The Uses of GFP in Plants.

GFP: Green Fluorescent Protein Strategies and Applications.

Jim Haseloff and Kirby R. Siemering

Division of Cell Biology

MRC Laboratory of Molecular Biology

Hills Road.

Cambridge. England.

Marker genes have proved extremely useful for reporting gene expression in transformed plants. The ß-glucuronidase (GUS) gene (Jefferson *et al.* 1987) has been used extensively. Transformed cells or patterns of gene expression within plants can be identified histochemically, but this is generally a destructive test and is not suitable for assaying primary transformants, nor for following the time course of gene expression in living plants, nor as a means of rapidly screening segregating populations of seedlings. The green fluorescent protein (GFP) from *Aequorea victoria* shares none of these problems, as its intrinsic fluorescence can be seen in living cells, and there has been intense interest in its use as a marker for transgenic plants.

Unmodified *gfp* has been successfully expressed at high levels in tobacco plants using the cytoplasmic RNA viruses potato virus X (Baulcombe *et al.* 1995) and tobacco mosaic virus (Heinlein *et al.* 1995). In these experiments, the gene was directly expressed as a viral mRNA in infected cells, and spectacularly high levels of GFP fluorescence were seen.

In contrast to the efficient RNA virus-mediated expression of GFP, variable results have been obtained with transformed cells and plants. Although green fluorescence has been seen in *gfp* transformed protoplasts of citrus (Niedz *et al.* 1995) and maize (Hu *et al.* 1995; Sheen *et al.* 1995), we and others have seen no fluorescence in *Arabidopsis* and other transformed plant species (Haseloff *et al.* 1995; Haseloff *et al.* 1997). Hu and Cheng have reported that no signal was seen in *gfp* transformed *Arabidopsis thaliana* protoplasts. Reichel and colleagues also failed to dectect fluorescence in *gfp* transformed *Arabidopsis*, tobacco or barley protoplasts (Reichel *et al.* 1996). Sheen and colleagues also saw no expression of a CAB2-driven *gfp* gene in transgenic *Arabidopsis* plants (Sheen et al. 1995), and Pang *et al.* (Pang *et al.* 1996) saw little or no expression in *gfp* transformed wheat, corn, tobacco and *Arabidopsis* plants. There appears to be a need for substantial improvement of the wild-type *gfp*

gene for use in plants. In this chapter, we describe some of the pitfalls affecting *gfp* expression and detection in plants, and describe modified forms of the gene and new techniques which have helped to overcome these problems.

Cryptic splicing of *gfp* mRNA in *Arabidopsis*.

Useful expression of the *gfp* cDNA in plants requires that: (i) the GFP apoprotein be produced in suitable amounts within plant cells, and (ii) the non-fluorescent apoprotein undergoes efficient post-translational modification to produce the mature GFP. The high levels of GFP fluorescence seen in plants infected with suitable RNA virus vectors (Baulcombe et al. 1995; Heinlein et al. 1995) demonstrate that the protein can undergo efficient post-translational maturation in plants. However the expression of integrated copies of the gene has proved problematic. We have used *Agrobacterium* mediated transformation to produce transgenic *Arabidopsis* plantlets containing a cauliflower mosaic virus 35S promoter-driven *gfp* cDNA (Haseloff et al. 1997). However, at no stage during the transformation procedure did we detect GFP-related fluorescence, using UV lamp illumination and epifluorescence microscopy. Therefore we used PCR-based methods to verify the correct insertion of the gene, and to check mRNA transcription and processing in these transformed plantlets. DNA and mRNA samples were separately extracted, and *gfp* sequences were amplified via PCR from the separate extracts and analysed. While the expected full-length *gfp* product was obtained after amplification of the integrated gene, RT-PCR of *gfp* mRNA sequences gave rise to a truncated product.

The shortened RT-PCR product was cloned and sequenced, and a deletion of 84 nucleotides was found between residues 380-463 of the GFP coding sequence (Fig. 1). The missing sequence bears close similarity to known plant introns and it is likely that expression of *gfp* in *Arabidopsis* is curtailed by aberrant mRNA splicing, with an 84 nucleotide sequence being recognised as a cryptic intron. This explanation would also account for the efficient expression of *gfp* from RNA virus vectors which replicate in the cytoplasm, and thus evade splicing. The nucleotide sequences bordering the deletion are shown in Figure 1, and demonstrate similarity to known plant introns. Matches were found for sequences that are conserved at the 5' and 3' splice sites of plant introns (reviewed in (Luehrsen *et al.* 1994), and for conserved branchpoint nucleotides in plant introns (Liu *et al.* 1996; Simpson *et al.* 1996). The excised *gfp* sequence contains a high AU content (68%) that has also been shown to be important for recognition of plant introns (Hanley *et al.* 1988; Wiebauer *et al.* 1988; Goodall *et al.* 1989; Goodall *et al.* 1991) . It is likely that this 84 nucleotide region of the jellyfish *gfp* cDNA sequence is efficiently recognised as an intron when transcribed in *Arabidopsis*, resulting in an in-

frame deletion and the production of a defective protein product, which is predicted to be 28 amino acids shorter. Subsequently, an artifical neural network program has been used to correctly predict the presence of the *Arabidopsis* cryptic intron in the *gfp* coding sequence (Hebsgaard *et al.* 1996), and similar cryptic splicing has been seen now in other plant species (Mary Schuler, personal communication). It should be noted that the borders of the cryptic intron do not coincide with any of the natural spliced junctions found after processing of the *gfp* mRNA in *A. victoria* (Prasher *et al.* 1992). No full-length *gfp* mRNA is detectable by RT-PCR, and so misprocessing must be close to complete in transformed *Arabidopsis* plantlets. It has been claimed that *gfp* fluorescence has been detected in bombarded *Arabidopsis* tissues (Sheen et al. 1995) . However in these experiments, leaf tissue was treated with methanol prior to microscopic examination, where methanol causes rapid and irreversible bleaching of GFP (Ward *et al.* 1980). Local wounding due to particle bombardment can cause punctate patterns of bright autofluorescence, and this type of experiment needs to be interpreted with care. The same authors saw no expression of a CAB2-driven *gfp* gene in transgenic *Arabidopsis* plants (Sheen et al. 1995). It is likely that elimination of cryptic splicing is essential for proper expression of the *gfp* gene in *Arabidopsis*.

Removal of the cryptic intron.

It has proved necessary to destroy this cryptic intron to ensure proper expression in plants. We have altered the codon usage for GFP, deliberately mutating recognition sequences at the putative 5' splice site and branchpoint and decreasing the AU content of the intron. All of the sequence modifications affected only codon usage, and this modified gene, *mgfp4*, encodes a protein product which is identical to that of the jellyfish (Fig. 2). When the *mgfp4* sequence was inserted behind the 35S promoter and introduced into *Arabidopsis* using the root transformation technique (Valvekens *et al.* 1988), bright green fluorescent plant cells were detected within 2-3 days of cocultivation. As cell proliferation continued, the brightest clumps of callus and developing shoot tissue were so intensely fluorescent that they were clearly visible by eye, using a 100 Watt long wavelength hand-held UV lamp (UV Products, B100AP). We have also adapted an inverted fluorescence microscope (Leitz DM-IL) to allow more sensitive, higher magnification observation of cells in sterile culture during transformation and regeneration. The microscope was fitted with a filter set (Leitz-D excitation BP355-425, dichroic 455, emission LP460) suitable for the main 395nm excitation and 509nm emission peaks of GFP, and we have used a 7mm threaded extension tube with a 4x objective (EF 4/0.12) to give a greater working distance above the microscope stage. This allows the convenient direct observation of transformed tissues and plantlets within sealed inverted petri dishes.

The ease with which fluorescent proteins can be monitored in living tissues allows new approaches for improving transformation and regeneration of intractable or slow-growing plant species. During our own regeneration experiments, we observed a wide range of GFP fluorescence intensities in 35S-*mgfp4* transformed plantlets, which we expect arose from position-dependent modulation of gene expression in different transformants. It proved difficult to regenerate fertile plants from the brightest transformants, with cells remaining as a highly fluorescent callus or mass of shoots after several months of culture. It is possible that high levels of GFP expression were mildly toxic or interfered with differentiation. This is of special concern with a fluorescent molecule such as GFP, which would be expected to generate free radicals upon excitation, and which undergoes oxidative modification and could possess catalytic properties. The conditions that we have used for plant regeneration should provide a stringent test for any deleterious effect due to GFP. The 35S promoter was used to drive expression of the protein at high levels throughout the plant, including meristematic cells, and regeneration took place under continual illumination, allowing the possibility for GFP mediated phototoxicity. Despite poor regeneration of the brightest transformants, we managed to obtain over 50 separate transgenic *Arabidopsis* lines, most of which contained levels of GFP that were easily detectable by microscopy.

Expression of GFP in other plants.

Some expression of wild-type *gfp* has been seen in plant protoplasts of tobacco (Reichel et al. 1996), *Citrus sinensis* (Niedz et al. 1995) and maize (Hu et al. 1995; Sheen et al. 1995; Pang et al. 1996), and so aberrant splicing of *gfp* mRNA may not be as efficient in other plant species, as in transgenic *Arabidopsis*. However, the *mgfp4* gene has proved useful for expression studies in other plants, which share features involved in intron recognition (Luehrsen et al. 1994). Experiments with tobacco and barley protoplasts (Reichel et al. 1996) have demonstrated that *mgfp4* derived sequences are expressed at much higher levels than the wild-type gene in these species. The *mgfp4* is also expressed efficiently in soybean cells (Plautz *et al.* 1996). There have been reports of improved GFP expression in mammalian cells after alteration of gene codon usage (Haas *et al.* 1996; Zolotukhin *et al.* 1996). Increased levels of expression have been attributed to improved rates of translation due to optimised codon usage. However, this "humanization" of *gfp* also leads to alteration of the cryptic intron sequence, and expression of sGFP (Haas et al. 1996) been shown to result in 20-fold increased fluorescence in maize protoplasts (Chiu *et al.* 1996). The increased levels of expression may be due, at least in part, to an effect on RNA processing. Other workers have also found it necessary to deliberately alter the codon usage of *gfp* for efficient

expression in plants. Transient expression of the synthetic *pgfp* gene gave rise to about 20-fold more fluorescence than wild-type *gfp* in maize and tobacco protoplasts (Pang et al. 1996)).

It is possible that altered mRNA sequences affect post-transcriptional processing in animal cells also. However, introns found in animals, including *A. victoria* (Prasher et al. 1992), share a conserved polypyrimidine tract adjacent to the 3' splice site, reviewed in (Green 1991), and introns in yeast cells possess a requirement for additional conserved sequences (UACUAAC) located at the branch point (Langford *et al.* 1984). The lack of these additional features may help to minimise recognition of the cryptic intron and aberrant processing of *gfp* mRNA in fungal and animal cells.

Localization of GFP in plant cells.

In transgenic *Arabidopsis* cells, GFP is found throughout the cytoplasm, but appears to accumulate within the nucleoplasm (Haseloff et al. 1995; Chiu et al. 1996; Grebenok *et al.* 1997; Haseloff et al. 1997; Kohler *et al.* 1997). It is excluded from vacuoles, organelles and other bodies in the cytoplasm, and is excluded from the nucleolus (Fig 3). A similar subcellular distribution of GFP was seen in all *Arabidopsis* cell types examined in our experiments, and red autofluorescent chloroplasts provide an effective counter fluor for GFP in the upper parts of the plant. Cytoplasmic streaming and the movement of organelles could be observed in these living cells. In addition to cell ultrastructure, the architecture of the intact tissue was also clearly discernible, and the arrangement of different cell types could be seen in longitudinal optical sections of root tips and cotyledons. For example, cells within the epidermis of the cotyledon contain few mature chloroplasts and could be distinguished from layers of neighbouring mesophyll cells, and files of developing cells around the primary root meristem are clearly evident (Fig. 3).

While the *mgfp4* gene was proving useful as a marker in transgenic *Arabidopsis*, it was also clear from the initial studies that it could bear improvement. While we were able to generate 35S-*mgfp4* transformed cells and calli that were intensely fluorescent, and easily detectable by eye under long wavelength UV illumination, it proved difficult to regenerate fertile plants from the brightest transformants. It is possible that very high levels of GFP expression are mildly toxic or interfere with regeneration, perhaps due to the fluorescent or catalytic properties of the protein. In jellyfish photocytes, where high levels of GFP are well tolerated, the protein is found sequestered in cytoplasmic granules (Davenport *et al.* 1955). In contrast, the mature protein is found throughout the cytoplasm and accumulates within the

nucleoplasm of transformed *Arabidopsis* cells. If GFP is a source of fluorescence-related free radicals, for example, it might be advisable to target the protein to a more localised compartment within the plant cell.

Subcellular targeting of GFP

We have fused several targeting peptides to GFP, and directed the protein to different subcellular compartments. The targeted forms of the *mgfp4* gene were initially tested by expression in *Saccharomyces cerevisiae*. The modified genes were introduced into yeast cells on a multicopy vector and expressed fluorescent protein was visualised using confocal microscopy (Fig. 4). Unmodified protein is normally found throughout the cytoplasm and nucleoplasm of yeast cells. The addition of a peptide containing the SV40 T-antigen nuclear localisation signal (NLS, amino acids APKKKRKVEDPR) to the N- or C-terminus of the protein does little to alter its distribution (not shown). However, if the NLS-GFP protein is fused to a larger protein, such as that encoding ß-galactosidase, the fusion protein is exclusively found in the nucleoplasm (Fig.4D). We have also fused to GFP a mitochondrial targeting sequence from the yeast cytochrome C oxidase IV protein (amino acids MLSLRQSIRFFKPATRTLCSSR). This sequence confers mitochonrial localisation to GFP in both yeast (Fig 4B) and *Arabidopsis* cells. Kohler *et al.* (Kohler et al. 1997) have fused a similar localization sequence to *mgf4* and demonstrated the utility of the encoded fluorescent protein as a precise marker for mitochondria in *Arabidopsis*. In addition, we have fused the yeast carboxypeptidase Y (amino acids MKAFTSLLCGLGLSTTLAKA) and *Arabidopsis* basic chitinase (amino acids MKTNLFLFLIFSLLLSLSSA) signal sequences to GFP, and have sucessfully targeted the protein to the secretory pathway (Figs. 4C & 5).

It would be highly advantageous to produce relatively high levels of fluorescence for routine screening of GFP expression in transgenic plants and we tested the targeted forms of GFP in *Arabidopsis*. The modified genes were placed behind the 35S promoter, introduced into *Arabidopsis* by *Agrobacterium* mediated root transformation (Valvekens et al. 1988), and we tested for localisation of the protein and fluorescence intensity in regenerated plants. The one variant that showed a substantial improvement over unmodified GFP was one that was targeted to the endoplasmic reticulum (ER) (Haseloff et al. 1997). This targeted form of GFP contains an N-terminal signal peptide derived from an *Arabidopsis* vacuolar basic chitinase and the C-terminal amino acid sequence HDEL (Fig. 2), to ensure entry into the secretory pathway and retention of the protein within the lumen of the ER. Using this modified gene (*mgfp4-ER*), it has been possible to regenerate intensely fluorescent and fertile plantlets consistently. Fluorescence within these plants could be readily observed by eye

using a long wavelength UV lamp. The *mgfp4-ER* expressing plants were examined by confocal microscopy, and fluorescent protein was found mainly within the endomembrane system. The protein is excluded from the nucleus, shows a perinuclear distribution, and is found associated with the ER which forms a characteristic reticulate network in highly vacuolate cells. In highly cytoplasmic meristematic cells, the nuclei and orientation of cell divisions can be clearly distinguished. Localisation of the modified protein to cytoplasmic organelles was also evident, to what appear to be large leucoplasts or proplastids. For example, an optical section of a hypocotyl epidermal cell is shown in figure 5 and this includes a thin portion of cytosol which is pressed between the cell wall and vacuole. Such hypocotyl cells in *mgfp4-ER* transformed seedlings appear to contain a spectrum of developing plastids that range from the brightly green fluorescent to those which take on a yellow, orange or red appearance in dual channel confocal micrographs (Fig. 6). We presume that this is due to increasing chlorophyll synthesis, and that the green fluorescent plastids may be the maturing precursors of chloroplasts in these cells. These green fluorescent plastids are also found within the chloroplast-free epidermal cells of leaves and cotyledons, but are not found within the underlying mesophyll cells that are packed with mature chloroplasts. It seems likely that these organelles are proplastids and are capable of developing into chloroplasts, but we cannot exclude the possibility that they are some specialised form of leucoplast.

The accumulation of *mgfp4-ER* protein within leucoplasts or developing proplastids, in addition to its entry into the secretory pathway and retention in the endoplasmic reticulum, may indicate misrecognition of the N-terminal signal peptide. Proplastid accumulation of GFP is not seen in the 35S-*mgfp4* transformed plants. If the *mgfp4-ER* encoded signal peptide is inefficiently recognised prior to docking and cotranslational transport of the protein into the lumen of the ER, a proportion of GFP bearing fused terminal sequences may be produced in the cytoplasm. If so, it is possible that the neglected signal peptide may act as a transit sequence for plastid entry. Alternatively, there may be some direct exchange between developing plastids and the endomembrane system. We see no free cytoplasmic fluorescence, and the protein is sorted very efficiently to the ER or to plastids.

It is unclear whether the beneficial effects of targeting GFP to the ER are due to increased levels or safer accumulation of mature GFP within cells. For example, if accumulation of fluorescent protein leads to the generation of free radicals in illuminated cells, it is conceivable that removing GFP from the nucleus could protect cells from DNA damage due to such short-range highly reactive species. However, it is also possible that the fusion of peptide targeting sequences may improve the properties of the protein itself, or that the localisation of GFP to the lumen of the ER may improve

its maturation and accumulation. The maturation of the GFP apoprotein is sensitive to temperature, and the apoprotein readily misfolds under certain conditions(Siemering *et al.* 1996). The lumen of the ER is known to contain components, such as chaperones and peptidyl prolyl isomerases which aid protein folding (Fischer 1994), and secretion and retention of GFP within the ER may allow improved formation and accumulation of the mature fluorescent protein. These improvements have allowed us to routinely generate transgenic *Arabidopsis* plants that contain high levels of GFP fluorescence (Fig. 7).

Improved maturation of GFP

The green fluorescent protein is normally produced within photocytes of the jellyfish *Aequorea victoria,* and must undergo a series of post-translational maturation steps to produce the fluorescent form of the protein. Expression of GFP in a number of heterologous systems has been descibed as poor or variable. For example, strong promoters and decreased incubation temperatures have been required for efficient expression of *gfp* in mammalian cells (Kaether *et al.* 1995; Ogawa *et al.* 1995; Pines 1995). Other researchers have found that development of fluorescence is favoured by a lower incubation temperature during expression of GFP in bacteria (Webb *et al.* 1995) and yeast (Lim *et al.* 1995). These observations suggested that expression of GFP in heterologous cells may be far from optimal. We have clearly demonstrated that maturation the wild-type GFP is temperature sensitive, due to a defect in the folding of the GFP apoprotein. We have produced mutant forms of GFP that have improved folding and spectral properties (Siemering et al. 1996; Haseloff et al. 1997). These new GFPs are cured of the cryptic intron and are expressed brightly in plant cells.

The *mgfp4* gene was subjected to random mutagenesis, expressed in *E. coli* at 37°C and colonies were screened for increased fluorescence. Brighter mutants were isolated, and mapped by recombination with the wild-type *mgfp4* gene. Sequencing of the brightest mutant (GFPA) revealed two amino-acid differences, V163A and S175G. The mutant GFP produced up to 35-fold increased fluorescence in bacterial cells, while the difference in protein levels was not nearly enough to account for this. The result suggested that a large proportion of wild-type GFP that is expressed in cells at 37°C is non-fluorescent. Experiments with a GFP-nucleoplasmin fusion protein have indicated that maturation of GFP to the fluorescent form may be sensitive to temperature during expression in the yeast *Saccharomyces cerevisiae* (Lim et al. 1995). To test whether the same could be true of expression in *E. coli* and whether the substitutions present in GFPA enhance maturation by suppressing any such sensitivity, we examined expression of GFP and GFPA over a range of different temperatures. Strains

expressing GFP or GFPA were grown overnight at temperatures ranging between 25°C and 42°C. For each culture, the fluorescence values were measured and normalised against the amount of recombinant protein present in the cells to give a measure of the proportion of intracellular GFP that is fluorescent at different temperatures. The proportion of GFP that is fluorescent steadily decreases with increasing incubation temperature (Figure 8), indicating that either mature GFP or the maturation pathway leading to its formation is temperature sensitive. Mature GFP is a highly stable protein whose fluorescence *in vitro* is unaffected by temperatures up to 65°C (Bokman *et al.* 1981), and we have confirmed that the fluorescence of the mature protein is unaltered in bacterial cells at 42°C. Therefore, higher incubation temperatures must interfere with the post-translational maturation of GFP, rather than causing inactivation of the mature protein. We have confirmed that expression of GFP is also temperature-sensitive in yeast and demonstrated that this is suppressed by the substitutions present in GFPA. These results indicate that the thermosensitivity of GFP maturation may be a common phenomenon that can be suppressed by the amino acid substitutions present in GFPA (Siemering et al. 1996).

The post-translational maturation of GFP presumably involves initial folding of the apoprotein into an active conformation, to allow the cyclization and oxidation reactions that form the chromophore (Cody *et al.* 1993; Heim *et al.* 1994; Cubitt *et al.* 1995). The mature protein must then be correctly folded to maintain its fluorescent properties, to protect the chromophore from solvent effects(Ward et al. 1980). In principle, any of these processes could be sensitive to temperature and thus be responsible for the observed thermosensitivity of GFP during maturation.

Since the oxidation reaction involved in GFP chromophore formation appears to require molecular oxygen (Heim et al. 1994), oxidation rates can be measured after growth under anaerobic conditions, by measuring the development of fluorescence after admission of air. We measured the rates of oxidation of GFP and GFPA expressed in anaerobically grown yeast at both 25°C and 37°C. The time constant measured for the oxidation of GFP at 37°C (5.9 ± 0.1 min) was found to be approximately 3-fold faster than that measured at 25°C (16.2 ± 0.3 min), indicating that the post-translational oxidation of the GFP chromophore is not the step responsible for the temperature sensitivity of maturation. In confirmation of this conclusion, the time constants derived for GFPA were somewhat slower than those measured for GFP (Siemering et al. 1996).

In contrast, we have found that the folding of GFP is clearly temperature sensitive and the substitutions present in GFPA enhance proper folding at increased temperatures. We examined the solubilities of the two proteins during expression in *E.coli* at 25°C and 37°C. Fluorescence was found almost exclusively in the soluble

fraction. At 25°C, both GFP and GFPA were found predominantly in the soluble fraction, indicating that proper folding of both proteins is relatively efficient at this temperature. At 37°C, however, the majority of GFP was found as non-fluorescent protein in the insoluble fraction, whereas most of GFPA was still present in the soluble fraction. To obtain information on which species in the maturation pathway of GFP misfolds at higher temperatures, we examined the absorption spectrum of denatured protein isolated from inclusion bodies. If GFP undergoes cyclisation of the chromphore prior to aggregation, protein from inclusion bodies should show an absorption in the near UV/blue region that is characteristic of the GFP chromophore in either the mature or chemically reduced state (Ward et al. 1980; Inouye *et al.* 1994). On the other hand, if unmodified GFP (apo-GFP) is the aggregating species, no such absorption should be observable in this region. GFP was purified from the inclusion bodies of bacterial cells grown at 37°C and, as a positive control, from the soluble fraction of cells grown at 25°C. Protein derived from cells grown at 25°C showed a characteristic absorption peak similar to that of acid-denatured GFP (Ward *et al.* 1982). By contrast, protein purified from inclusion bodies of cells grown at 37°C showed no such absorption, indicating that the aggregating species has not formed a chromophore. Taken together, the results indicate that the temperature sensitivity of GFP maturation is due primarily to the failure of the unmodified apoprotein to fold into its catalytically active conformation at higher temperatures. Furthermore, the amino acid substitutions present in GFPA suppress this defect by enhancing proper folding at elevated temperatures.

Modification of fluorescence spectra.

The fluorescence excitation spectrum of GFP and GFPA exhibits peaks at wavelengths of 395nm and 475nm, with the 395nm peak predominating. This is a useful property for simple detection of the protein using a longwavelength UV source. UV illumination is not efficiently detected by the human eye and a suitable longwavelength UV lamp can be used to excite GFP for simple observation of transformed plant material without obscuring the green emission. However, efficient blue light excitation (around 470nm) is essential for use with imaging devices such as confocal microscopes or cell sorters which are equipped with argon laser sources.

Recently, it has been demonstrated that the relative amplitudes of the excitation peaks of GFP can be altered by means of mutagenesis (Heim et al. 1994; Delagrave *et al.* 1995; Ehrig *et al.* 1995; Heim *et al.* 1996). These mutations appear to affect the microenvironment of the chromophore so as to influence the equilibrium between two spectroscopic states of the chromophore (Heim et al. 1994; Ehrig et al. 1995). One of these mutations, I167T, has been shown to increase the amplitude of the 475 nm excitation peak relative to that of the 400 nm peak (Heim et al. 1994). We have

recombined the I167T substitution with the substitutions present in GFPA to increase the amplitude of the 475 nm peak relative to the 395 nm excitation peak, to produce a variant (GFP5) which has two excitation peaks (maxima at 395 nm and 473 nm) of almost exactly equal amplitude and an emission spectrum (λ_{max} = 507 nm) largely unchanged from that of wild-type. GFP5 retains a thermotolerant phenotype, and bacterial cells grown at 37ºC fluoresce 39-fold and 111-fold more intensely than cells expressing GFP, when excited at 395 nm and 473 nm, respectively.

The broad excitation spectrum of GFP5 allows both efficient UV and blue light excitation of the protein. For example, the expression of GFP5 gene fusions can be rapidly scored after transformation of microbial colonies or plant tissues by simple inspection with a UV lamp. The same material is well suited for laser scanning confocal microscopy. In addition, plants are highly autofluorescent and the use of a dual wavelength excitation mutant like GFP5 also enables faint signals to be easily distinguished from autofluorescence during microscopy, by alternating the excitation sources. For example chloroplasts are intensely fluorescent but are less efficiently excited by UV light. We routinely use long wavelength UV excitation for visual and microscopic screening of transformed tissues. Autofluorescence can also be an advantage. For example UV light excites a faint blue fluorescence in *Arabidopsis* cell walls, and this "counterstain" allows roots growing in agar culture to be easily located and scored for GFP fluorescence. We have recently screened several thousand *Arabidopsis* transformants for root specific "enhancer-trap" expression patterns, and this feature was very useful (J.H. & Sarah Hodge, unpublished results). In contrast, widely used GFP variants which contain the S65T mutation (Heim *et al.* 1995; Cormack *et al.* 1996) provide optimised properties for blue light excitation, but are not useful for detection by longwavelength UV light.

It is possible to manipulate the fluorescence spectra of GFPA by introducing additional substitutions into the protein without deleteriously affecting its improved folding characteristics. The Y66H substitution dramatically blue-shifts both the excitation and emission spectra of GFP to give a "blue fluorescent protein" (Heim et al. 1994). GFPA containing the Y66H substitution, was found to have identical fluorescence spectra to those of the corresponding GFP(Y66H) protein (excitation maximum = 384 nm, emission maximum = 448 nm), and gave rise to 29-fold more fluorescence when expressed at 37°C and 3-fold more fluorescence when expressed at 25°C. In addition, a number of workers have obtained GFP variants that show brighter fluorescence in heterologous cell types, and it is likely that the improved properties of these proteins is due largely to improved folding. For example, the V163A mutation present in GFPA has also been generated independently by at four different groups (Crameri *et al.* 1996; Davis *et al.* 1996; Heim et al. 1996; Kohler et al. 1997) and this

residue may play a pivotal role in folding of the protein. Cormack et al. (1996) have introduced random amino acid substituitions throughout the 20 residues flanking the chromophore of GFP. They used fluorescence activated cell sorting to select variants that fluoresced 20- to 35-fold more intensely than wild-type, and noted that the mutant proteins had improved solubility during expression in bacteria. The mutant proteins presumably have improved folding properties. One of these variants (GFPmut1, (Cormack et al. 1996)) contains two amino acid differences, F64L and S65T, located within the central α-helix of the protein, adjacent to the chromophore. The V163A and S175G mutations that we have isolated are positioned on the outer surface of the protein (Ormo *et al.* 1996; Yang *et al.* 1996). and recombination of these two sets of mutations appears to result in markedly improved fluorescence in bacterial, plant and animal cells (Zoenicka-Goetz *et al.* 1996; Zoenicka-Goetz *et al.* 1997) (J.H. & K.R.S., unpublished results). It is possible that the mutations affect separate steps of the folding or maturation process, and that their benefit is additive.

Modified GFP genes for plant expression.

Expression of the wild-type *gfp* gene has given poor results in a number of plant systems, and we have found it necessary to alter the gene for our experiments with transgenic *Arabidopsis* plants. As outlined above, we have (i) altered codon usage to remove a cryptic plant intron, (ii) added peptide sequences to allow targeting of the protein to the lumen of the endoplasmic reticulum, and mutated the protein to (iii) improve folding of the apo-GFP during post-translational maturation (V163A, S175G), and (iv) provide equalised UV and blue light excitation (I167T). These alterations have all been incorporated into a single highly active form of the gene (*mgfp5-ER*) which we now routinely use for monitoring gene expression and marking cells in live transgenic plants (Siemering et al. 1996; Haseloff et al. 1997).

Removal of the cryptic intron appears to be essential for *gfp* expression in *Arabidopsis*, and other workers have observed improved expression in plants using *gfp* genes containing "humanized" or synthetic codon usage (Chiu et al. 1996; Pang et al. 1996) Altered codon usage of the *gfp* gene appears to be a crucial requirement for efficient expression in plant cells. Improvements in the folding, spectral properties and subcellular localization of the protein provide secondary improvements which allow the accumulation of high levels of fluorescent protein in plant cells.

Imaging GFP in plant cells.

GFP can be visualised directly in living plant tissue, unlike commonly markers such as ß-glucuronidase, which require a prolonged and lethal histochemical staining procedure (Jefferson et al. 1987). GFP is therefore finding application in three broad

areas (1) for the dynamic visualization of labelled protein within the cells, and at a larger scale, (2) for the selective labelling and monitoring of whole plant cells within growing plant tissue, and (3) for the identification of individual transgenic plants expressing GFP. For example, different peptide domains can be fused to GFP to allow the decoration of particular structures within cells and/or to observe the subcellular distribution of the fusion protein. In addition, use of an active GFP marker gene allows transgenic cells to be scored by simple observation during a plant transformation experiment, throughout regeneration to the adult plant and its progeny. The use of tissue specific promoters to drive expression of GFP also allows the selective labelling of particular cell types within intact transformed plants. In these cases it is beneficial to express GFP at high levels within the marked cells to aid detection, and to minimise any deleterious effects of GFP expression. We have found the optimised *mgfp5-ER* gene very useful for this type of experiment. The dynamic properties of labelled cells or subcellular features can be resolved at high resolution in whole plant tissues using fluorescence microscopy techniques, however the use of intact tissue imposes some additional constraints on the imaging process.

Intact plant tissue proves a difficult subject for fluorescence microscopy as it consists of deep layers of highly refractile cell walls and aqueous cytosol and contains various autofluorescent and light scatttering components. There are two approaches to the difficulties imposed by these conditions: to fix and to clear the tissue with a high refractive index mounting medium, or to directly image living tissue using suitably corrected microscope optics. In our experience, has proved difficult to effectively clear *Arabidopsis* wholemounts without causing artifacts or losing GFP fluorescence, and there are considerable advantages to working with living tissues, so we have mainly pursued the second approach. The natural autofluorescence and depth of intact plant tissue means that out of focus blur often obscures high magnification views obtained with a conventional epifluorescence microscope. However, the technique of laser scanning confocal microscopy can be used to optically section GFP-expressing plant tissues. Confocal imaging allows precise visualisation of fluorescent signals within a narrow plane of focus, with exclusion of out-of-focus blur, and the technique permits the reconstruction of three dimensional structures from serial optical sections. *Arabidopsis* seedlings can simply be mounted in water for microscopy, and examined using a long-working distance water immersion objective to minimise the effects of spherical aberration when focusing deep into an aqueous sample (Haseloff et al. 1995). Young seedlings (3-7 days old) can be grown on agar culture media, and then placed in a drop of water (100-200ul) on a glass slide. A glass coverslip is lowered gently to flatten and cover the seedling. Even with the use of a specialised water immersion objective such as the Nikon 60x planapochromat, N.A. 1.2 (working distance 220

μm), image quality degrades rapidly for optical sections deeper than 60-80 μM within the tissue. Ideally, the tissue of interest should be positioned immediately below the cover slip and depression slides should be avoided unless this is ensured. Despite these limitations, the small size of *Arabidopsis* seedlings allows very useful imaging and, for example, median longitudinal optical sections can be easily obtained from intact root tips (e.g. Fig. 9).

The blue 488nm wavelength line of the commonly used argon ion or krypton-argon lasers is ideal for exciting GFP, and this can be used in combination with a fluorescein/rhodamine or texas red filter set for dual channel imaging of GFP and chlorophyll for photosynthetic tissues. For non-photosynthetic tissues, a fluorescent counterstain that can be distinguished from GFP is often very useful. For example *Arabidopsis* seedlings can be placed in a solution of 10ug/ml propidium iodide for 5-20 minutes, before being directly mounted in water for confocal microscopic examination of roots. Propidium iodide is red fluorescent and highly charged and does not enter living cells. It stains the walls of living cells within the root tip and fills dead cells (van der Berg *et al.* 1995) (Fig. 10). In a similar way the red fluorescent dye Nile Red can be used to stain neutral lipids, and rhodamine 6B can be used to stain the casparian strip and lignified cells within living roots (J.H., unpublished results).

Visualising subcellular dynamics

The expression of GFP within an organism produces an intrinsic fluorescence that colours normal cellular processes, and high resolution optical techniques can be used non-invasively to monitor the dynamic activities of these living cells. For time-lapse studies, it is very important that GFP fluorescence be bright, to minimize levels of illumination that can cause phototoxicity and photobleaching during observation. The modified *mgfp5-ER* gene that is described above has proved very useful for generating highly fluorescent transgenic *Arabidopsis* plants that are suitable for intensive time-lapse studies. During confocal microscopy experiments, we have routinely observed high rates of cytoplasmic streaming within living specimens, and we have used short term time-lapse observations to gain a better understanding of the relative movements of cellular components. *Arabidopsis* seedlings that expressed the *mgfp5-ER* gene at high levels were simply mounted in water for confocal microscopy, which allowed observation for up to two hours. Hypocotyl epidermal cells form ideal specimens for viewing the various components of the cytoplasm. The cells are large, highly vacuolate and surface borne. An extremely thin layer of cytoplasm is squeezed between the wall and the vacuole of these cells. This greatly limits the movement of cytoplasmic components to within a single plane of focus of the microscope, and objects can be rapidly tracked across a portion of the cell without the need for refocusing. A seedling

can easily be mounted so that the hypocotyl is pressing closely against the microscope coverslip, and the layer of cytoplasm beneath the outer wall of an epidermal cell will be only a few microns from the surface, allowing high optical resolution.

To follow rapid movements in cells it is necessary to use a correspondingly fast sampling rate. We have collected time-lapse confocal images at up to 2 frames per second, which requires almost continual laser scanning with a Biorad MRC-600 microscope. Living specimens have been examined for up to an hour without appreciable phototoxic or bleaching effects, but this is only possible with bright samples which allow attenuation of the exciting laser light. A short segment of a time-lapse experiment is shown in figure 11. A section of hypocotyl epidermis was monitored at a rate of 0.5 frames per second for about 20 minutes and representative confocal images are shown for a 4.5 minute period. Cellular components are clearly recognisable in the optical sections, and their identity is indicated in a schematic diagram (Fig. 11A). The cells contain green fluorescent proplastids and highly reticulate endomembranes. The nuclei are outlined due to the peripheral distribution of the ER, and the reticulate surface of a nucleus can be seen in the cell that is central to the field of view. A cross-section of a nucleus can also be seen in the adjacent lower cell. Chloroplasts are red autofluorescent, and characteristically small and spheroid in these hypocotyl epidermal cells.

When a time course of images is played at video rate proplastids and what appears to be vesicular material move vigorously and erratically through the cells. The distribution of all proplastids was plotted frame by frame though this experiment and the path of one example is shown in figure 11 B. These plastids move with uneven velocities, up to 20 microns per second, along irregular paths that may correspond to underlying cytoskeletal elements such as actin. In contrast, the endoplasmic reticulum, which is presumably associated with cortical microtubules, undergoes relatively slower rearrangement. A relatively stable feature of the ER is indicated with an arrow in figure 11, panels 0:00 to 0:25, while nearby proplastids undergo substantial movement. Chloroplasts and nuclei moved only slowly during the 20 minute time course of the experiment. These cells contain an ER retained form of GFP, and we expect the protein to be cycled in vesicles between the lumen of the ER and the *cis* golgi. A rapid and irregular movement of small vesicle-like particles is seen throughout cells during the time course. Although these small movements are difficult to see in still images, we also see the transient formation of extended filamentous structures (Fig. 11, panel 3:40), which are comprised of a larger amount of this fluorescent material, and are associated with rapid movement of both vesicular-like material and proplastids. The location of the *cis* golgi in these micrographs is unclear, although small regions of punctate fluorescence can be seen associated with endomembranes.

Marking cells within the plant.

The expression of GFP can be limited to particular cell types within a plant, to provide a means for visualizing the behaviour of individual cells within the living organism. We have developed a scheme for targeted gene expression in plants, which is based on a method widely used in *Drosophila* (Brand *et al.* 1993). We have randomly inserted a gene for a foreign transcription activator (a derivative of the yeast GAL4 gene) into the *Arabidopsis* genome using *Agrobacterium tumefaciens*-mediated transformation. We have thus generated a library of *Arabidopsis* lines which each express the GAL4 derivative in a particular pattern, dependent on adjacent genomic DNA sequences. The inserted DNA has also been engineered to contain a GAL4-responsive *mgfp5-ER* gene, and so interesting patterns of GAL4 gene expression are immediately and directly visible, with each GAL4-expressing cell marked by green fluorescence within the endoplasmic reticulum. Importantly, GAL4 expression within these lines allows precise targeted ectopic gene expression. A chosen target gene can be cloned under the control of a GAL4 responsive promoter, separately transformed into *Arabidopsis*, and maintained silently in the absence of GAL4. Genetic crossing of this single line with any of the library of GAL4-containing lines allows specific activation of the target gene in particular tissue and cell types, and the phenotypic consequences of mis-expression, including those lethal to the organism, can be conveniently studied (J.H., Sarah Hodge, Howard M. Goodman, unpublished results).

We have used *in vivo* detection of GFP to develop a new and efficient enhancer-trap screening procedure. As our particular interest is in the cells of the *Arabidopsis* root tip, we have modified the plant transformation protocol to include an auxin induction of roots from regenerating shootlets. More than 7500 transformants were then generated, planted in grid patterns in sterile culture dishes and directly screened by fluorescence microscopy for GAL4-driven GFP expression within roots. Several hundred lines with interesting patterns of root expression were choosen, documented, transferred to soil and grown to seed, to both amplify and self-hybridise the lines. As a result, we have a collection of 250 *Arabidopsis* lines with distinct and stable patterns of GAL4 and GFP expression in the root. These GAL4-GFP lines provide a valuable set of markers, where particular cell types are tagged and can be visualised with unprecedented ease and clarity in living plants.

The GAL4-GFP enhancer-trap screen was designed to yield markers for the *Arabidopsis* root meristem, which is our choice of a model system. The simple and well characterised architecture of the root (Dolan *et al.* 1993) enables simple analysis of GAL4-mediated perturbations of cell fate within the meristem. The *Arabidopsis* root

meristem consists of a plate of quiescent cells surrounded by initials that divide to produce distal root cap cells, and also lay down continuous cell files proximally. Behind the tip, the newly formed cells of the root undergo differentiation and expansion to build a conserved arrangement of cell types within the mature root. We have generated GAL4-GFP lines which precisely mark particular cell types within the meristem, and one example is shown in Fig. 11. *Arabidopsis* line J0571 exhibits GAL4-directed expression of GFP within the cortex and endodermis of the root, including the initials shared by these two cell files. 5 day old seedlings can be briefly counterstained with 10 µg/ml propidium iodide and mounted in water for confocal microscopy. Optical sectioning allows very simple and precise imaging of the GFP-labelled cells within the root meristem (Fig. 11a). The behaviour of these cells within the developing root meristem can be observed using time-lapse techniques. GFP-expressing seedlings can be planted in sterile agar media and grown in coverslip-based vessels. The roots grow down through the media and then along the surface of the coverslip. The roots are then ideally positioned for microscopic imaging through the base of the vessel. A series of images are shown in Fig. 11b that illustrate 2 hours during the growth of a root tip of *Arabidopsis* line J0571. Confocal optical sections were collected at 2 minute intervals. The cortical and endodermal cell files and their initials are clearly seen due to the expression of GAL4-driven *mgfp5-ER* in this line. The localization of GFP to the endoplasmic reticulum, and its consequent perinuclear distribution, ensures that the cell nuclei are clearly evident in these meristematic cells. In addition, the processes of cell division can be seen within the living plant. The breakdown of the nuclear membrane, segregation of chromosomes and formation of the daughter nuclei and cell wall plate are reflected in changes of the distribution of the ER-localized GFP. Also, the cell nuclei appear to possess a larger volume prior to cell division, consistent with an extra, newly replicated DNA complement. This may be a useful character for scoring DNA replication within living tissues.

Such GFP-expressing lines allow the simple, non-invasive observation of events within living plants at an unprecedented level of detail. GFP can now be used as a cellular marker to illuminate the defective behaviour of mutant plants, or the perturbations induced by reverse genetic techniques.

Conclusions

In order to overcome problems with the expression of GFP in plant cells, and with the safe accumulation and detection of GFP in whole *Arabidopsis* plants, we have engineered improvements to the *gfp* gene. The modified gene contains (i) altered codon

usage to remove a cryptic plant intron, (ii) added peptide sequences to allow targeting of the protein to the lumen of the endoplasmic reticulum, and mutations which (iii) improve folding of the apoprotein during post-translational maturation (V163A, S175G), and (iv) provide equalised UV and blue light excitation (I167T). This highly modified variant (*mgfp5-ER*) is proving useful as a safe and bright marker in transgenic plants. We expect that the *mgfp5-ER* gene and its derivatives will also be useful in work with transgenic fungi and animals, where at least some similar problems may be encountered.

A major use for green fluorescent protein will be as a replacement for the ß-glucuronidase gene, which is widely used as a reporter for promoter and gene fusions in transformed plants. The GUS gene product can be localised or quantified using histochemical techniques, but these are generally destructive tests (Jefferson et al. 1987). In contrast, GFP can be directly seen in living tissues. For example, high levels of fluorescence intensity are obtained in GFP-transformed bacterial and yeast colonies, allowing simple screening for GFP expression with the use of a hand-held UV lamp. Such an assay for gene expression in living plants will be a very useful tool for plant transformation and breeding experiments. Many transformation techniques give rise to regenerating tissues which are variable or chimeric, and require testing of the progeny of the primary transformants. Potentially, GFP expressing tissues could be monitored using *in vivo* fluorescence, avoiding any need for destructive testing, and the appropriate transformants could be rescued and directly grown to seed. Similarly, *in vivo* fluorescence will be an easily scored marker for field testing in plant breeding, allowing transgenes linked to the GFP gene to be easily followed, and provide a potential alternative to antibiotic resistance markers.

Unlike enzyme markers, green fluorescent protein can be visualised at high resolution in living cells using confocal microscopy. The images are not prone to fixation or staining artifacts, and can be of exceptional clarity. Moreover, the activities of living cells, such as cytoplasmic streaming, are clearly evident during microscopy. Ordinarily, movement within a sample is a nuisance, placing constraints on the use of sometimes lengthy techniques for noise reduction during confocal microscopy, such as frame averaging. However,we have shown that it is also possible to monitor dynamic events by time-lapse confocal microscopy, and this combination of a vital fluorescent reporter with high resolution optical techniques shows much promise for use in cell biological and physiological experiments.

Genetic systems such as that of *Arabidopsis* provide a large resource of potentially informative mutants, and there has been much recent improvement in techniques for determining the molecular basis of a particular phenotype. The use of fluorescent proteins will provide a further tools for examining the biology of mutant cells. The ability to simply and precisely monitor both particular cells and subcellular structures that have been highlighted with a fluorescent signal will improve both the screening for particular abnormal phenotypes and the characterisation of dynamic processes.

References.

Baulcombe, D.C., Chapman, S. and Cruz, S.S. 1995. Jellyfish green fluorescent protein as a reporter for virus infections. *Plant Journal* 7:1045-1053.

Bokman, S.H. and Ward, W.W. 1981. Renaturation of *Aequorea* green fluorescent protein. *Biochemical and Biophysical Research Communications* 101:1372-1380.

Brand, A.H. and Perrimon, N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118:401-415.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W. and Prasher, D.C. 1994. Green fluorescent protein as a marker for gene expression. *Science* 263:802-805.

Chiu, W.L., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H. and Sheen, J. 1996. Engineered GFP as a vital reporter in plants. *Current Biology* 6:325-330.

Cody, C.W., Prasher, D.C., Westler, W.M., Prendergast, F.G. and Ward, W.W. 1993. Chemical-structure of the hexapeptide chromophore of the aequorea green-fluorescent protein. *Biochemistry* 32:1212-1218.

Cormack, B.P., Valdivia, R.H. and Falkow, S. 1996. FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* 173:33-38.

Crameri, A., Whitehorn, E.A., Tate, E. and Stemmer, W.P.C. 1996. Improved green fluorescent protein by molecular evolution using dna shuffling. *Nature Biotechnology* 14:315-319.

Cubitt, A.B., Heim, R., Adams, S.R., Boyd, A.E., Gross, L.A. and Tsien, R.Y. 1995. Understanding, improving and using green fluorescent proteins. *Trends Biochem. Sci.* 20:229-233.

Davenport, D. and Nichol, J.A.C. 1955. Luminescence in Hydromedusae. *Proceedings of the Royal Society, Series B.* 144:399-411.

Davis, S. and Viestra, R. 1996. Soluble derivatives of green fluorescent protein (GFP) for use in *Arabidopsis thaliana*. *Weeds World* 3:43-48.

Delagrave, S., Hawtin, R.E., Silva, C.M., Yang, M.M. and Youvan, D.C. 1995. Red-shifted excitation mutants of the green fluorescent protein. *Bio-Technology* 13:151-154.

Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K. and Scheres, B. 1993. Cellular organisation of the *Arabidopsis thaliana* root. *Development* 119:71-84.

Ehrig, T., Okane, D.J. and Prendergast, F.G. 1995. Green fluorescent protein mutants with altered fluorescence excitation spectra. *Febs Letters* 367:163-166.

Fischer, G. 1994. Peptidyl-prolyl isomerases and their effectors. *Angew. Chem. Int. Ed. Engl.* 33:1415-1436.

Goodall, G.J. and Filipowicz, W. 1989. The AU-rich sequences present in the introns of plant nuclear pre-mRNAs are required for splicing. *Cell* 58:473-483.

Goodall, G.J. and Filipowicz, W. 1991. Different effects of intron nucleotide composition and secondary structure on pre-mRNA splicing in monocot and dicot plants. *The EMBO Journal* 10:2635-2644.

Grebenok, R.J., Pierson, E., Lambert, G.M., Gong, F.C., Afonso, C.L., Haldeman-Cahill, R., Carrington, J.C. and Galbraith, D.W. 1997. Green fluorescent protein fusions for efficient characterization of nuclear targeting. *Plant J.* 11:573-586.

Green, M.R. 1991. Biochemical mechanisms of constitutive and regulated pre-mRNA splicing. *Annual Reviews of Cell Biology* 7:559-599.

Haas, J., Park, E.C. and Seed, B. 1996. Codon usage limitation in the expression of hiv-1 envelope glycoprotein. *Current Biology* 6:315-324.

Hanley, B.A. and Schuler, M.A. 1988. Plant intron sequences: evidence for distinct groups of introns. *Nucleic Acids Research* 16:7159-7176.

Haseloff, J. and Amos, B. 1995. GFP in plants. *Trends in Genetics* 11:328-329.

Haseloff, J., Siemering, K.R., Prasher, D.C. and Hodge, S. 1997. Removal of a cryptic intron and subcellular localisation of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. *Proc. Natl. Acad. Sci. U.S.A.* 94:2122-2127.

Hebsgaard, S.M., Korning, P.G., Tolstrup, N., Engelbrecht, J., Rouze, P. and Brunak, S. 1996. Splice site prediction in *Arabidopsis thaliana* pre-mRNA by combining local and global sequence information. *Nucleic Acids Research* 24:3439-3452.

Heim, R., Cubitt, A.B. and Tsien, R.Y. 1995. Improved green fluorescence. *Nature* 373:663-664.

Heim, R., Prasher, D.C. and Tsien, R.Y. 1994. Wavelength mutations and posttranslational autoxidation of green fluorescent protein. *Proceedings Of the National Academy Of Sciences Of the United States Of America* 91:12501-12504.

Heim, R. and Tsien, R.Y. 1996. Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy-transfer. *Current Biology* 6:178-182.

Heinlein, M., Epel, B.L., Padgett, H.S. and Beachy, R.N. 1995. Interaction of tobamovirus movement proteins with the plant cytoskeleton. *Science* 270:1983-1985.

Hu, W. and Cheng, C.L. 1995. Expression of *Aequorea* green fluorescent protein in plant cells. *FEBS Letters* 369:331-334.

Inouye, S. and Tsuji, F.I. 1994. Evidence for redox forms of the *Aequorea* green fluorescent protein. *Febs Letters* 351:211-214.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. 1987. GUS fusions: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO Journal* 6:3901-3907.

Kaether, C. and Gerdes, H.H. 1995. Visualization of protein-transport along the secretory pathway using green fluorescent protein. *Febs Letters* 369:267-271.

Kohler, R.H., Zipfel, W.R., Webb, W.W. and Hanson, M.R. 1997. The green fluorescent protein as a marker to visualize plant mitochondria *in vivo. Plant J.* 11:613-621.

Langford, C.J., Klinz, F.-J., Donath, C. and Gallwitz, D. 1984. Point mutations identify the conserved, intron-contained TACTAAC box as an essential splicing signal in yeast. *Cell* 36:645-653.

Lim, C.R., Kimata, Y., Oka, M., Nomaguchi, K. and Kohno, K. 1995. Thermosensitivity of green fluorescent protein fluorescence utilized to reveal novel nuclear-like compartments in a mutant nucleoporin nsp1. *Journal Of Biochemistry* 118:13-17.

Liu, H.X. and Filipowicz, W. 1996. Mapping of branchpoint nucleotides in mutant pre-mRNAs expressed in plant cells. *The Plant Journal* 9:381-389.

Luehrsen, K.R., Taha, S. and Walbot, V. 1994. Nuclear pre-mRNA processing in higher plants. *Progress in Nucleic Acid Researc and Molecular Biology* 47:149-193.

Niedz, R.P., Sussman, M.R. and Satterlee, J.S. 1995. Green fluorescent protein - an in-vivo reporter of plant gene- expression. *Plant Cell Reports* 14:403-406.

Ogawa, H., Inouye, S., Tsuji, F.I., Yasuda, K. and Umesono, K. 1995. Localization, trafficking, and temperature-dependence of the aequorea green fluorescent protein in cultured vertebrate cells. *Proceedings Of the National Academy Of Sciences Of the United States Of America* 92:11899-11903.

Ormo, M., Cubitt, A.B., Kallio, K., Gross, L.A., Tsien, R.Y. and Remington, S.J. 1996. Crystal structure of the *Aequoria victoria* green fluorescent protein. *Science* 273:1392-1395.

Pang, S.Z., DeBoer, D.L., Wan, Y., Ye, G., Layton, J.G., Neher, M.K., Armstrong, C.L., Fry, J.E., Hinchee, M.A.W. and Fromm, M.E. 1996. An improved green fluorescent protein gene as a vital marker in plants. *Plant Physiol.* 112:893-900.

Pines, J. 1995. GFP in mammalian cells. *FEBS Lett.* 11:326-327.

Plautz, J.D., Day, R.N., Dailey, G.M., Welsh, S.B., Hall, J.C., Halpain, S. and Kay, S.A. 1996. Green fluorescent protein and its derivatives as versatile markers for gene expression in living *Drosophila melanogaster*, plant and mammalian cells. *Gene* 173:83-87.

Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G. and Cormier, M.J. 1992. Primary structure of the aequorea-victoria green-fluorescent protein. *Gene* 111:229-233.

Reichel, C., Mathur, J., Eckes, P., Langenkemper, K., Reiss, B., Koncz, C., Schell, J. and Maas, C. 1996. Enhanced green fluorescence by the expression of an *Aequorea victoria* green fluorescent protein mutant in mono- and dicotyledonous plant cells. *Proceedings of the National Academy of Sciences USA* 93:5888-5893.

Sheen, J., Hwang, S.B., Niwa, Y., Kobayashi, H. and Galbraith, D.W. 1995. Green-fluorescent protein as a new vital marker in plant-cells. *Plant Journal* 8:777-784.

Siemering, K.R., Golbik, R., Sever, R. and Haseloff, J. 1996. Mutations that supress the thermosensitivity of green fluorescent protein. *Current Biology* 6:1653-1663.

Simpson, C.G., Clark, G., Davidson, D., Smith, P. and Brown, J.W.S. 1996. Mutation of putative branchpoint consensus sequences in plant introns reduces splicing efficiency. *The Plant Journal* 9:369-380.

Valvekens, D., Van Montagu, M. and Van Lijsebettens, M. 1988. *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proceedings of the National Academy of Sciences U.S.A.* 85:5536-5540.

van der Berg, C., Willemsen, V., Hage, W., Wiesbeek, P. and Scheres, B. 1995. Cell fate in the *Arabidopsis* root meristem determined by directional signalling. *Nature* 378:62-65.

Ward, W.W. and Bokman, S.H. 1982. Reversible denaturation of *Aequorea* green-fluorescent protein: physical separation and characterization of the renatured protein. *Biochemistry* 21:4535-4540.

Ward, W.W., Cody, C.W., Hart, R.C. and Cormier, M.J. 1980. Spectrophotomeric identity of the energy transfer chromophores in Renilla and Aequorea green fluorescent proteins. *Photochemistry and Photobiology* 31:611-615.

Webb, C.D., Decatur, A., Teleman, A. and Losick, R. 1995. Use of green fluorescent protein for visualization of cell-specific gene expression and subcellular protein localization during sporulation in *Bacillus subtilis*. *J. Bacteriol.* 177:5906-5911.

Wiebauer, K., Herrero, J.-J. and Filipowicz, W. 1988. Nuclear pre-mRNA processing in plants: distinct modes of 3'-splice-site selection in plants and animals. *Molecular & Cellular Biology* 8:2042-2051.

Yang, F., Moss, L.G. and Phillips, G.N.J. 1996. The molecular structure of green fluorescent protein. *Nature Biotech.* 14:1246-1251.

Zoenicka-Goetz, M., Pines, J., Ryan, K., Siemering, K.R., Haseloff, J., Evans, M.J. and Gurdon, J.B. 1996. An indelible lineage marker for *Xenopus* using a mutated green fluorescent protein. *Development* 122:3719-3724.

Zoenicka-Goetz, M., Pines, J., Siemering, K.R., Haseloff, J. and Evans, M.J. 1997. Following cell fate in the living mouse embryo. *Development* 124:1133-1137.

Zolotukhin, S., Potter, M., Hauswirth, W., Guy, J. and Muzyczka, N. 1996. A "humanized" green fluorescent protein cDNA adapted for high level expression in mammalian cells. *Journal of Virology* 70:4646-4654.

Fig. 1. Cryptic splicing of *gfp* transcripts in transgenic *Arabidopsis thaliana*.

(*A*) Restriction endonuclease digestion of PCR fragments derived from *gfp* DNA and mRNA sequences. Sequences corresponding to the integrated *gfp* gene and to mRNA transcripts were isolated and separately amplified using PCR techniques, and incubated with various restriction endonucleases. The radiolabelled fragments were fractionated by electrophoresis in a 5% polyacrylamide gel, and are shown labelled with the source of the amplified sequences (DNA or mRNA) and the name of the restriction endonuclease used for digestion, or not (uncut). The mRNA derived sequences appeared to lack sites for *Dra* I and *Acc* I, and to contain a corresponding deleted region of 80-90 nucleotides. Restriction endonuclease fragments that are smaller than those expected of the gene sequence have been indicated with a white asterisk.

(B) Sequence analysis of cloned *gfp* mRNAs. Autoradiograph and sequence of the amplified *gfp* mRNA sequence. Nucleotides 380-463 are absent from the transcribed sequence, and the site of this 84 nucleotide deletion is arrowed.

(C) Schematic diagram of the gfp gene sequence which shows the positions of restriction endonuclease sites used for the analysis of PCR amplified mRNA transcripts, and the location of the cryptic intron, shown with dark shading. Sequences that are similar to those normally found at splice sites and branchpoints of plant introns. are shown below. Splice sites are arrowed, and the putative lariat branchpoint is shown in reverse type (Haseloff et al. 1997)

Fig. 2. Sequence comparison of *gfp* and the modified *mgfp5-ER* gene.

The sequence of *gfp* is as described for the *gfp10* cDNA (Prasher et al. 1992), except that codon 80 contains a change from CAG to CGG resulting in replacement of a glutamine with arginine, as noted by Chalfie *et al.* (Chalfie *et al.* 1994). Both the *gfp* and *mgfp5-ER* gene cassettes are flanked by restriction endonuclease sites for *Bam*HI and *Sac*I, a ribosome binding site (RBS) for bacterial expression and the sequence AACA upstream of the start codon for improved plant translation. The cryptic plant intron present in *gfp* (Haseloff et al. 1997) is shown

underlined with the 5' and 3' splice sites arrowed. Nucleotide sequence alterations present in *mgfp5-ER* are shown outlined in grey. Most alterations are silent and all amino acid substitutions are shown in reverse type below the nucleotide sequence. The *mgfp5-ER* gene cassette contains additional sequences shown in bold type, which comprise a 5' terminal signal peptide and 3' HDEL sequence. An *Eco*RI site was used to link the signal peptide and coding sequences.

Fig. 3. Confocal images of 35S-*mgfp4* transformed *Arabidopsis* plants.

35S-*mgfp4* transformed seedlings were grown in sterile agar culture and mounted intact in water for confocal microscopy. Images were collected using a Biorad MRC-600 instrument equipped with Nikon Optiphot microscope and Nikon planapo 60x water immersion lens. GFP and chlorophyll were excited using the 488nm and 568nm lines, respectively, of a 25nm krypton-argon ion laser. The green and red emissions were collected in separate channels and combined using Adobe Photoshop.

(A). The shoot apical meristem is shown. Individual vacuolate cells that each contain a layer of green fluorescent cytoplasm containing red autofluorescent chloroplasts, can be distingished. (B). A emerging leaf primordia, positioned at the shoot apex between two cotyledons. (C). Mesophyll cells within a cotyledon, show large numbers of mature chloroplasts. (D). An optical section of a single hypocotyl cortex cell, showing mature chloroplasts. (E). Cells from within the root meristem. GFP accumulates within nuclei, but is excluded from nucleoli, and is found throughout the cytoplasm where various endomembrane compartments are shown in negative relief. (F). Median longitudinal optical section of a root tip.

Fig. 4. Localisation of GFP in yeast.

Various peptide targeting sequences were fused to GFP in order to direct the protein to different subcellular compartments. The modified proteins were expressed in yeast cells and visualised by confocal microscopy. (A). Unmodified GFP is found throughout the cytoplasm and nucleoplasm. (B). Fusion of N-terminal sequences from yeast

cytochrome oxidase B subunit IV **(aa seq)** results in mitochondrial localisation of GFP. (C). N-terminal fusion of the signal sequence from yeast carboxypeptidase Y **(aa seq)** and C-terminal fusion of the amino acids HDEL results in retention of GFP within the endoplasmic reticulum. (D). N-terminal fusion of a nuclear localisation sequence (NLS) from the SV40 T-antigen **(aa seq)** ensures nuclear import of GFP, however only larger forms of GFP are efficiently retained within the nucleus. In this case NLS-GFP has been fused to *E. coli* ß-galactosidase. This results in exclusive localisation of the fusion protein within nuclei. The outlines of the yeast cells, obtained using phase contrast optics, are superimposed in this image

Fig. 5. Images of 35S-*mgfp4-ER* transformed seedlings.

The ER-localised form of GFP was visualized in transgenic *Arabidopsis* seedlings using the procedures described for Figure 2. (A). An optical section of the apical meristem showing the junction of the hypocotyl and cotyledons, and (B). an emerging first leaf. (C). Cells within the mesophyll of a cotyledon, packed with mature red autofluorescent chloroplasts. (D). A view of epidermal cells within the hypocotyl, showing the reticulate distribution of GFP within the endomembrane system and the appearance of green fluorescence within maturing plastids. Mature chloroplasts are brightly red autofluorescent in these cells. (E). Cells within the root meristem clearly display the characteristic perinuclear distribution expected for the ER-localised GFP. This is also seen in the shoot (panel A). (F). Median longitudinal optical section of a root tip.

Fig. 6. Subcellular distribution of mgfp4-ER.

The distribution of GFP and GFP-ER is shown in cells of the hypocotyl epidermis of transformed *Arabidopsis*. The cytosol forms a thin layer at the periphery of these highly vacuolate cells. The cytoplasmic form (*mgfp*) is excluded from endomembrane components and plastids within the cytosol, and forms a negative stain for these components. A single chloroplast, with its red fluorescent chlorophyll contents, can be

seen in this image. Several non-fluorescent cigar-shaped bodies that appear to be some kind of plastids are also evident. In contrast, *mgfp-ER* is found within the endoplasmic reticulum and unexpectedly within the plastid-like organelles. The distribution of these labelled plastids is mainly limited to epidermal cells of the shoot, and varying degrees of chlorophyll fluorescence can be detected within the organelles, indicating that they may be developing pro-plastids.

Fig. 7. Transgenic 35S-*mgfp4-ER Arabidopsis* seedlings. 5-day old wild-type (left) and 35S-*mgfp4-ER* transgenic (right) seedlings were mounted in water on a Leitz DM-IL inverted fluorescence microscope and illuminated with long wavelength UV light (Leitz-D filter set, excitation BP355-425, dichroic 455, emission LP460). Seedlings were visualized using a 4x objective (EF 4/0.12) and a Sony DXC-930P video camera with F100-MPU framestore. A montage of the entire seedlings was assembled from collected video images using Adobe Photoshop.

Fig. 8. Improved mutants of GFP.

(A). Improved thermotolerance of GFP. Bacterial cells expressing GFP and GFPA (V163A, S175G) were grown at different temperatures. GFP fluorescence values were measured and normalized with respect to the amount of intracellular recombinant protein for cultures grown at 25°C, 30°C, 37°C and 42°C. (Siemering et al. 1996)

(B). Excitation and emission spectra of GFP, GFPA (V163A, S175G) and GFP5 (V163A, I167T, S175G). Protein concentrations were 23.5 µg/ml in PBS (pH 7.4). All spectra have been normalised to a maximum value of 1.0. (Siemering et al. 1996)

Fig. 9. Dual channel imaging of GFP/chlorophyll/propidium iodide

A Biorad MRC-600 confocal microscope equipped with an argon-krypton mixed gas laser and K1/K2 filter blocks was used for greeen and red fluorescence imaging of GFP expressing Arabidopsis seedlings. (A). Separate images of a 35S-*mgfp4* transformed cotyledon mesophyll cell showing the green fluorescence channel with the

GFP signal distributed throughout the nucleoplasm and cytoplasm, the red fluorescent signal of the chloroplasts, and the combined dual channel image. There is little spillover between the two channels. (B). Separate green, red and combined fluorescence images are also shown for a propidium iodide stained root tip of a GFP-expressing enhancer trap line J0571 (J. Haseloff & S. Hodge, unpublished results). The *mgfp5-ER* gene is expressed strongly in the root cortex and endodermis of this line. Propidium iodide provides a distinct counterfluor which outlines cells in the living root tip.

Fig. 10. Time-lapse confocal microscopy of subcellular processes.

A transgenic *Arabidopsis* seedling expressing the *mgfp5-ER* gene was mounted in water and a small segment of the hypocotyl epidermis was observed using a Biorad MRC-600 laser scanning confocal microscope. The laser light was attenuated by 99% using a neutral density filter, and the confocal aperture was stopped down. Two channel, single scan images were collected at the rate of 1 per 2 seconds for 20 minutes, and transferred to an Apple Macintosh computer. The large data file was then converted to full-colour numbered PICT files using the program PicMerge, and finally converted to a Quicktime movie for analysis and video rate playback. A section corresponding to 4.5 minutes of the original observation was chosen and representative frames are presented here. Each frame is marked with the time (minutes:seconds) that had elapsed from the first chosen frame. Two schematic diagrams are shown. A key for the identities of cellular structures and organelles is shown in digram A. Nuclei (N), chloroplasts (C) endoplasmic reticulum (ER), proplastids or leucoplasts (P) and the position of the cell wall (CW) are shown (scale bar = 10 microns). In the second digram (B), The positions of proplastids throughout the 4.5 minute period of the experiment is shown. The plastids were located in each frame, and their cumulative positions within the cell were plotted frame by frame, indicated by black dots. The position of one plastid is also plotted, with a series of red lines representing the successive orientations of the long axis of the plastid. This particular organelle is indicated by a white cross on the timed confocal images, from its appearance in the

field of view at 0:20. The images 0:00 to 0:25 each contain an arrow which indicates a ring-like feature within the ER that provides a morphological landmark. Image 3:40 contains an arrow which indicates the formation of a transient filamentous structure that appears associated with rapid vesicular and plastid traffic.

Fig. 11. Time-lapse confocal microscopy of root development.

Seeds of the *Arabidopsis* enhancer trap line J0571 (J Haseloff & S. Hodge, unpublished results) were germinated and grown in agar medium on a coverglass. After 10 days of growth, an emerging lateral root was visualized by confocal time-lapse microscopy. The root tip was imaged through the coverglass of the tissue culture vessel. A median longitudinal optical section was collected every 2 minutes over a 6 hour period. (A). Representative frames from a 320 minute period are shown, labelled with the time of collection (min: sec). Cell divisions and growth of the labelled cortex and endodermis cell layers are evident. Individual cells in the process of mitotic division are arrowed. One endodermis cell is marked with an asterisk, and its behaviour is shown in more detail. (B). Frames collected at shorter intervals are shown for the marked cell. The times of image collection are indicated (min: sec). The ER-localization of the GFP marker allows clear visualization of nuclear division and phragmoplast formation in these cells.

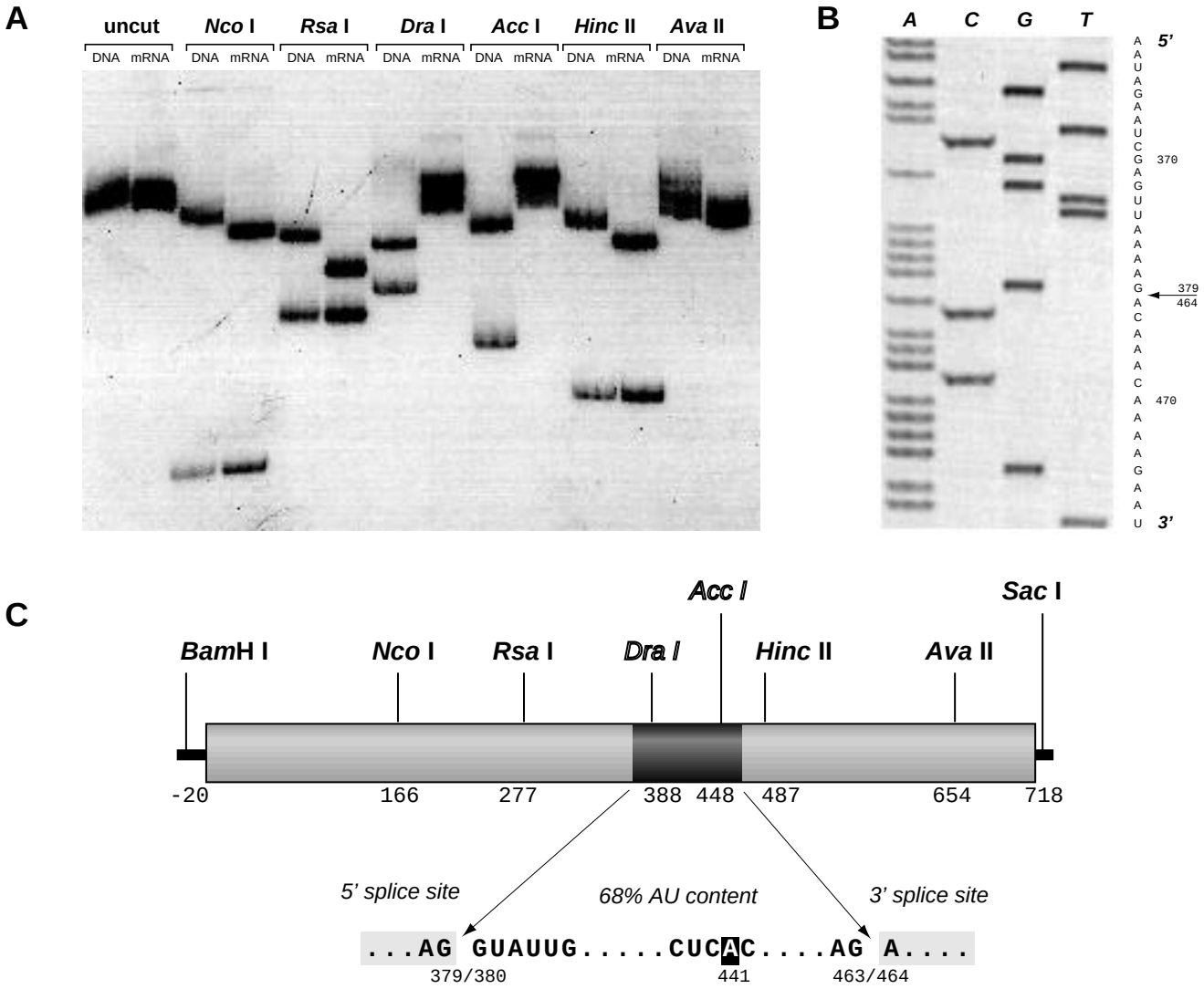
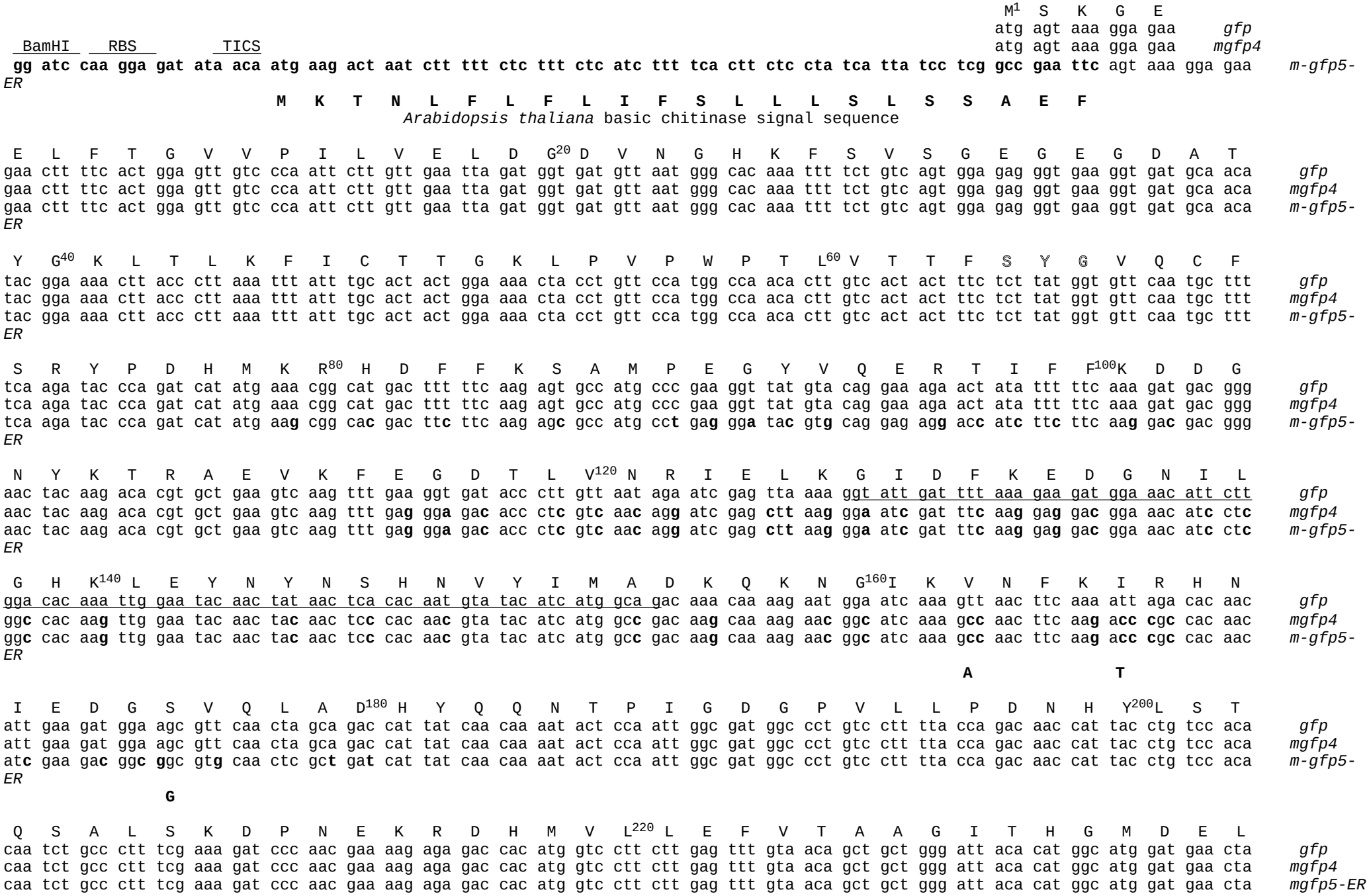
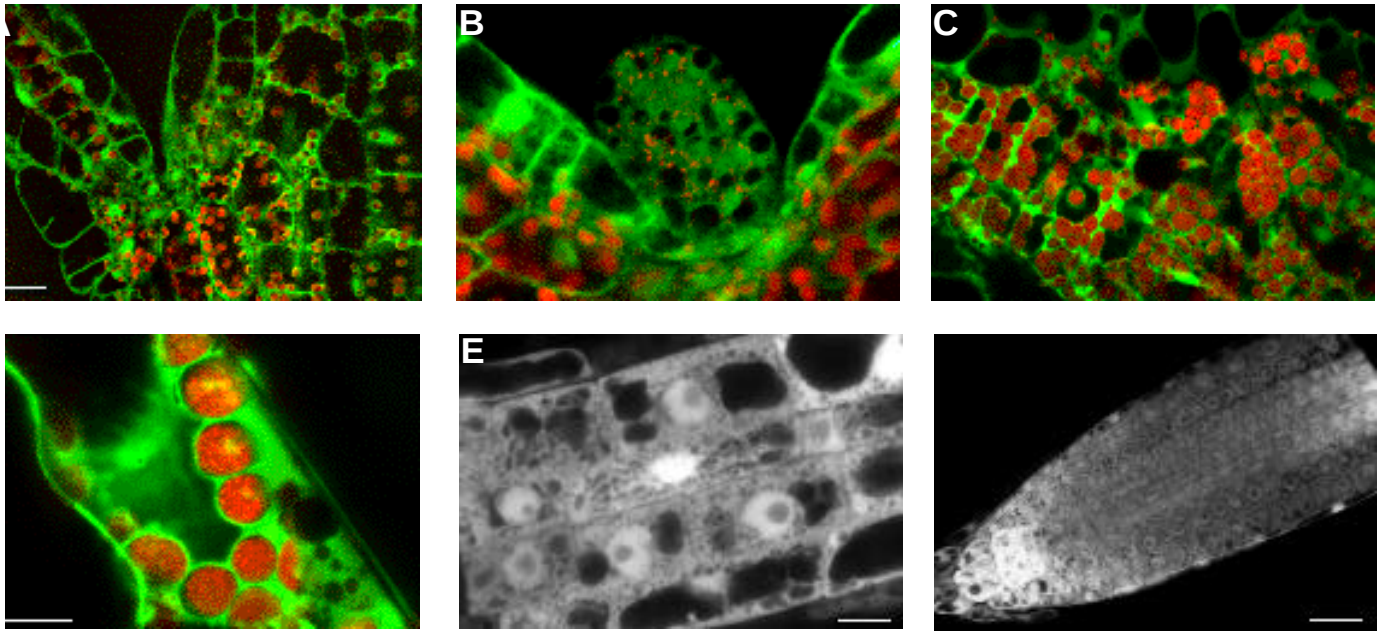
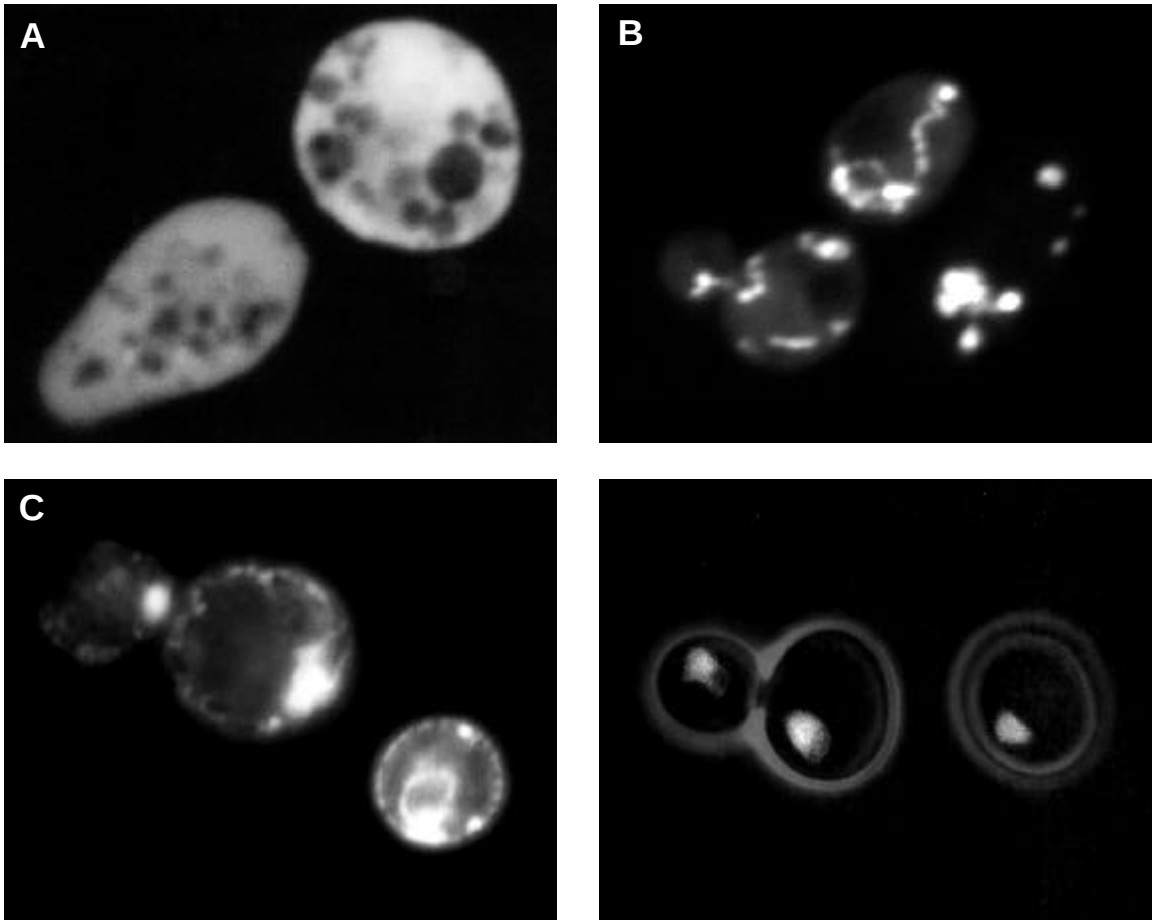
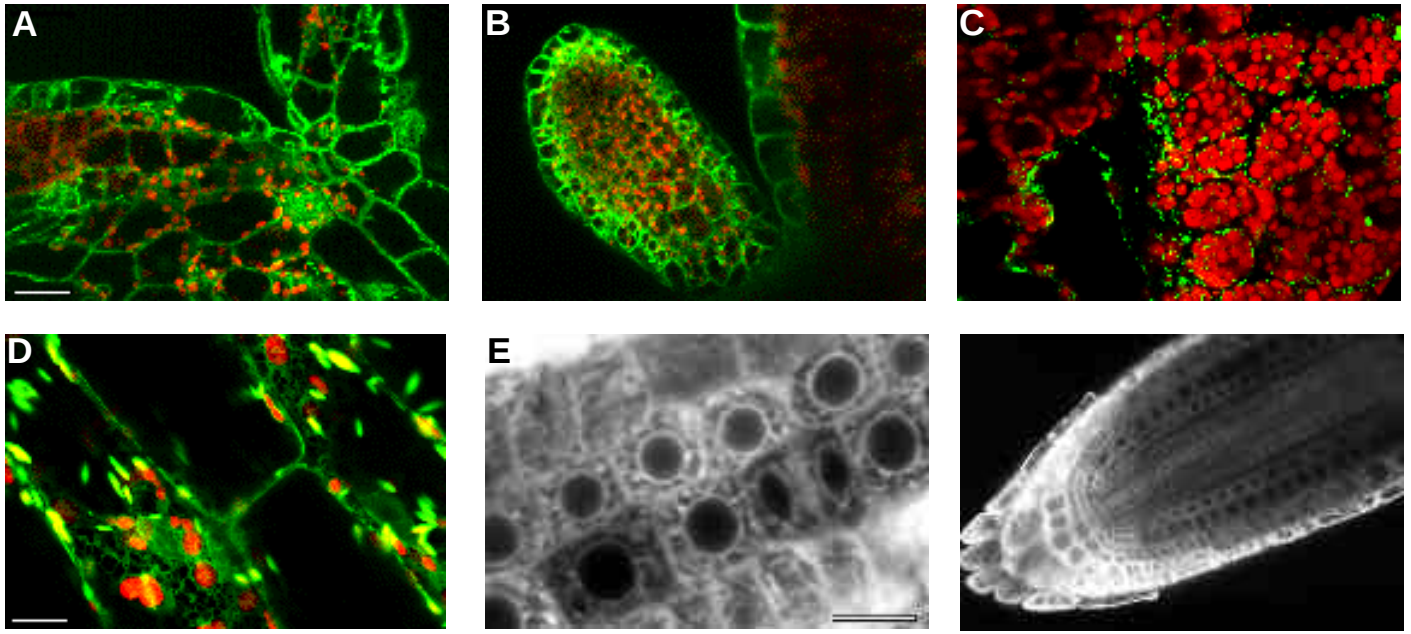

A
uncut
Nco I
Rsa I
Dra I
Acc I
Hinc II
Ava II
DNA mRNA
B
A C G T
5'
370
379
464
470
3'
C
Acc I
Sac I
BamH I
Nco I
Rsa I
Dra I
Hinc II
Ava II
-20
166
277
388 448
487
654
718
5' splice site
68% AU content
3' splice site
...AG GUAUUG.....CUCAC....AG A....
379/380
441
463/464

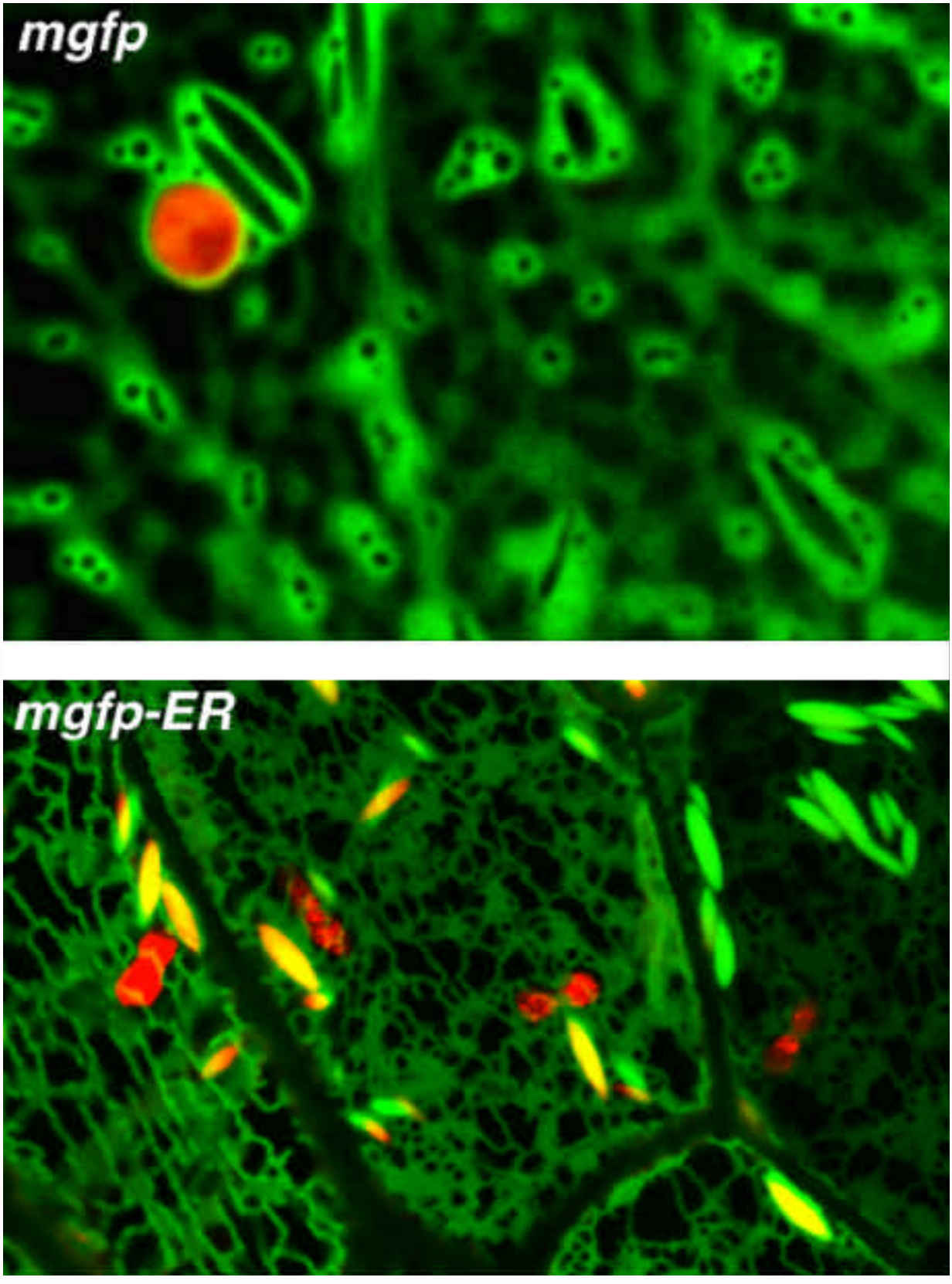
FIG. 2

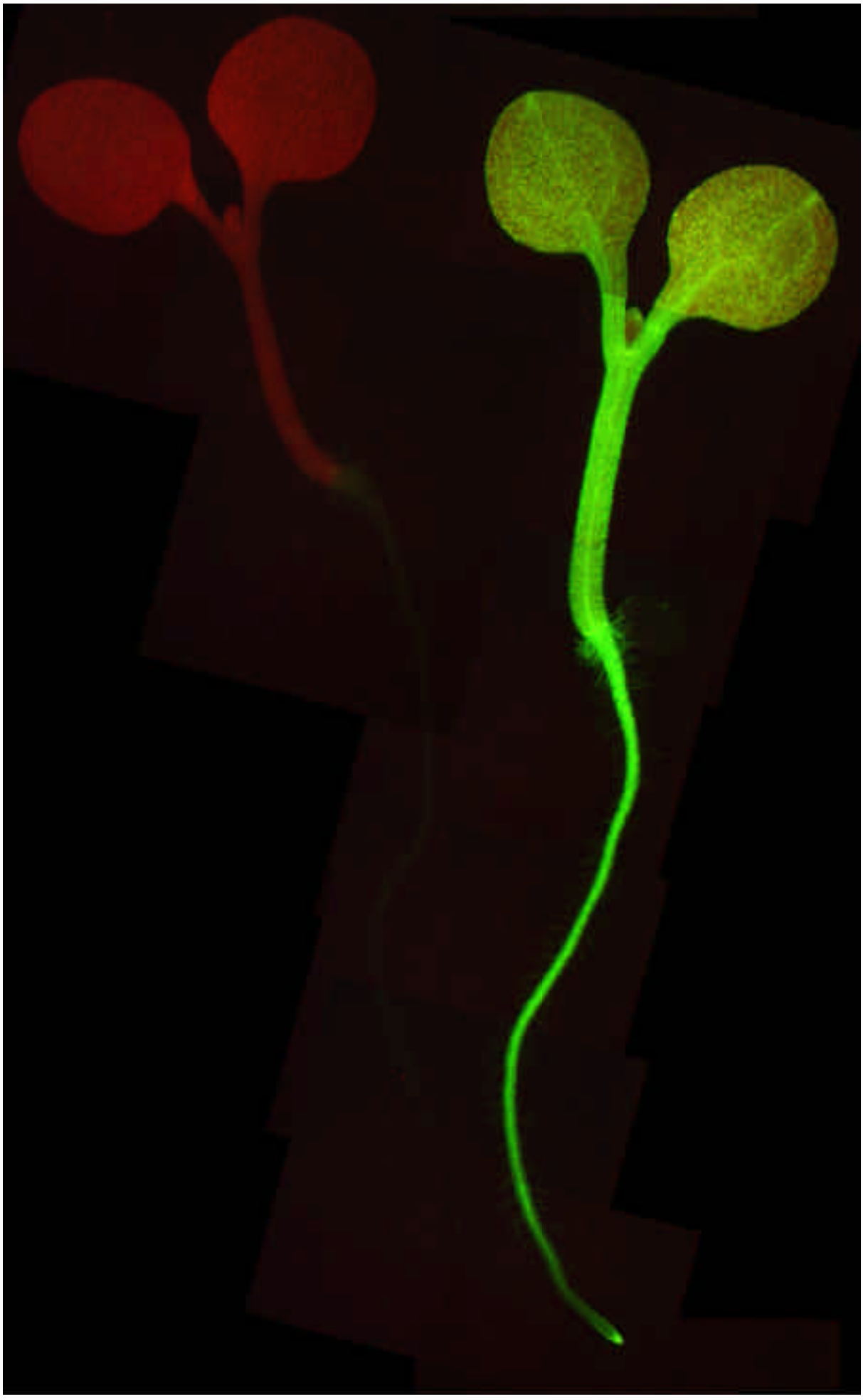

BamHI
RBS
TICS
M1 S K G E
atg agt aaa gga gaa gfp
atg agt aaa gga gaa mgfp4
gg atc caa gga gat ata aca atg aag act aat ctt ttt ctc ttt ctc atc ttt tca ctt ctc cta tca tta tcc tcg gcc gaa ttc agt aaa gga gaa m-gfp5-ER
M K T N L F L F L I F S L L L S L S S A E F
Arabidopsis thaliana basic chitinase signal sequence
E L F T G V V P I L V E L D G20 D V N G H K F S V S G E G E G D A T
gaa ctt ttc act gga gtt gtc cca att ctt gtt gaa tta gat ggt gat gtt aat ggg cac aaa ttt tct gtc agt gga gag ggt gaa ggt gat gca aca gfp
gaa ctt ttc act gga gtt gtc cca att ctt gtt gaa tta gat ggt gat gtt aat ggg cac aaa ttt tct gtc agt gga gag ggt gaa ggt gat gca aca mgfp4
gaa ctt ttc act gga gtt gtc cca att ctt gtt gaa tta gat ggt gat gtt aat ggg cac aaa ttt tct gtc agt gga gag ggt gaa ggt gat gca aca m-gfp5-ER
Y G40 K L T L K F I C T T G K L P V P W P T L60 V T T F S Y G V Q C F
tac gga aaa ctt acc ctt aaa ttt att tgc act act gga aaa cta cct gtt cca tgg cca aca ctt gtc act act ttc tct tat ggt gtt caa tgc ttt gfp
tac gga aaa ctt acc ctt aaa ttt att tgc act act gga aaa cta cct gtt cca tgg cca aca ctt gtc act act ttc tct tat ggt gtt caa tgc ttt mgfp4
tac gga aaa ctt acc ctt aaa ttt att tgc act act gga aaa cta cct gtt cca tgg cca aca ctt gtc act act ttc tct tat ggt gtt caa tgc ttt m-gfp5-ER
S R Y P D H M K R80 H D F F K S A M P E G Y V Q E R T I F F100K D D G
tca aga tac cca gat cat atg aaa cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa aga act ata ttt ttc aaa gat gac ggg gfp
tca aga tac cca gat cat atg aaa cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa aga act ata ttt ttc aaa gat gac ggg mgfp4
tca aga tac cca gat cat atg aag cgg cac gac ttc ttc aag agc gcc atg cct gag gga tac gtg cag gag agg acc atc ttc ttc aag gac gac ggg m-gfp5-ER
N Y K T R A E V K F E G D T L V120 N R I E L K G I D F K E D G N I L
aac tac aag aca cgt gct gaa gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt att gat ttt aaa gaa gat gga aac att ctt gfp
aac tac aag aca cgt gct gaa gtc aag ttt gag gga gac acc ctc gtc aac agg atc gag ctt aag gga atc gat ttc aag gag gac gga aac atc ctc mgfp4
aac tac aag aca cgt gct gaa gtc aag ttt gag gga gac acc ctc gtc aac agg atc gag ctt aag gga atc gat ttc aag gag gac gga aac atc ctc m-gfp5-ER
G H K140 L E Y N Y N S H N V Y I M A D K Q K N G160I K V N F K I R H N
gga cac aaa ttg gaa tac aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat gga atc aaa gtt aac ttc aaa att aga cac aac gfp
ggc cac aag ttg gaa tac aac tac aac tcc cac aac gta tac atc atg gcc gac aag caa aag aac ggc atc aaa gcc aac ttc aag acc cgc cac aac mgfp4
ggc cac aag ttg gaa tac aac tac aac tcc cac aac gta tac atc atg gcc gac aag caa aag aac ggc atc aaa gcc aac ttc aag acc cgc cac aac m-gfp5-ER
A T
I E D G S V Q L A D180 H Y Q Q N T P I G D G P V L L P D N H Y200L S T
att gaa gat gga agc gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc cct gtc ctt tta cca gac aac cat tac ctg tcc aca gfp
att gaa gat gga agc gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc cct gtc ctt tta cca gac aac cat tac ctg tcc aca mgfp4
atc gaa gac ggc ggc gtg caa ctc gct gat cat tat caa caa aat act cca att ggc gat ggc cct gtc ctt tta cca gac aac cat tac ctg tcc aca m-gfp5-ER
G
Q S A L S K D P N E K R D H M V L220 L E F V T A A G I T H G M D E L
caa tct gcc ctt tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt gta aca gct gct ggg att aca cat ggc atg gat gaa cta gfp
caa tct gcc ctt tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt gta aca gct gct ggg att aca cat ggc atg gat gaa cta mgfp4
caa tct gcc ctt tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt gta aca gct gct ggg att aca cat ggc atg gat gaa cta mgfp5-ER

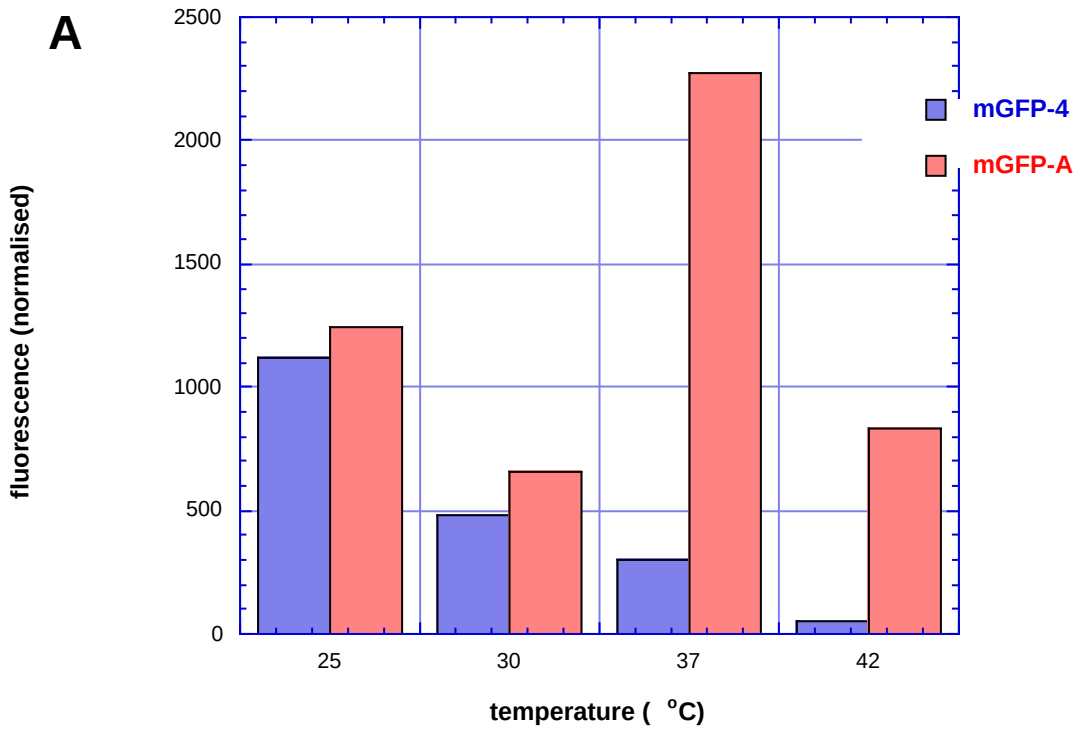

B
C
E

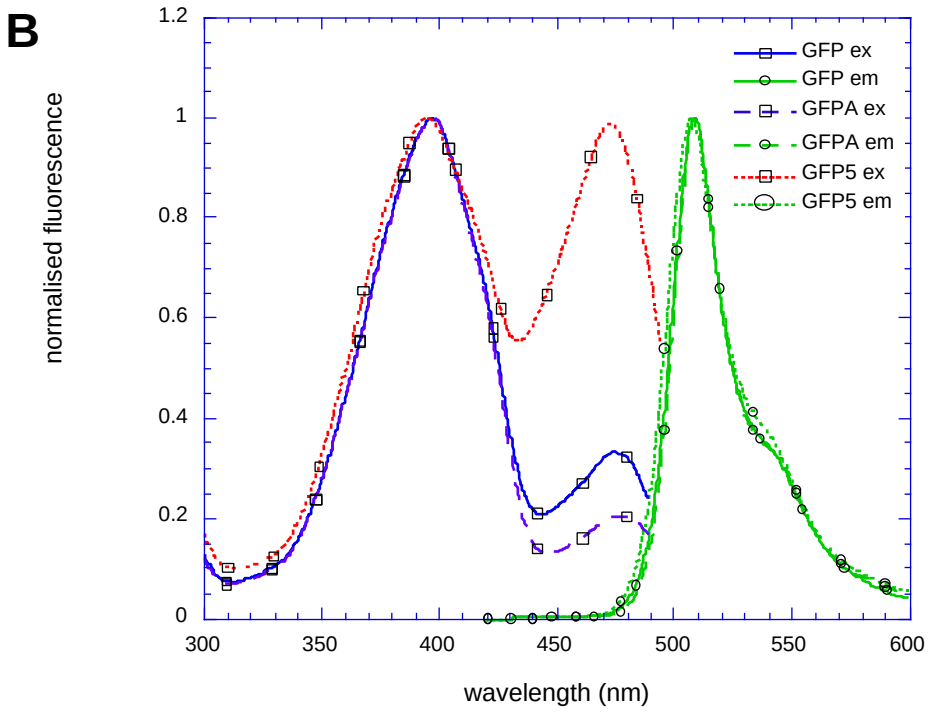

A
B
C

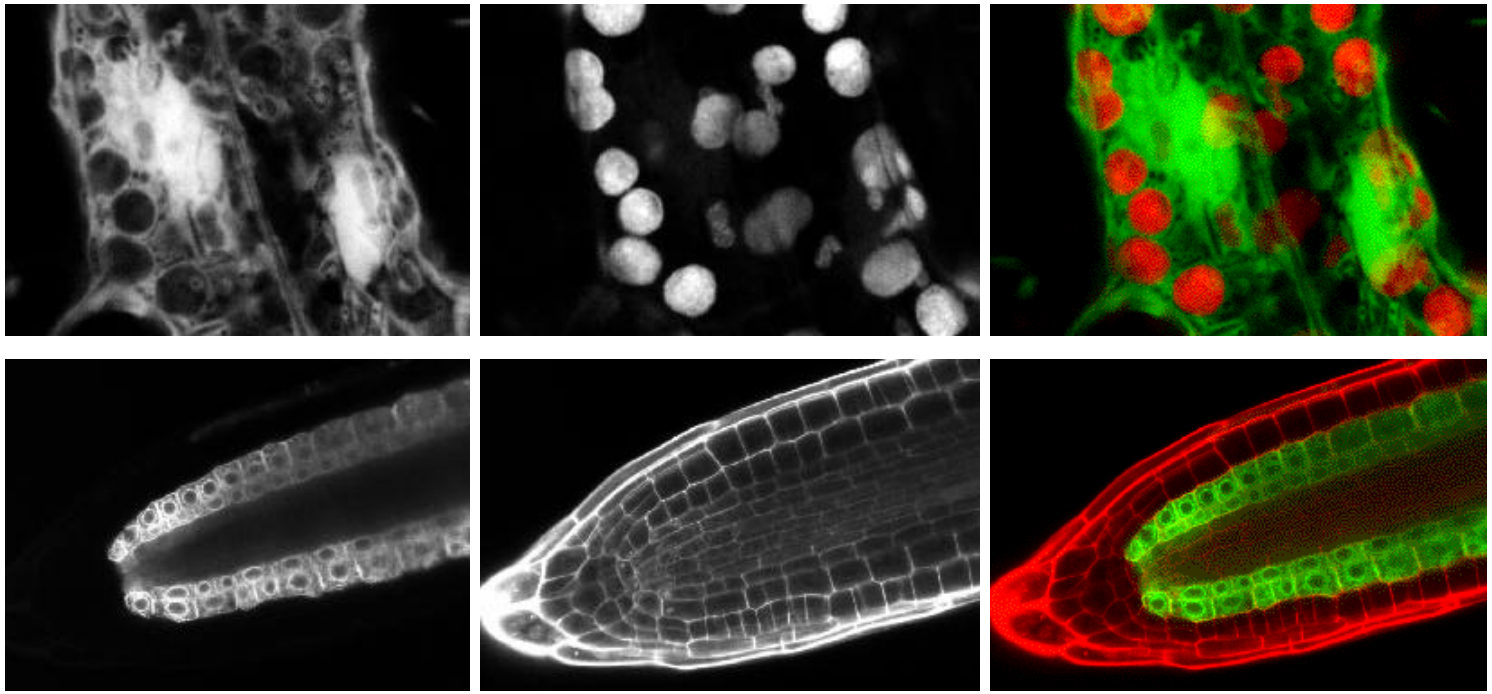

A
B
C
D
E

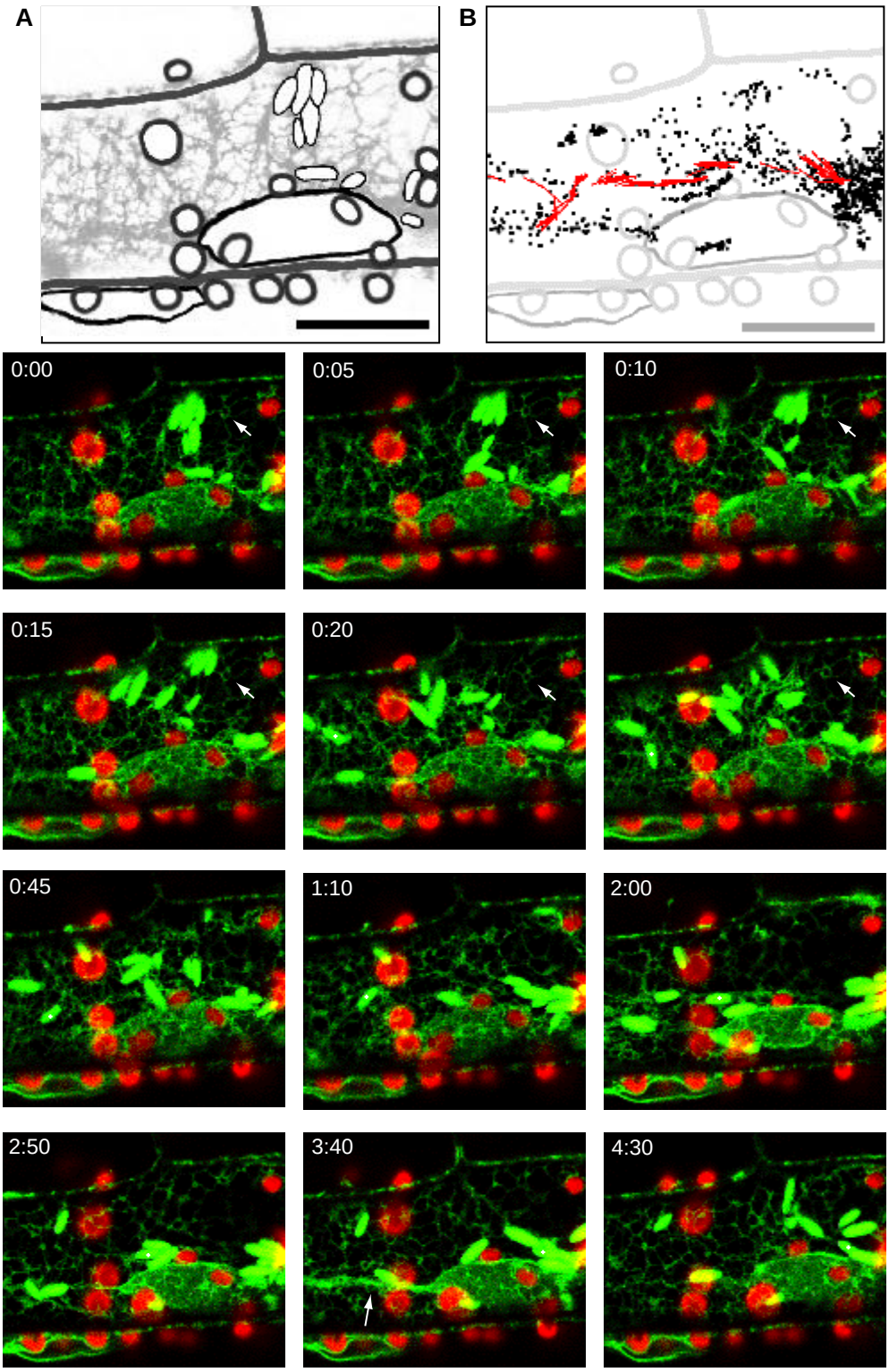

mgfp
mgfp-ER



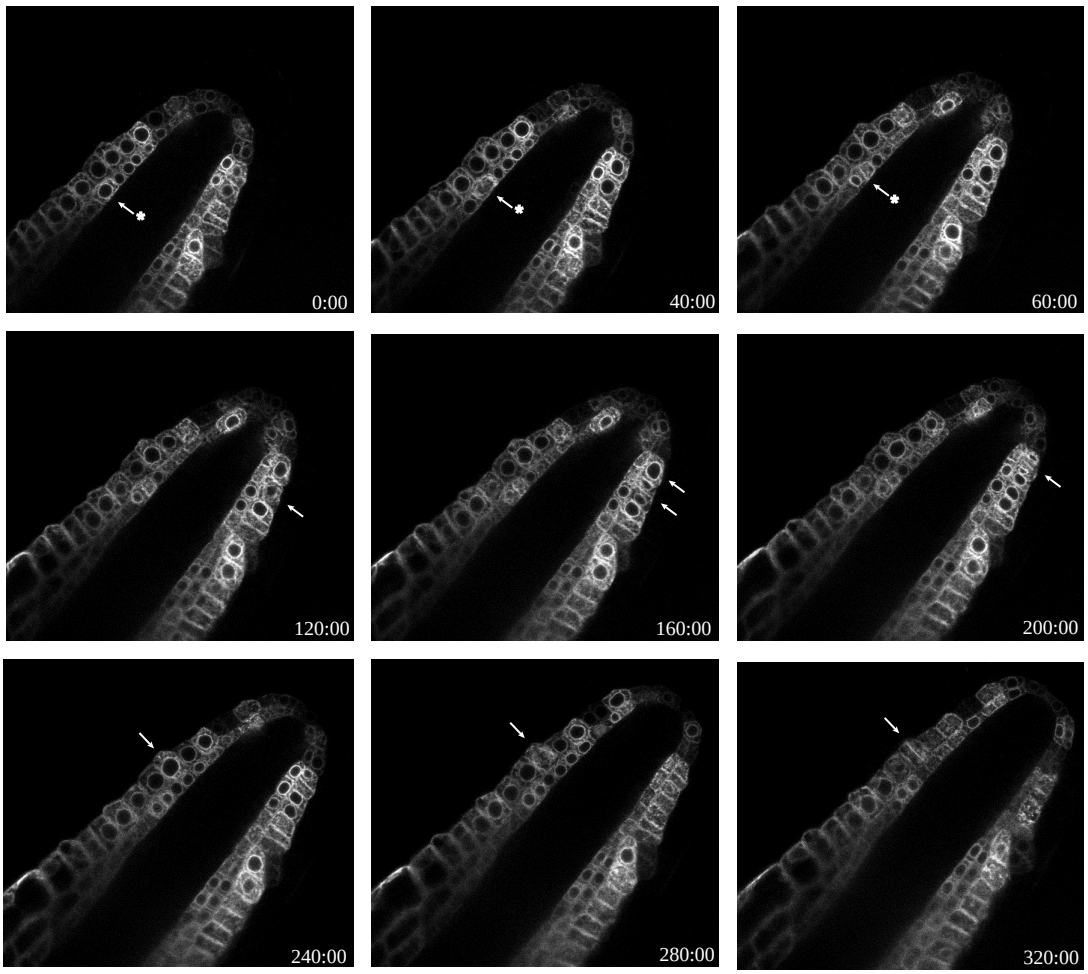

A
2500
2000
1500
1000
500
0
fluorescence (normalised)
mGFP-4
mGFP-A
25
30
37
42
temperature (°C)


B
1.2
1
0.8
0.6
0.4
0.2
0
normalised fluorescence
GFP ex
GFP em
GFPA ex
GFPA em
GFP5 ex
GFP5 em
300
350
400
450
500
550
600
wavelength (nm)

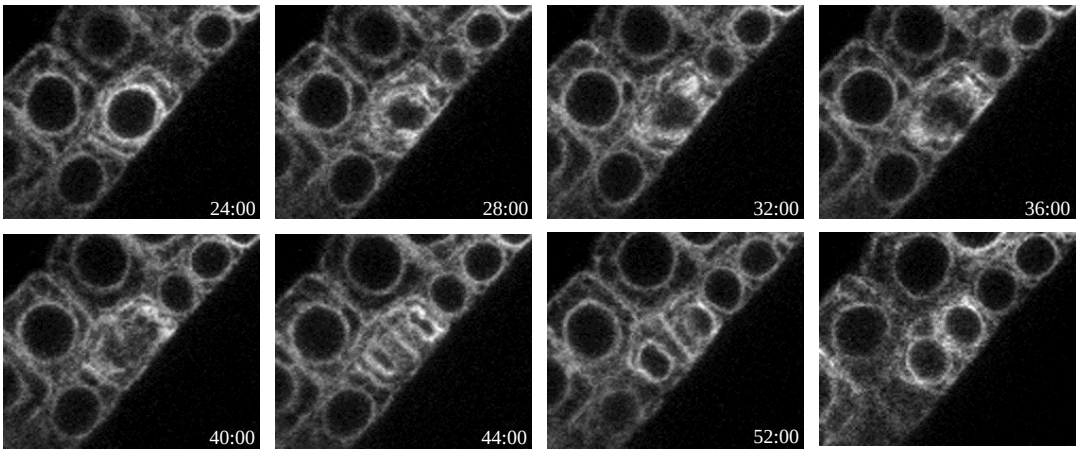



A
B
0:00
0:05
0:10
0:15
0:20
0:45
1:10
2:00
2:50
3:40
4:30

A


0:00
40:00
60:00
120:00
160:00
200:00
240:00
280:00
320:00

B


24:00
28:00
32:00
36:00
40:00
44:00
52:00