The illuminated plant cell
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Jaideep Mathur

Laboratory of Plant Development and Interactions, Department of Molecular and Cellular Biology, College of Biological Science, University of Guelph, 588 Gordon Street, Guelph, Ontario, N1G 2W1, Canada

The past decade has provided biologists with a palette of genetically encoded, multicolored fluorescent proteins. The living plant cell turned into a ‘coloring book’ and today, nearly every text-book organelle has been highlighted in scintillating fluorescent colors. This review provides a concise listing of the earliest representative fluorescent-protein probes used to highlight various targets within the plant cell, and introduces the idea of using the numerous multicolor, subcellular probes for the development of an early intracellular response profile of plants.

Visualizing the plant-cell interior

The wall encasing the plant cell has been the largest barrier to the visualization and understanding of subcellular processes in living plants. Traditionally, plant scientists have relied on squashing, maceration, sectioning or enzyme-mediated degradation of the cell wall to gain access to the inner compartments of the plant cell. Although detailed observations of the plant cell constitute the foundations of plant biology, the fact remains that many of the descriptions are extrapolations of observations made on fixed, dead plant tissue. By comparison, the analysis of live-cell phenomena, such as cytoplasmic streaming and organelle interactions, has been rather limited (1) and references cited therein) because only those plant tissues that allow light to be transmitted through are amenable to non-invasive visualization techniques involving time-lapse video recordings. With the advent of fluorescence microscopy and the availability of cell permeant dyes, such as the nucleotide-binding SYTO stains, the endomembrane-staining DIOC6 and FM4–64 dyes and the mitochondria-specific mitotracker dyes (2), plant biologists have obtained a short time window for live-cell imaging before toxicity-related concerns become pertinent. Also, microinjection of specific stains and fluorescent-protein analogs into living plant cells has become a powerful tool for the observation of subcellular processes (3). Unfortunately, microinjection procedures do require skilled researchers, are often labor intensive and are limited in terms of useful cell types, observable cell numbers and experimental reproducibility. Thus, only a handful of researchers could explore their potential and, for plant biologists, they never reached the status of a routine technique.

Here, I provide a brief overview of how our ability to look inside the plant cell received a tremendous boost in the early 1990s, with the cloning and rapid availability of a 27-kDa green fluorescent protein (GFP) from the jellyfish Aequorea victoria [4].

Fluorescent proteins light up the plant cell interior

The genetically encoded GFP swept away many of the cell wall-imposed limitations on live imaging of the plant cell interior because, in stark contrast to the cumbersome external loading of stains and dyes, GFP and its derivative fluorescent proteins (FPs) are produced by the cells themselves and do not require exogenous substrates, cofactors or chemical treatments for their activity [5,6]. Through their fusion to specific nucleotide sequences, FPs can be targeted to literally any compartment or component of the cell. Once introduced into a plant cell, either for transient or for stable transgene expression after integration in the plant genome, FPs follow the general rules governing subcellular protein dynamics, localization and interactions. The fusion proteins are thus able to respond to both cell-intrinsic and external environmental cues. Through concomitant advancements in non-invasive, CCD-based epifluorescent and confocal laser scanning microscopy, FPs can be readily visualized in living plant cells [6].

Whereas, many modern laboratories using FPs in their research trace their initial acquisition of GFP clones to Douglas Prasher and Martin Chalfie [4], Roger Tsien [7] or to the commercial source Clontech (http://www.clontech.com), a large portion of the credit for modifying GFP for optimal expression in plants and popularizing its use among fellow plant scientists through its unconditional sharing goes to Jim Haseloff and his research team [8]. By late 1997, many plant research laboratories had introduced the cDNAs for mGFP5 and mGFP5-ER (targeted to the endoplasmic reticulum) into their plant dissection strategies involving transcriptional and translational fusion proteins.

The rapid acceptance of GFP as a live reporter protein provided a strong motivation for the creation of newer versions of FPs with altered spectral characteristics [9]. Today, multicolored FPs spanning the visible spectrum have been obtained from a variety of organisms [9,10]. In plants, two complementary FP-based investigative strategies have been adopted:

(i) those creating chimeric translational and transcriptional constructs using FPs to gain spatiotemporal information about gene activity in the plant developmental context;
(ii) those that specifically target FPs to organelles and vesicles or to the cell boundary components to understand subcellular dynamics and interactions (Table 1).
Whereas this review focuses largely on subcellular markers for use in plants, the visualization of FPs within a tissue/organ has been pivotal in educating us about various important aspects of plant development including those related to signaling [11,12] and patterning [13,14]. Many of the stable transgenic lines of *Arabidopsis thaliana* exhibiting tissue- or cell-type-specific expression patterns, such as the GAL4–GFP enhancer trap lines from Jim Haseloff and from Scott Poethig, have been created using the mGFP5-ER construct and are now available through public domain seed stock centers, such as NASC (http://arabidopsis.info/).

In addition to their uses as marker lines in research and in helping to elucidate gene function within the developmental context, *Arabidopsis* lines exhibiting tissue-specific GFP expression are turning out to be excellent teaching material.

**Understanding subcellular dynamics and interactions through targeted FPs**

The ss-GFP–HDEL fusion construct, one of the first subcellular targeted probes to be created, fluorescently

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### Table 1. A non-comprehensive list of different targeted fluorescent protein probes available for plants

<table>
<thead>
<tr>
<th>Target compartment</th>
<th>Fusion protein* – Brief description</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoplastic space</td>
<td>secGFP – Secretory GFP created by fusing a chitinase signal peptide to GFP; transits through the ER lumen</td>
<td>[29]</td>
</tr>
<tr>
<td>Cell wall</td>
<td>CFP::PP2Z – A cyan fluorescent protein fused to <em>A. thaliana</em> full-length proline-rich protein 2A; RBCS1A::GFP – <em>A. thaliana</em> ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit 1A N-terminal transit peptide (amino acid residues 1–55) fused to GFP</td>
<td>[41]</td>
</tr>
<tr>
<td>Chloroplast</td>
<td>RECA::GFP – <em>A. thaliana</em> recombinase-A N-terminal transit peptide (amino acid residues 1–15) fused to GFP</td>
<td>[16]</td>
</tr>
<tr>
<td>Plastid nucleoids</td>
<td>YEND::GFP – N-terminal region from plastid envelope DNA-binding protein fused to GFP</td>
<td>[67]</td>
</tr>
<tr>
<td>Amyloplast</td>
<td>Tt::YFP – Transit peptide from wheat granule bound starch synthase fused to YFP and placed under a rice actin promoter</td>
<td>[88]</td>
</tr>
<tr>
<td>Cytosol</td>
<td>GFP/smGFP – Soluble, modified GFP. Non-targeted GFP accumulates in the cytosol</td>
<td>[8,69]</td>
</tr>
<tr>
<td>Endoplasmic reticulum (ER)</td>
<td>ss-GFP::KDEL – GFP fused to a potato patatin storage protein N-terminal signal sequence (amino acid residues 1–23) and <em>Catharanthus roseus</em> heat-shock protein 90 C-terminal KDEL ER retrieval sequence (amino acid residues 814–817)</td>
<td>[70]</td>
</tr>
<tr>
<td>ER-membrane</td>
<td>ss-GFP::HDEL – GFP fused to an <em>A. thaliana</em> basic chitinase N-terminal signal sequence (amino acid residues 1–21) and a C-terminal HDEL ER retrieval sequence</td>
<td>[8,36]</td>
</tr>
<tr>
<td>ER-body</td>
<td>ARA6::GFP – <em>Arabidopsis</em> full-length Rab isoform 6 fused to GFP</td>
<td>[31]</td>
</tr>
<tr>
<td>Early-type endosome</td>
<td>ERD2::GFP – <em>Arabidopsis</em> full-length ER retention-defective-2 protein (HDEL-ER retrieval signal receptor protein) fused to GFP</td>
<td>[23]</td>
</tr>
<tr>
<td>Late-type* endosome</td>
<td>cis Golgi</td>
<td>GmMAN1::GFP – (1,2)-mannosidase I, a resident Golgi protein from <em>Glycine max</em> fused to GFP</td>
</tr>
<tr>
<td>Median Golgi</td>
<td>XyIT36::GFP – First 36 amino acids of β(1,2)-xylosyltransferase from <em>A. thaliana</em> fused to GFP</td>
<td>[73]</td>
</tr>
<tr>
<td>trans Golgi</td>
<td>Smta::GFP – Rat α(2,6)-sialyltransferase N-terminus (amino acid residues 1–55) including the single transmembrane domain) fused to GFP</td>
<td>[23]</td>
</tr>
<tr>
<td>Golgi body</td>
<td>Microfilaments (F-actin)</td>
<td>GFP::mTalin – GFP fused to the F-actin-binding domain (amino acid residues 2345–2541) from mouse Talin gene</td>
</tr>
<tr>
<td></td>
<td>Microtubules</td>
<td>GFP::MAP4 – GFP fused to the microtubule-binding domain (amino acid residues 935–1084) from mouse microtubule-associated protein 4 fused to GFP</td>
</tr>
<tr>
<td></td>
<td>Microtubule+ end</td>
<td>GFP::EB1 – GFP fused to the full length cDNA of microtubule plus-binding protein (EB1a/EB1b) from <em>A. thaliana</em></td>
</tr>
<tr>
<td>Mitochondrion</td>
<td>Coxl::GFP – Cytochrome oxidase subunit IV N-terminal presequence (amino acid residues 1–29) from <em>Saccharomyces cerevisiae</em> fused to GFP</td>
<td>[74]</td>
</tr>
<tr>
<td></td>
<td>Nucleus</td>
<td>SM40::GFP – Mammalian simian virus 40 large T-antigen nuclear-localization signal (amino acid residues 126–132) fused to GFP</td>
</tr>
<tr>
<td></td>
<td>Peroxisome</td>
<td>GFP::PTS1 – GFP fused to pumpkin hydroxypropionate reductase isoform 1 C-terminus (amino acid residues 377–386 of peroxisomal targeting signal type 1)</td>
</tr>
<tr>
<td>Nuclear envelope</td>
<td>LBR::GFP – The first 238 amino acids of the human lamin B-Receptor fused to GFP</td>
<td>[71]</td>
</tr>
<tr>
<td>Nuclear pore</td>
<td>MOS3::GFP – Full-length cDNA for <em>A. thaliana</em> MOS3 (MODIFIER OF SNC1) fused to GFP</td>
<td>[76]</td>
</tr>
<tr>
<td>Nucleolus</td>
<td>AtFor1::smGFP – Full-length cDNA for <em>A. thaliana</em> FIBRILLARIN1 gene fused to GFP</td>
<td>[77]</td>
</tr>
<tr>
<td>Chromatin</td>
<td>H2B::YFP – <em>A. thaliana</em> cDNA for histone H2B fused to YFP</td>
<td>[78]</td>
</tr>
<tr>
<td>Oil body</td>
<td>Oleosin::GFP – <em>A. thaliana</em> full-length oleosin isoform S3 fused to GFP</td>
<td>[79]</td>
</tr>
<tr>
<td>Peroxisome</td>
<td>GFP::PTS1 – GFP fused to pumpkin hydroxypropionate reductase isoform 1 C-terminus (amino acid residues 377–386 of peroxisomal targeting signal type 1)</td>
<td>[80]</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>GPP::ROP6 – GFP fused to <em>A. thaliana</em> full-length RhO of plants isoform 6</td>
<td>[81]</td>
</tr>
<tr>
<td>Plasmodesmata</td>
<td>MP::GFP – Full-length tobacco mosaic tobamovirus movement protein fused to GFP</td>
<td>[15]</td>
</tr>
<tr>
<td>Precursor-accumulating vesicle</td>
<td>SP::GFP::PV72C – Pumpkin 2S albumin signal peptide sequence (residues 1–22) fused to GFP with the C-terminus (amino acid residues 557–624) of pumpkin precursor-accumulating vesicle 72-kDa protein</td>
<td>[82]</td>
</tr>
<tr>
<td>Protoplast</td>
<td>PAF::GFP – Tobacco full-length protoplastase α 6(F) subunit of 20S proteasome fused to GFP</td>
<td>[64]</td>
</tr>
<tr>
<td>Ribosomes</td>
<td>TH1::GFP – The N-terminus of <em>A. thaliana</em> TH1 cDNA (nucleotides 1–315) fused to GFP</td>
<td>[63]</td>
</tr>
<tr>
<td>Lytic-type vacuole</td>
<td>Aleurain::GFP – N-terminus of barley aleurain thiol protease precursor (amino acid residues 1–143) including an ER-targeting signal sequence and vacuolar targeting propeptide) fused to GFP</td>
<td>[83]</td>
</tr>
<tr>
<td>Storage-type vacuole</td>
<td>ss-GFP::VSS – N-terminal signal sequence (amino acid residues 1–23) of tobacco chitinase A fused to GFP and C-terminal vacuolar sorting signal (amino acid residues 318–324) fusion construct</td>
<td>[84]</td>
</tr>
</tbody>
</table>

*The late-type endosome is also referred to as a prevacuolar compartment or multivesicular body [31].

*The apoplastic space and the wall are not considered subcellular components but are intimately related to the plant cell.*
highlights the endoplasmic reticulum (ER) [8] and has been used by many laboratories to obtain their first glimpse of green fluorescence in plant cells. Subsequently, this probe has been used as a useful control when targeting FPs to other organelles (Figure 1). Although every discovery relating to the inner workings of the plant cell should be considered important and find a mention in this overview, a listing of the myriad of discoveries and the numerous probes created during the past decade has to be curtailed owing to space limitations. However, in recognition of the rapid growth of the field and the necessity of keeping it frequently updated, a new online resource devoted to ‘the Illuminated Plant Cell’ (http://www.illuminatedcell.com/) is being created. This website aims to provide a comprehensive listing of probes and related information to the community. Nevertheless, certain findings, such as the elucidation of mechanisms of plasmodesmatal functioning and viral movement [15], the rediscovery of plastid stromules [16], the visualization of the intricate cytoskeletal organization [17,18] and unraveling of its functioning [19–22], the recognition of novel actin-based mechanisms for organelle motility [23–25] and subcellular interactions [26–28], the recognition of exocytosis- [29] and endocytosis-mediated [30,31] mechanisms in plant development, the visualization of cellulase synthase (CESA6) organization and in situ activity [32], and the use of targeted GFP in microscope-based mutant screens [33–35], have resulted directly from the use of targeted fluorescent proteins.

One of the benefits of using FPs for live imaging is the observation of transient subcellular phenomena, such as the conditional highlighting of spindle-shaped ER bodies [8] in response to defense-inducing conditions (e.g. herbivory [36]) and the quick evaluation of organelle behavior in response to stress and apoptotic signals. Conditional dual targeting of certain probes, such as the ERD2–GFP, which can accumulate specifically in Golgi bodies (Figure 1) or be localized in both the ER and Golgi stacks [23], has also

![Diagram of plant cell with labeled subcellular components](image-url)
been possible using targeted FPs. Furthermore, although (based on similar localization patterns) a large number of proteins are known to highlight peroxisomes and mitochondria [37,38], the need for multiple probes has been felt for the labeling of F-actin [17,39] and microtubules [18,40] following concerns that a single probe might not label the entire gamut of arrays displayed by these ubiquitous cytoskeletal elements. Thus, the number of targeted probes created sometimes out of dire necessity but often through routine experiments designed to localize a gene product, continues to grow steadily. An approach that has been particularly useful in generating probes has been the creation of random cDNA::FP fusions to identify new subcellular structures in plant cells [37,41–43]. The increased diversity in probes targeted towards the same subcellular structure provide the researcher with a range of protein tools to suit specific experimental requirements, act as the much-needed independent probes for controls and confirmation of observations, and are leading to more detailed dissection of suborganelar properties [21,22,25,44]. Because the creation, merits, demerits and numerous uses of probes targeted to specific organelles have been reviewed in detail [44–47], Table 1 and Figure 1 do not provide a comprehensive listing of every targeted fusion protein created to-date. Rather, they serve to emphasize the fact that a vast majority of subcellular compartments and components of the plant cell have become fluorescently highlighted.

The following sections briefly explore the long-term implication of the availability of multiple subcellular probes for achieving a better understanding of the plant cell.

**A target within reach: the multicolored plant cell**

Most early organelle-targeted probes used different versions of GFP. These probes allowed the clear visualization of the targeted organelle (Table 1) and the dissection of interactions occurring between similar organelles, such as chloroplast–chloroplast interactions through stromules [16] or the ‘kiss and run’ transient interaction between mitochondria [45]. However, they did not allow the visualization of interactions occurring between two or more different organelles. Single FP-based observations thus reveal only a small part of the dynamic subcellular world. The vital subcellular cooperation and coordination of interactions can best be pursued when more than one FP tags are used for targeting different structures [24,27,48]. Therefore, there is a growing trend to replace GFP with other compatible, colored FPs to achieve the simultaneous, multicolor visualization of multiple organelles and their interactions. For example, the GFP–MAP4 (MBD) probe, which has served as a very useful label for cortical microtubule arrays [18], is now also available in cyan (CFP–MAP4) [48], yellow (YFP–MAP4) [27] and red (DsRed–MAP4) [49] versions. The availability of these different FP-tagged versions of MAP4 has allowed the fine analysis of microtubule dynamics and led to numerous new insights [19,20,26,27,48,49]. The multicolor visualization approach is also resulting in a slow reversal of the tendency to exclude chlorophyll autofluorescence in green tissues by creating specific narrow band filters for GFP. In fact, in many live visualization strategies, not only does the orange-red chlorophyll provide a bright counter-fluorescence to GFP but it also allows the simultaneous visualization of chloroplasts with other FP-labeled organelles (Figure 2a,h). Most importantly, the photobleaching of chloroplasts can be used as an internal control for photodamage-induced artifacts (Box 1). Figure 2 provides a few examples of multicolor probes that are being used for the simultaneous visualization of different organelles in plant cells. In the simplest strategy for creating a multicolor line, two stable transgenic lines carrying dissimilar FP-probes targeted to separate organelles can be crossed. By including chloroplasts as autofluorescent structures, this strategy easily allows the visualization of three different organelles within the same cell (Figure 2a,h,i). Although subject to limitations imposed by gene-silencing mechanisms, the two-color FP-containing plant can be further transformed and selected for inclusion of an additional FP-marker (Figure 2b–e). Although the expression of four FPs [e.g. ECFP (emission max. ca. 475 nm), EGFP (emission max. 509 nm), YFP (emission max. ca. 527 nm) and RFP (monomeric DsRed/mRFP, emission max. ca. 607 nm) [9,10,50] can be achieved, the actual visualization and convincing separation of more than four colors within a cell is still a technically challenging task. Theoretically, very fine spectral separation can be achieved by confining data collection to peak emission wavelengths for different

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**Box 1. Knowing the ‘darker side’ of FP-based technology**

The FP-based method for studying plants does require a cautionary note. Because each FP has a specific size and characteristic folding properties, the addition of an FP-tag to determine the subcellular localization and behavioral properties of another protein of interest must be mindful of issues related to alterations of protein mobility, turnover and stability, in addition to the possible alterations in subcellular localization patterns. The size of the protein being fused to the FP, the information related to the folding of the fusion protein, the shielding of a signal sequence or the inadvertent snipping off of a portion of the C-terminal sequence, the introduction of a ‘hinge’ between the protein of interest and the FP, the unintentional introduction of a mutation in a PCR-based cloning approach, are all considerations that should be matched by adequate controls. Artifacts might also result from using multimeric versus monomeric versions of a given FP or by relying overly on transient over-expression data versus stable expression (or vice versa) for a particular fusion-protein probe. Although transient expression experiments, including those resulting in a sudden subcellular flooding of a FP-probe through the use of inducible promoters, can result in major misinterpretations of protein behavior and localization, even stable transgenic lines should be carefully screened for the range of protein expression, plasmid insertion related effects and the possible developmental consequences before putting forward an opinion on gene function. The most common misinterpretations result from faulty imaging methods and conditions, especially where broadband filters that allow a bleed-through of native autofluorescence are used. This specific imaging artifact is a major concern in conclusions based on FP-colocalization or FRET interactions. Great caution must be exercised when observing motile organelles over time, because even transient changes in high-intensity laser-induced photobleaching as well as membrane damage can greatly skew motility data. Again, data overextraction through the use of non-transparent algorithms and data extrapolation software are concerns associated with FP-technology. More detailed discussions on the advantages and disadvantages of FP-based probes and the limitations of light microscopy are available [5–7,9,10,47,50,60].
probes. Whereas this is possible for brightly fluorescent cells where the different FPs display roughly similar levels of fluorescence intensities, the large overlaps in excitation and emission spectra for most commonly used FPs, combined with the subcellular motility of organelles, frequently create confusing color-overlaps. Approaches aimed at increasing wavelength resolution for multicolor imaging range from spectrophotometric separation or/and algorithm-based FP-specific spectral profiling for protein discrimination. Alternatively, FPs possessing more stringent spectral characteristics are available for use [9,10]. Recently, the creation of several inducible promoters has introduced another exciting range of possibilities whereby the FP remains unexpressed until the chimeric gene is triggered by the exogenous application of an inducing chemical or change of temperature regime [21,51,52].

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addition, several photo-inducible FPs have become available. These include PA-GFP (photoactivable-GFP), which becomes activated by 405 nm-light to produce a many-fold increase in fluorescence [53]. PA-GFP has been targeted to the ER to understand protein dynamics within this compartment [28]. Similarly EosFP [54] and Kaede [55], two FPs that rapidly change color from green to red upon activation by near-UV light (ca. 390 nm) have been used for the visualization of endocytosis events at the plasma membrane [30] and to improve our understanding of mitochondrial fusion and division [56], respectively. FPs that accumulate in the cytosol and respond to specific activator molecules, such as reactive oxygen species [57] or to changes in the status of various ions, including H⁺, Ca²⁺, Cl⁻ and NO₃⁻ (reviewed in Ref. [58], and destabilized versions of FP [59] have been developed and are valuable additions to the fluorescent protein tool kit.

Although not discussed here, advanced imaging techniques, such as bimolecular fluorescence complementation (BiFC [60]), biluminescence resonance energy transfer (BRET), Forster or fluorescence resonance energy transfer (FRET), fluorescence lifetime imaging (FLIM) and fluorescence recovery after photobleaching (FRAP [61]) (reviewed in Refs [5–7,9,10,50]), are all off-shoots of FP-based technology whose application in plant research is gathering momentum.

Targeted FPs and EIRPing of plants: an emerging concept

As discussed earlier, FPs have provided us with the ability to look inside living plant cells and have revealed that, within their rigid walls, plant cells actually display rapid subcellular dynamics. To ensure survival, a rooted plant needs to respond very quickly to diverse environmental cues. Interestingly, our understanding of a plant’s response to a given stimulus comes from observations that are usually made long after the causal event has occurred. For example, although we know that, like all other living organisms, plants suffer from stress, we do not know the earliest subcellular indications of stress shown by a plant cell. Similarly, we recognize that plants are susceptible to pests and diseases but have only a hazy idea about the earliest responses of a plant cell to the invasion of its epidermal surface.

The fluorescently illuminated plant cell thus has a lot of new information to offer through its rapid response to environmental cues. In the long term, this information can be judiciously combined with molecular-genetic strategies to devise better strategies for plant improvement and management. These thoughts and the availability of numerous targeted fluorescent protein probes and transgenic lines have led to the idea of generating an early intracellular response profile for plants (EIRPP, http://www.uoguelph.ca/~jmathur/research/EIRP.html). Proof of concept studies for EIRPP using the model plant Arabidopsis thaliana are already underway.

Acknowledgements

An overview on FP-based contributions to understanding plants merits the inclusion of many more references than has been possible here. I hope that the comprehensive online resource being created will serve as a suitable atonement for the omissions. I thank Allison Sinclair and Preetinder Dhanoa for help in compiling the initial list of FP probes that appeared in a similar mini-review [62], Anshudeep Mathur for the creation of the Interactive flash animation and the design of the webpage, David Logan, Sean Cutler, Geoff Wasteneys, Rob Mullen and Hugo Zhang for their critical inputs. The Natural Sciences and Engineering Research Council of Canada (NSERC) fund the EIRPP program initiated by my laboratory.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tplants.2007.08.017.

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