Description of Binary Plasmids

Each binary vector (pBI) harbours a transfer DNA gene cassette (tDNA) between the left and right borders to be integrated into the genome of higher plants. The tDNA carries a gene intended to express a fusion of a GFP variant and an Aequorin variant. Expression of the fusion protein is targeted either to the cytoplasm or to the cell wall. Successful expression allows monitoring of pH and calcium concentrations in the targeted cellular compartment.

Historical Context of the Variants Used

GFP Variants

A modified GFP (mGFP4) was developed for expression in plants by the Jim Haselhoff lab (Haselhof et al., 1997) and improved for better solubility by the Richard Vierstra lab (Davies and Vierstra, 1998) giving soluble modified GFP4 (smGFP4). This was further improved to give smGFP5 (GenBank U87973). The mutations of smGFP5 that enable optimised expression in plants are encoded in the first half of the gene (nucleotides 1–393).

pHluorins, pH-sensitive variants of GFP, were developed by directed evolution in the James Rothman lab (Miesenböck et al.,1998). All mutations that convey the pH-sensitivities to the pHluorins are encoded in the second half of the genes (nucleotides 394–717). The final variants were called 'ratiometric pHluorin' (GenBank AF058694) and 'ecliptic pHluorin' (GenBank AF058695). However, pHluorins were originally derived from a GFP gene that was unsuitable for expression in plants.

Fusing the first half of the smGFP5 gene with the second half of the pHluorin gene, allowed the expression of pHluorins in plants (e.g., in *Arabidopsis thaliana* see current study and Schulte et al., 2006). They were called 'ratiometric At-pHluorin' and 'ecliptic At-pHluorin' and abbreviated as smratioGFP and smeclipGFP, respectively.

Aequorin Variants

The aequorin gene, *AEVAEQA*, abbreviated AQ (GenBank M16103), is described in Prasher et al. (1987). It was first expressed in the cytoplasm of plants in 1991 by Marc Knight in the Trewavas lab (Knight et al., 1991). The Anthony Campbell lab (Kendall et al., 1992) reported a mutation, D119A, with reduced affinity for calcium. This low-affinity aequorin variant is abbreviated LAAQ.

Final Binary Constructs

The genes of aequorin variants, AQ and LAAQ, were combined with N-terminal At-pHluorins for expression as fusion proteins in plants. For expression of the indicator in the apoplast (i.e., targeting export of the gene product to the cell wall), a 20 amino acid chitinase signal sequence (MKTNLFLFLIFSLLLSLSSA) was fused to its N-terminus (Samac et al., 1990; GenBank AB023461). The resulting gene cassettes, for expression either in the plant cytoplasm (At-pHluorin:AQ) or in the plant cell wall (chit:At-pHluorin:LAAQ), were finally subcloned via XbaI and SacI into the tDNA of the binary vector pBI121 (GenBank AF485783) for integration via agrobacterium-mediated floral dip method (Clough and Bent, 1998) into the plant genome. The final gene constructs are depicted in Figure 1 of the Gao et al., 2004 paper.

Fig. 1B* pBI121::chitsmGFP5:AQ (14467 bp)				
	Fragments	Start	End	Category
	right Border	2478	2454	Miscellaneous
	CaMV 35S promoter	4974	5802	Promoter
	Chitinase signal	5844	5900	Localization Sequence
	smGFP5	5907	6617	Gene
	Aequorin	6633	7196	Reporter Gene
	Terminator	7212	7413	Terminator
	left Border	8355	8331	Miscellaneous
	Restriction Sites cuts once / cuts twice	Location		Sequence
	BamHI	5822, 7155		ggatcc
	Cla I	4538, 6287		aagctt
	HindIII	4951		aagctt
	KpnI	7419		ggtacc
	NotI	14372		gcggccgc
	SacI (SstI)	7425		gagctc
	SalI	6618		gtcgac
	XbaI	5816		tctaga
	XhoI	6628		ctcgag

*refers to Figure 1 in Gao et al. 2004 (PubMed 15020753; DOI: 10.1104/pp.103.032508)

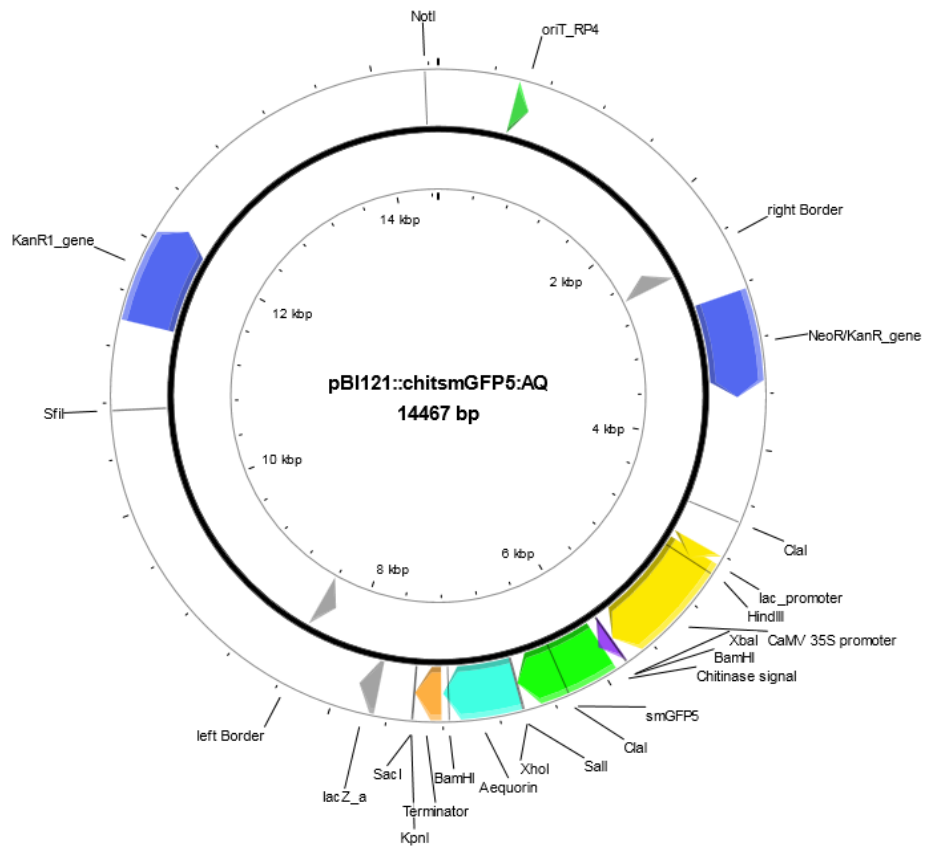


Fig. 1C* pBI121::chitRatioGFP:LAAQ				
	Fragments	Start	End	Category
	right Border	2478	2454	Miscellaneous
	CaMV 35S promoter	4974	5802	Promoter
	Chitinase signal	5844	5900	Localization Sequence
	ratiometric At-pHluorin	5907	6617	Gene
	low affinity Aequorin	6633	7196	Reporter Gene
	Terminator	7212	7413	Terminator
	left Border	8355	8331	Miscellaneous
	Restriction Sites cuts once / cuts twice	Location		Sequence
	BamHI	5822, 7155		ggatcc
	Cla I	4538, 6287		aagctt
	HindIII	4951		aagctt
	KpnI	6386, 7419		ggtacc
	NotI	14372		gcggccgc
	SacI (SstI)	7425		gagctc
	SalI	6618		gtcgac
	XbaI	5816		tctaga
	XhoI	6628		ctcgag

*refers to Figure 1 in Gao et al. 2004 (PubMed 15020753; DOI: 10.1104/pp.103.032508)

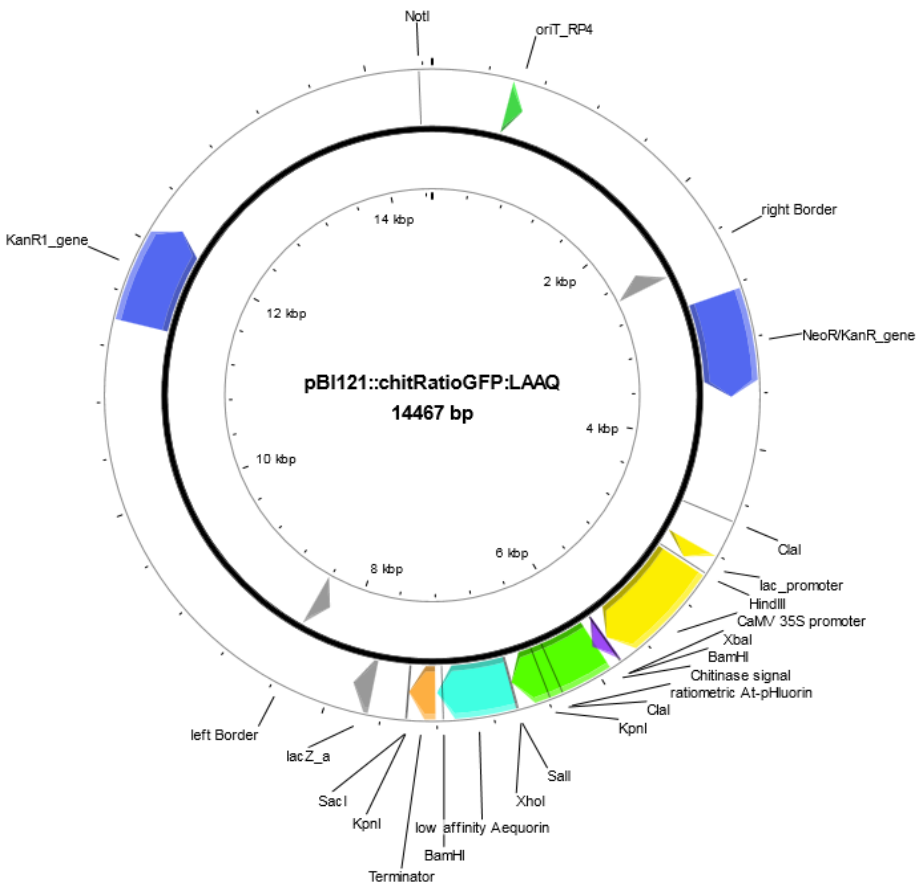


Fig. 1D* pBI121::chitEclipGFP:LAAQ				
	Fragments	Start	End	Category
	right Border	2478	2454	Miscellaneous
	CaMV 35S promoter	4974	5802	Promoter
	Chitinase signal	5844	5900	Localization Sequence
	ecliptic At-pHluorin	5907	6617	Gene
	low affinity Aequorin	6633	7196	Reporter Gene
	Terminator	7212	7413	Terminator
	left Border	8355	8331	Miscellaneous
	Restriction Sites cuts once / cuts twice	Location		Sequence
	BamHI	5822, 7155		ggatcc
	Cla I	4538, 6287		aagctt
	HindIII	4951		aagctt
	KpnI	7419		ggtacc
	NotI	14372		gcggccgc
	SacI (SstI)	7425		gagctc
	SalI	6618		gtcgac
	XbaI	5816		tctaga
	XhoI	6628		ctcgag

*refers to Figure 1 in Gao et al. 2004 (PubMed 15020753; DOI: 10.1104/pp.103.032508)

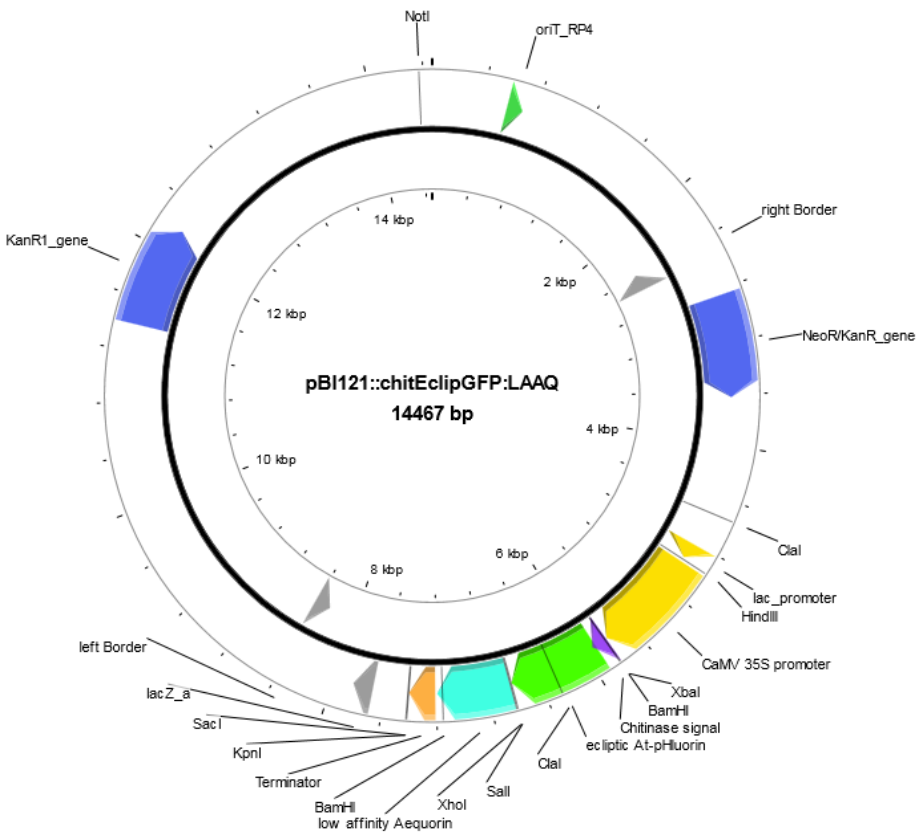


Fig. 1E* pBI121::smRatioGFP:AQ				
	Fragments	Start	End	Category
	right Border	2478	2454	Miscellaneous
	CaMV 35S promoter	4874	5802	Promoter
	ratiometric At-pHluorin	5825	6538	Gene
	Aequorin	6554	7117	Reporter Gene
	Terminator	7133	7334	Terminator
	left Border	8276	8252	Miscellaneous
	Restriction Sites cuts once / cuts twice	Location		Sequence
	BamHI	7076		ggatcc
	Cla I	4538, 6208		aagctt
	HindIII	4951		aagctt
	KpnI	6307, 7340		ggtacc
	NotI	14293		gcggccgc
	SacI (SstI)	7346		gagctc
	SalI	6539		gtcgac
	XbaI	5816		tctaga
	XhoI	6549		ctcgag

*refers to Figure 1 in Gao et al. 2004 (PubMed 15020753; DOI: 10.1104/pp.103.032508)

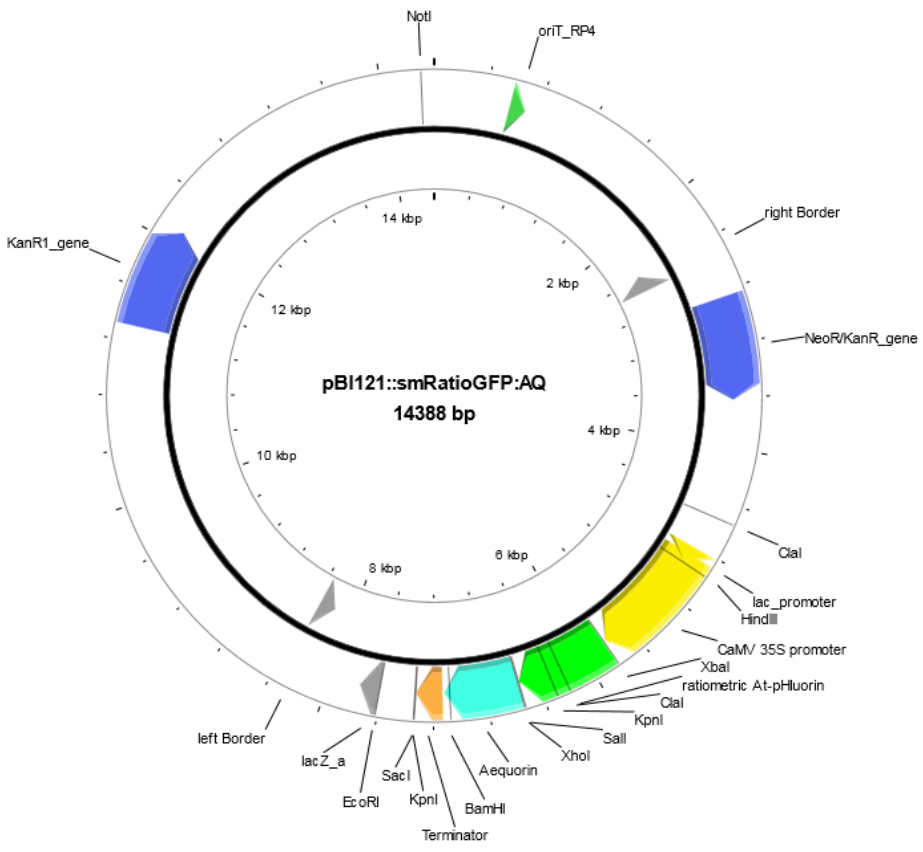
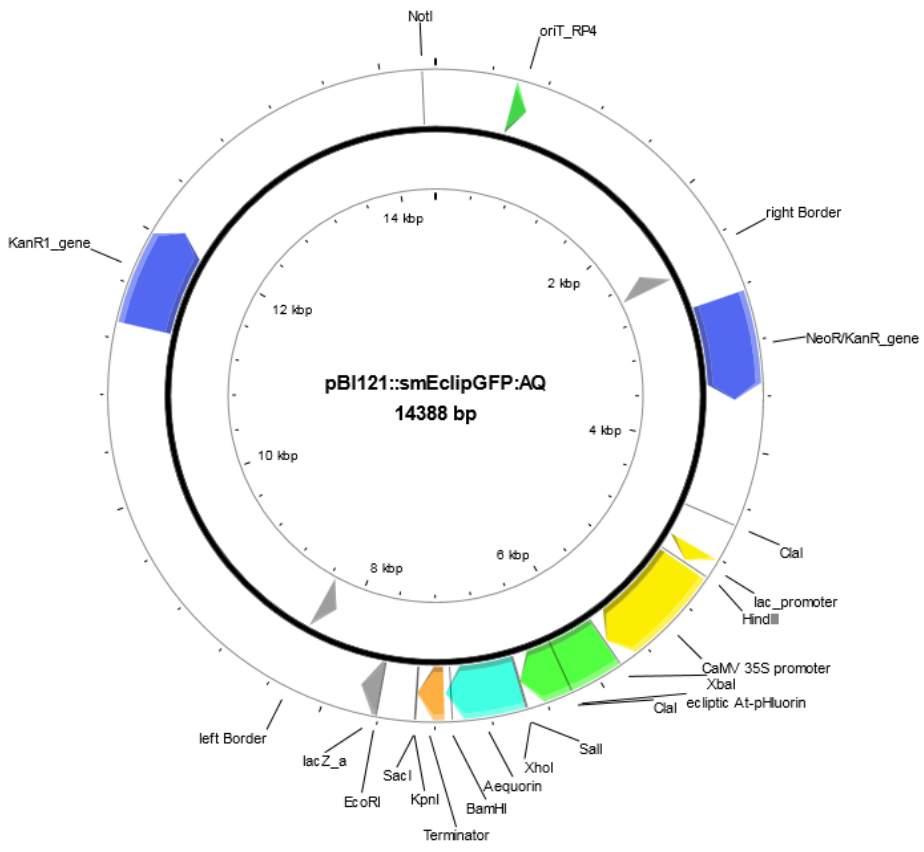


Fig. 1F* pBI121::smEclipGFP:AQ				
	Fragments	Start	End	Category
	right Border	2478	2454	Miscellaneous
	CaMV 35S promoter	4974	5802	Promoter
	ecliptic At-pHluorin	5825	6538	Gene
	Aequorin	6554	7117	Reporter Gene
	Terminator	7133	7334	Terminator
	left Border	8276	8252	Miscellaneous
	Restriction Sites cuts once / cuts twice	Location		Sequence
	BamHI	7076		ggatcc
	Cla I	4538, 6208		aagctt
	HindIII	4951		aagctt
	KpnI	7340		ggtacc
	NotI	14293		gcggccgc
	SacI (SstI)	7346		gagctc
	SalI	6539		gtcgac
	XbaI	5816		tctaga
	XhoI	6549		ctcgag

*refers to Figure 1 in Gao et al. 2004 (PubMed 15020753; DOI: 10.1104/pp.103.032508)



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