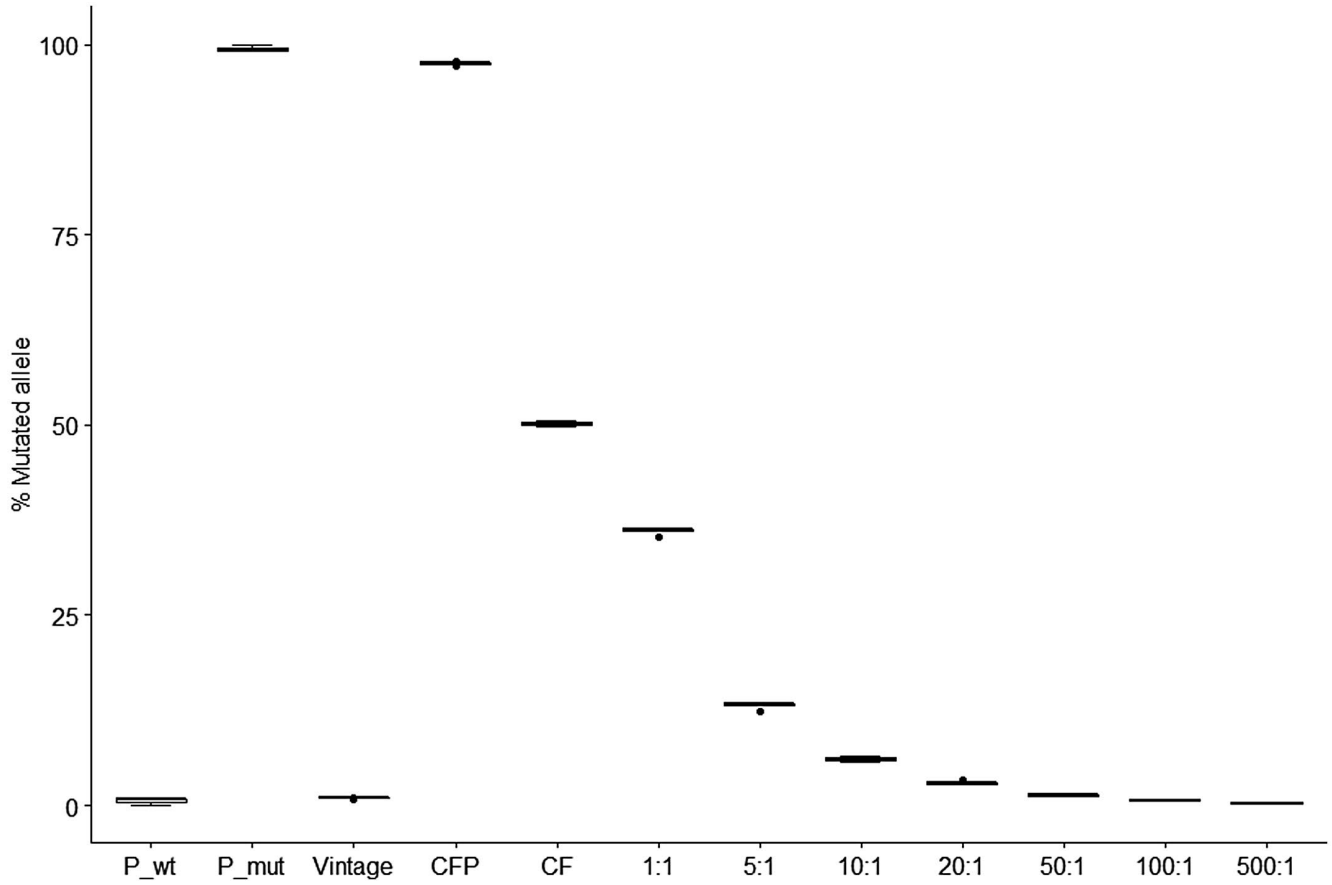

FIGURE 4 Detection of the *Bract1* mutated allele in amplicon-sequencing (amplicon-seq) experiments. Boxplots for the plasmids *35S::Bract1* (P_wt) and *35S::Bract1_mut* (P_mut) were calculated with three independent replicates. Boxplots for the poinsettia samples and serial dilutions were calculated with five independent replicates. CF, 'Christmas Feelings' variety; CFP, 'Christmas Feelings Pearl' variety

$p \leq .05$) for the detection level of the mutated allele, whereas the dilution 100:1 showed no differences, and the 500:1 dilution fell under the threshold of the assay (Figure 5a). The dilutions 20:1 and 50:1 were also significantly different (Kruskal–Wallis, $p \leq .05$) from the 'Vintage' variety, whereas the dilutions 100:1 and 500:1 fell under the detection threshold (Figure 5b). These results show that the amplicon-seq approach can accurately detect the presence of the *Bract1* mutation at dilutions of at least 50:1 (wild-type allele:mutant allele).

4 | DISCUSSION

Ionizing radiation is an important and frequently used tool for mutation breeding for bract colour variants in poinsettia. In contrast to ethyl methanesulphonate (EMS) chemical mutagenesis, which produces point mutations with high frequency (Greene et al., 2003), ionizing radiation (e.g., X-rays and γ-rays) induces DNA oxidative damage, such as double-strand breaks (DSBs), base substitutions, deletions and chromosomal alterations, at a lower frequency, thereby commonly resulting in the loss of gene function (Jo & Kim, 2019; Kazama et al., 2011; Morita et al., 2009). We have previously identified a short 4-bp repeat in the poinsettia anthocyanin-related *GST* gene (name *Bract1*). Upon X-ray treatment, progenies of irradiated plants show a deletion of one repeat (4 bp) in the *Bract1* locus at very high frequencies, leading to a frame shift in the coding region and a loss of function of the *Bract1* gene. This mutation is associated with the appearance of a white phenotype (Vilperte et al., 2020).

SSRs and short repeats are among the most variable and highly dynamic types of repetitive sequences in the genome (Ellegren, 2004; Verstrepen et al., 2005). These sequences are defined as short tandemly repeated DNA sequences that involve a repetitive unit of 1–9 bp and lengths of up to 100 nucleotides (nt; Fan & Chu, 2007; Gemayel et al., 2010). SSR regions are highly unstable, where mutations occur in the form of the addition or deletion of repeat units at considerably higher rates than in other parts of the genome (10 to 100,000 times higher; Gemayel et al., 2010; Schlötterer, 2000). The most evident source of SSR diversity is the slippage of DNA polymerases during SSR replication (Baptiste & Eckert, 2012; Brandström et al., 2008; Webster & Hagberg, 2007). Such slippage also occurs in in vitro SSR PCR amplification, which results in erroneous SSR alleles, thereby making accurate SSR genotyping challenging. In addition, gel electrophoresis, the most commonly used approach to detecting SSR PCR products, does not enable high-throughput applications and is not accurate due to its low resolution (Li et al., 2017). Although we were able to apply our gel electrophoresis approach to

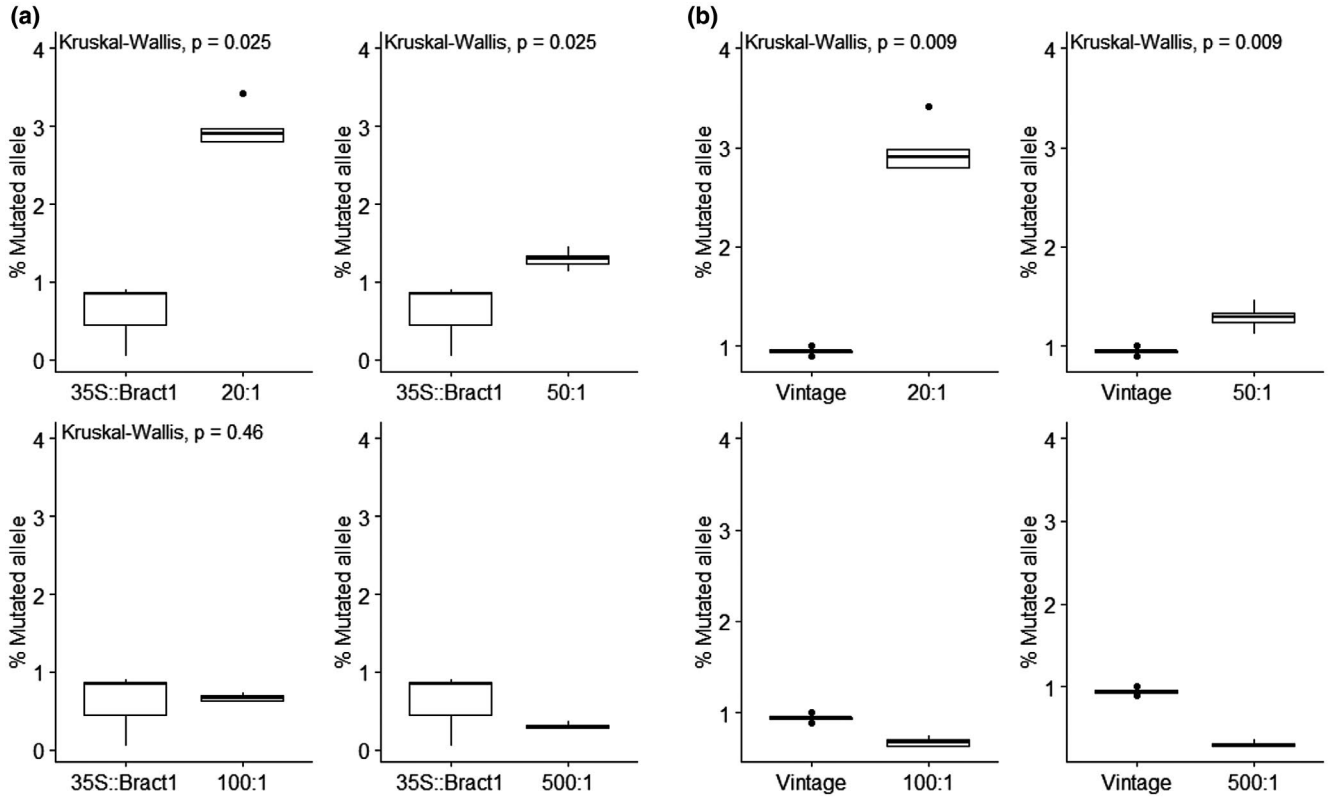

FIGURE 5 Limit of detection of heterozygous mutations using amplicon sequencing. (a) Comparison of amplicon-sequencing coverage of the mutant allele in serial dilutions with the *35S::Bract1* plasmid. (b) Comparison of amplicon-sequencing coverage of the mutant allele in serial dilutions of DNA from the homozygous dominant variety 'Vintage' mixed with DNA from the white variety 'Christmas Feelings Pearl'. Boxplots for the plasmids *35S::Bract1* and *35S::Bract1_mut* were calculated with three independent replicates. Boxplots for the poinsettia samples and serial dilutions were calculated with five independent replicates. The Kruskal–Wallis test for non-parametric data ($p \leq .05$) for pairwise comparisons between samples was performed using in-house R scripts

detect SSR *Bract1* mutations, it was not sensitive enough to detect heterozygous mutations at low frequencies. Moreover, only a small number of samples could be screened at a time.

Amplicon-seq is an increasingly frequently utilized genotyping approach that provides a cost-effective strategy; that is, it is time efficient and less laborious than conventional electrophoresis-based approaches to profile short targeted gene sequences (Early et al., 2019; Li et al., 2019). High-throughput approaches based on next-generation sequencing (NGS) have only rarely been applied to SSR genotyping but have nonetheless yielded helpful outputs. Yang et al., (2019) applied an approach called target SSR-seq, which combines the multiplexed amplification of SSRs with high-throughput sequencing and can be used for genetic background selection, gene and QTL mapping, and molecular breeding of cucumber varieties. A similar approach (AmpSeq-SSR) was used for fingerprinting rice varieties, as well as for mapping resistance genes (Li et al., 2017). Our amplicon-seq approach is similar to both Target SSR-seq and AmpSeq-SSR, and it was determined to be reliable and accurate in detecting low-frequency heterozygous mutations in poinsettia.

Despite the importance of SSR genotyping by NGS and its recent applications in many fields of molecular biology, employing this method, especially for small insertions and deletions (*indels*) analysis, remains challenging and error-prone (Albers et al., 2011; Treangen & Salzberg, 2011; Wang et al., 2011). In our amplicon-seq experiments, both negative controls for the *Bract1* mutated allele (*35S::Bract1* plasmid and 'Vintage' variety) and the positive controls (*35S::Bract1* and 'Christmas Feelings Pearl') showed a low background of mutated products, most likely the results of slippage that occurred during PCR. In addition to error introduced by the slippage of in vitro SSR PCR, there is an increased sequencing error in such regions due to imprecise base calling of NGS platforms (Albers et al., 2011). Studies using plasmids containing artificially inserted tandem repeat sequences have exhibited stutters due to slippage, which is strongly correlated with the number of repeat units and repeat unit length (Fungtammasan et al., 2015; Shinde, 2003). It is therefore possible that the amplicon variants observed in our study are likely attributable to polymerase slippage and/or base calling errors.

It is important to mention that the role of the *Bract1* mutation in the appearance of white phenotypes in poinsettia is not only relevant for the varieties analysed in this study. Poinsettia varieties with red bracts homozygous for the wild-type allele of *Bract1* differ for several other characteristics, for example, bract and shoot morphology, and production parameters. As poinsettia is a vegetatively propagated crop, introgression of the mutated allele of *Bract1* into elite varieties is not an option leaving de novo mutagenesis as the only option for breeding white variants of existing varieties. The high mutagenicity

of *Bract1* therefore provides the opportunity to obtain such mutants from commercially important homozygous red varieties. However, mutations, either natural or induced, can occur in several genes involved in the anthocyanin biosynthesis/accumulation and, therefore, lead to acyanic phenotypes. An insertion in a *leucoanthocyanidin dioxygenase* (*LDOX*) gene led to the appearance of pomegranates containing a white phenotype when the allele is a recessive state (Ben-Simhon et al., 2015). A frameshift mutation in a *dihydroflavonol 4-reductase* (*DFR*) gene is responsible for a rare white flower phenotype in *Mimulus lewisii* (Wu et al., 2013). The insertion of a retrotransposable element into the *F3H* 5′-upstream region caused a reduction in transcript expression and thus responsible for the white petal phenotype in *Torenia fournieri* (Nishihara et al., 2014). Analysing possible mutations in other anthocyanin-related genes in a wider range of varieties (e.g., from different breeding programmes) would generate valuable information on alternative targets that could be used in colour breeding in poinsettia.

Nevertheless, we were able to distinguish the background noise from true mutations down to a dilution of 50:1. This finding enables us to improve the generation of novel colour mutants in poinsettias in two important aspects. The first is that screens in homozygous red varieties may now become more feasible and considerably more effective compared with previous screens, as heterozygotes can be readily detected and used in a second round of mutagenic treatment, leading to white varieties. The second aspect is that considerably lower dosages of ionizing radiation may now be employed for the generation of heterozygotes from homozygotes, thereby reducing the mutational load that is associated with the application of ionizing radiation. In the long term, this approach avoids the accumulation of negative mutations in the poinsettia breeding pool.

5 | CONCLUSIONS

The amplicon-seq approach utilized in our study exhibited higher accuracy and sensitivity to detect heterozygous mutations in poinsettia DNA samples. In addition, this approach demonstrated the ability to detect low-frequency mutations at a sensitivity of up to one mutant allele in 50 nonmutated alleles, thereby increasing the possibility of high-throughput screenings. Because the current approach that is applied for mutagenic breeding in poinsettia is based on the use of heterozygous plants and the subsequent selection of white sports, the development of a strategy to detect heterozygous mutations arising from radiation mutagenesis of homozygous plants is a valuable tool to implement in the breeding process. This approach may enable an expansion of the genetic resources available for the development of new varieties, especially those related to colour traits, as well as enabling a reduction in the mutation load in poinsettia gene pools.

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CONFLICT OF INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

V. V., T. D. and R. B. conceived the research. V. V. and T. D. designed the experiments. R. B. provided the plant material. V. V. conducted the laboratory and bioinformatics analyses. V. V. and T. D. wrote the manuscript. All authors revised and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The DNA sequence from the *Bract1* gene is available in the GenBank repository under Accession Number MW718861. The CDS sequences from several poinsettia genotypes are available in the GenBank repository under Accession Numbers MW718847–MW718860. The NGS data generated in this study are available on request from the authors.

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SUPPORTING INFORMATION

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