Title: Blooming balloons: Searching for mechanisms of the Inflated Calyx.

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Abstract

Studying morphological novelties offers special insights into developmental biology and evolution. The Inflated Calyx Syndrome (ICS) is a largely unrecognized, but fascinating feature of flower development where sepals form balloon-like husks that encapsulate fruits. Despite its independent emergence in many lineages of flowering plants, the genetic and molecular mechanisms of ICS remain unknown. Early studies in the Solanaceae genus *Physalis* put forth key roles of MADS-box genes in ICS. However, recent work suggests these classical floral identity transcription factors were false leads. With newfound capabilities that allow rapid development of genetic systems through genomics and genome editing, *Physalis* has re-emerged as the most tractable model species for dissecting ICS. This review revisits current understanding of ICS and highlights how recent advancements enable a reset in the search for genetic and molecular mechanisms using unbiased, systematic approaches.

Introduction

The evolution of morphological novelties is an intriguing topic in biology, which provides insights into developmental genetics and mechanisms of adaptation and speciation. A prime example is the flower, whose remarkable diversification and adaptive relevance over 100 million years and across vast geographical and ecological domains has captivated generations of scientists. Despite the relative conserved

composition of four floral whorls (sepals, petals, stamens, and carpel), species-wide diversity of these organs is astounding, most evident in the identity, size, shape, number, symmetry and texture of petals, stamens and fruits [1,2]. For example, in the North American wildflower genus *Penstemon*, repeated floral syndrome evolution has been observed representing shifts from bee to hummingbird pollination, where flowers with bright red pigmentation, narrow tubular petals, elongated stamen filaments and styles have emerged [3]. There is also the detailed mechanistic dissection of flower development in the classical model snapdragon, which revealed central roles for the now widely-studied *TCP* and *MYB* transcription factor genes in asymmetrical petal morphology and patterning [4,5]. From these examples and many others, few would argue that petals don't deserve continued attention in plant biology. Yet, despite its known photosynthetic capacities and structurally supportive roles throughout flower and fruit development [6–8], the seemingly uninteresting outermost floral whorl – the sepal – has been relatively overlooked. In recent years, the *Arabidopsis* sepal has risen prominently as a system to study molecular and biophysical rules underlying organ size control [9]. However, one striking and still poorly understood syndrome where these rules appear to be broken is a morphological innovation known as the Inflated Calyx Syndrome (ICS).

Found in angiosperm families such as the Lamiaceae (mint family), Sapindaceae (soapberry family), Malvaceae (mallow family), and Solanaceae (nightshade family), ICS is characterized by the dynamic and excessive growth of sepals, forming balloon-like husks that encapsulate fruits. Historically, efforts to identify the genetic mechanisms of ICS were focused on the Solanaceae genus *Physalis*, most known by the fruit tomatillo (*P. philadelphica*) used in Mexican cuisine to make salsa verde. With few genomic resources or functional tools, tenuous evidence from two decades ago implicated two MADS-box genes were critical in the evolutionary emergence of ICS, extended to a seemingly solid mechanism involving heterotopic expression of one gene caused by modified *cis*-regulatory control of the other [10,11]. With the recent establishment of reference genomes and tools allowing genetic perturbation in multiple *Physalis* species, molecular and phylogenetic studies have revealed a more complex, and likely contradictory, path to ICS distinct from the canonical roles of MADS-box genes in floral organ development. Specifically, reevaluation of the roles of these MADS-box genes using CRISPR-Cas9 mutagenesis has demonstrated that they are not central to ICS [12]. With this backdrop, we review here our current understanding of the genetic and molecular mechanisms of ICS and highlight that it is now possible to revisit this curious morphological innovation from a fresh perspective using an unbiased and systematic approach.

A brief overview of ICS across flowering plants

As all floral organs are derived from the same founding organs, leaves, it is perhaps not surprising that, like petals, sepals can also experience hyper-variation in growth dynamics and final form during evolution, [13] [14]. ICS is best known from the genus *Physalis* where several species have long been cultivated as

regional crops [15]. These include tomatillo (*P. philadelphica* and *P. ixocarpa*), goldenberry (*P. peruviana*) and groundcherry (*P. grisea* and *P. pruinosa*). However, ICS has emerged independently in at least 18 families, such as *Silene vulgaris* (Bladder Campion), *Scutellaria Mexicana* (Bladder sage) and *Hibiscus sabdariffa L.* (Roselle), suggesting lability in sepal growth dynamics and the underlying mechanisms [16].

In *Physalis,* where ICS development has most been studied, the entire whorl of sepals undergoes a rapid and dramatic increase in size within a week from flower opening. However, the dynamics of this organ size increase is nuanced. The first stage is an expansion at the sepal base, which, within a few days, drives inward curving of the entire whorl towards the adaxial side. This causes the sepals to coalesce at their tips, followed by continued expansion of the middle and base portion of the sepal whorl, producing a fully inflated calyx surrounding the fruit. The result is a balloon-like structure, perhaps providing a protective micro-environment from the calyx [17] to the developing fruit.

The development of ICS illustrates a beautifully coordinated morphogenesis program that must require exquisite temporal and spatial control of cell division, cell size patterning and polarity specification. But not all inflated calyces are the same, and in fact, phenotypic variations of ICS are not well described in most species in which the syndrome has evolved. Three basic categories of calyx types were defined in the context of phylogenetic studies of ICS within Solanaceae: non-accrescent calyx when the calyx enlarges less than 50% from flower to fruit stage, appressed-accrescent calyx when the calyx enlargement exceeds 50% or covers the whole fruit with no space in between, and inflated when the calyx encloses the entire fruit with space in-between. (Figure 1A, [18,19]). Although, detailed microscopic and macroscopic developmental characterizations are lacking for the majority of ICS species, almost certainly there are subtle and substantial differences in ICS progression. These distinctions could be based on variation in the underlying mechanisms, regardless of whether the origins of the syndrome are shared or independent.

A detailed phylogenetic analysis of ICS within the Physalideae tribe of Solanaceae estimated the evolutionary trajectory of calyx inflation among 215 species, including *Physalis* and its close relatives [18]. This analysis estimated 24 gains of appressed-accrescence, 24 subsequent gains of inflation, and two apparent reversals back to appressed-accrescent, but no cases of complete loss of inflation. This indicates that ICS evolution likely proceeded in a directional fashion. To our knowledge, there are no documented losses of ICS in any angiosperm. The directionality of ICS emergence and its retention within the studied lineages is particularly informative, as it might suggest the underlying mechanisms are extremely tolerant to genetic perturbation, perhaps because ICS is highly adaptive or coupled with other developmental programs with fitness relevance. The diverse range of calyx inflation observed within Physalideae, as well as in other solanaceous species (Figure 1A) represented by over 52 mya of divergence [20] continues to offer the best opportunity to explore the mechanisms behind ICS within an evolutionary context.

Though there have been few investigations of ICS outside the Solanaceae, at least 18 angiosperm families spanning ~125 million years have species with ICS [18,21]. These instances of independent emergences capture a spectrum of variation among inflated calyces, including temporal development and final size, photosynthesis capacity, pigmentation (anthocyanins), and desiccation rate. For example, different from *Physalis* where calyx inflation occurs post anthesis, calyx inflation in the mint species *Scutellaria mexicana* appears to start in early floral bud stages long before flower petals fully elongate and open (Figure 1A). In another difference, while *Physalis* calyces retain a leaf-like texture before eventually desiccating as fruits mature, the ICS species *Hibiscus sabdariffa* develops a fleshy calyx resistant to desiccation and displays various colors (Figure 1B). These and previously mentioned examples of ICS, though poorly understood, illustrate the phenotypic convergence of a basal calyx inflation program over broader evolutionary timeframes. Extending the genetic dissection to species such as *Scutellaria Mexicana* and *Hibiscus sbdariffa* provides exciting opportunities to probe the genetic mechanisms underlying convergent evolution in general. Evolution of ICS across a deeper timescale, regulated by the same basal genetic factors and molecular mechanisms is an intriguing possibility. This prompts the additional question of whether the unique characteristics of ICS within different lineages represent modifications of potentially shared foundational programs or if they stem from entirely distinct processes.

A logical leap to MADS-box genes controlling ICS

The Solanaceae is the only family where genetic and molecular mechanisms of ICS can readily be pursued. More than two decades ago, mutants exhibiting the foliose-sepal-syndrome (FSS), characterized by abnormally large leaf-like sepals, were the inspiration for the first ICS studies [19,22]. One such mutant was the Tunicate mutation in *Z. mays*, where ectopic expression of a MADS-box gene, *ZMM19*, resulted in excessive growth of glumes, covering the entire kernel [23,24]. This ICS mimic phenotype was recapitulated by overexpressing *ZMM19* or its orthologs in *Arabidopsis* and tobacco [19]. These observations prompted the reasonable hypothesis that its *Physalis* ortholog, *MPF2*, could be responsible for ICS. RNAi-mediated knock-down lines of *MPF2* were generated in *P. floridana*, where multiple transgenic lines appeared to show a suppression of calyx inflation, which was correlated with impaired male fertility [10]. However, the abundance of *MPF2* transcripts in the RNAi lines did not always correlate with the severity of ICS suppression, raising questions on a direct role of *MPF2* in ICS development. Indeed, in a follow-up study of ICS evolution in Solanaceae, it was shown that in the sampled species from the Physaleae and Capsiceae, floral expression of *MPF2* was not sufficient to produce ICS [25]. This suggested at least additional factors are central to ICS development.

Building on the hypothesis that *MPF2* is a key regulator of ICS and reasonable supporting data given the technological approaches available at the time, a subsequent study described an attractive molecular mechanism involving a second MADS-box gene. In this mechanism, the *euAP1*-like gene, *MPF3*, plays an equally crucial role in ICS by directly regulating *MPF2* transcription. A further advance in the model, albeit supported by less convincing data, proposed that fine-tuning the ratio of these proteins could alter the balance between cell division and cell expansion, resulting in different calyx growth dynamics [11].

Altogether, a compelling molecular mechanism for ICS involving two critical MADS-box genes emerged. However, as indicated above, the supporting functional evidence was based on overexpression, along with RNAi and virus-induced gene silencing (VIGS) knockdown of expression. Given the common occurrence of pleiotropy with MADS-box genes, possibly due to their evolutionary history involving extensive duplications followed by sub- and neo-functionalizations, assessing the results from overexpression is challenging and requires cautious interpretation. RNAi and VIGS are also difficult to interpret due to variable knockdown efficiencies and potential off-target effects. Particularly in the conjecture of *MPF3* imposing *cis*-regulatory control of *MPF2*, the ratio between *MPF2* and *MPF3* became critical and altogether inconsistent with observations from the genetic perturbations. In fact, it would be reasonable to expect a range of calyx inflation phenotypes from independent *MPF2*/*MPF3* double knockdown lines, and potentially between flowers within the same plant, as the variability of knockdown efficiency could yield a wide range of *MPF2*/*MPF3* ratios. This was not observed, suggesting what was presented as conclusive at this point in the dissection of ICS analysis required a reassessment.

Fortunately, we now have powerful technologies in genome sequencing and genome editing. Within the last few years, high-quality genomes were established for three *Physalis* species: *P. floridana*, *P. grisea*, and *P. pruinosa* [12,26]. To provide a more robust genetic foundation for the mechanism of ICS, CRISPR-Cas9 genome editing of *MPF2* and *MPF3* was performed in *P. grisea*, and contrary to the previously suggested mechanisms, calyx inflation was not disrupted in multiple independent null mutants of *mpf2* and *mpf3*, or in their double mutant combination. An additional nine MADS-box genes were also knocked out based on their reported involvement in sepal development from other Solanaceae species. Despite a range of floral defects, including strong and predicted homeotic changes in floral organ identity, the calyx inflation program remained intact in all of these mutants [12]. Failure to disrupt ICS with these single loss-of-function mutations leaves open the possibility that double and higher-order mutants of MADS-box genes could eventually perturb ICS. Still, these robust genetic findings forced a reset in the investigation of ICS mechanisms, and suggested other gene(s) or regulatory mechanisms are likely central to this process.

A relationship between ICS and fertility, or not?

The collection of MADS-box mutants, particularly those exhibiting homeotic defects, provided an opportunity to test the proposed link to fertilization. The apparent correlation between fertility defects and the suppression of ICS, together with the fact that the onset of calyx inflation coincides with anthesis made it tempting to assume a critical role of fertilization in controlling ICS [10]. This hypothesis was strengthened with follow-up studies suggesting that the hormones cytokinin and gibberellin, both induced during pollination and fertilization, are also involved in inflation [27]. In addition, over-expression of another MADS-box gene, *MBP21,* in *P. floridana* resulted in poor male fertility and apparent smaller inflated calyces [26].

While these correlations suggested a physiological mechanism whereby fertilization drives ICS, the genetic evidence from our MADS-box mutants proved otherwise. Most strikingly, four of the MADS-box mutants all displayed severe floral organ homeotic transformation, resulting in various degrees of fruit development defects and no self-fertilization. [11] In further support, we generated a null mutation in the *P. grisea* ortholog of tomato *FALSIFLORA* (*FA;* arabidopsis *LEAFY*), a central regulator of floral specification. As expected, flower development is severely disrupted *Pglfy* mutants, with all floral whorls replaced by leaf-like structures, including release and proliferation of cryptic bracts [28–30]. Alongside these phenotypes, the outermost leaf-like organs of each single-flower inflorescence form lobed structures curved towards the adaxial side, akin to ICS (Figure 2B). That ICS remained intact indicates that ICS can be uncoupled from gametogenesis, pollen shedding, and fertilization ([12], Figure 2A). Thus, an innate inflated calyx developmental program proceeds regardless of fertilization, and even apparently independently of having precise, intact floral organ identity programs.

Classical and advanced approaches to dissect mechanisms of ICS

As has been done for dozens of traits in plant biology [31], intraspecific variation of ICS would greatly facilitate the identification of its genetic mechanism through genetic mapping and subsequence identification of causative genes and variants. Similarly, viable interspecific crosses between closely related ICS and non-ICS species could also provide valuable insights. However, such opportunities are thus far not available. Deeper sampling of species within the Physalideae tribe, where ICS has evolved repeatedly, is potentially a promising path, albeit requiring taxonomic expertise and considerable amount of effort. In this section, we discuss the approaches immediately available in the lab to interrogate the mechanisms of ICS.

Power and limitations of forward genetics

On the surface, the observation that ICS has rarely been lost in any lineage, unlike other morphological novelties that have disappeared due to natural mutations [32–34], could suggest no individual genes drove the evolution of ICS or underlie its mechanisms. Of course, natural losses may have occurred, but such

mutant individuals in a population might have been eliminated due to fitness disadvantages from losing the inflated calyx or due to the effects of developmental pleiotropy affecting fitness and survival. An attractive prospect from the literature on evolutionary novelties is that enhanced or novel *cis*-regulatory control of a gene drove ICS evolution [35]. However, identifying mutations that reverse putative gain-of-function effects in the responsible sequences would likely be rare. This rarity stems from the higher tolerance of *cis*-regulatory sequences to phenotypic effects, attributed to the inherent redundancy in *cis*-regulatory control. Additionally, the mutational space available to *cis*-regulatory sequences is significantly more restricted compared to that of coding sequences [36].

Forward genetics is a proven method that can help address these questions. However, from the few reports in which random mutagenesis was aimed at disrupting ICS, none led to the discovery of mutants where a non-inflated calyx remained intact or where inflation was even partially inhibited. For example, a forward genetics-derived *P. floridana GLOBOSA*-like MADS-box mutant transforms petals to sepals, creating two whorls of sepals that still undergo ICS [37]. More recently, using chemical mutagenesis on *P. grisea,* we identified the *huskless* (*hu*) mutant, which lacks an inflated calyx entirely due to the loss of function of an *AP2*-like transcription factor. However, *hu* mutants develop only three floral whorls, and the outermost whorl is clearly petal-like with some sepal identity, indicating this gene is unlikely to be directly involved in ICS. Importantly, none of these experiments were comprehensive (i.e. did not achieve saturation mutagenesis), leaving open that single gene mutants disrupting ICS could be found, with or without pleiotropic consequences. A much greater investment of time and resources would be required to find such mutants, especially for a phenotype that cannot be assayed early in development. And finally, forward mutagenesis fails to capture the contribution of multiple factors, for example, if redundancy or other higher-order gene or *cis*-regulatory functional relationships were responsible for the emergence of ICS.

Uniting functional genomics with genome editing brings scale and logic to reverse genetics

The use of CRISPR-Cas9 gene editing has proven far superior to traditional knock-down methods in studying gene functions. Besides the ability to generate numerous mutations in gene targets, off-target effects can be avoided through proper *in silico* guide design protocols, and easily eliminated by backcrossing if they occur. The implementation of CRISPR-Cas9 technology in *Physalis* has made it possible to unambiguously interrogate the roles of candidate genes through a robust reverse genetics approach. In the case of MADS-box genes, where logically many genes could serve as genome editing candidates according to their reported or predicted roles in other species, none of the CRISPR-Cas9 *P. grisea* mutants disrupted ICS. Higher order genetics of all possible gene combinations appears necessary to unambiguously determine their roles. However, given their large number, likely genetic redundancies and physical interactions with other members, it would not be surprising for pleiotropy to become a hurdle

to interrogating these relationships and interpreting the results. Potential pleiotropic effects could result in fertility or floral identity defects that make higher-order genetics impractical, or developmental epistasis where severe early developmental consequences preclude phenotypic evaluation of later phenotypes such as ICS. Thus, it becomes more important to devise logical experimental designs on which candidate genes should be targeted, in what combinations, and perhaps even with more delicate gene editing of *cis*-regulatory regions of some genes to address this issue. Success in this endeavor would benefit from a thorough understanding of molecular characteristics associated with ICS development.

As is widely appreciated, profiling gene expression over space and time provides an unbiased approach to reveal the molecular events of dynamic developmental processes and identify candidate genes for reverse genetics. ICS, like other organs that change in size and shape through developmental programs, is a coordinated maturation program involving precise spatial and temporal control of molecular and cellular processes leading to changes in cell number and tissue/organ morphometrics that translate into calyx phenotypes. Perceivable calyx expansion starts from the base of the sepal whorl and proceeds to enlarge and inflate at a noticeable rate each day. It stands to reason that the molecular events responsible for each phase, such as the initiation of ICS, are even more transient, likely within the order of hours. Thus, without clear developmental stage indicators, conventional RNA-seq of bulk samples to represent stages of calyx inflation is challenging and likely not sensitive enough to reveal the transient dynamics of key regulators.

A promising approach to address this challenge comes from recent work on a similarly transitory program, the floral transition of the tomato shoot apical meristem (SAM), which has been molecularly dissected through capturing the transcriptomes of precisely staged, individual SAMs. By sampling a large number of meristems throughout a critical transition point that takes place over 2-3 days, a dynamic transcriptome map of the floral transition was established [38]. This high-resolution reconstruction of the temporal expression map, from wild-type and flowering-time mutants, exposed previously hidden short-lived transition programs at the SAM, and follow-up genetics validated the critical roles of specific genes.

ICS is an ideal system for applying this methodology, aiming not only to enhance our understanding of the molecular events involved but also to enrich for highly promising candidate genes related to the temporal and spatial regulation of ICS. In wild type *Physalis*, calyx inflation occurs uniformly across the entire sepal whorl, implying all five sepals in each calyx should be synchronized in developmental stage and transcriptomic profile. Sepal whorls from hundreds of individual flowers across ICS developmental stages can be harvested for single organ transcriptome profiling at a fine scale. As for tomato single SAM profiling, the progression of ICS can be temporally sorted by molecular signatures of each individual calyx whorl. Referencing the molecular staging to both macroscopic and microscopic phenotyping would create an unprecedented and objective depiction of ICS development, as a critical first step towards understanding its genetic and molecular nature. This is particularly important given the uncoupling of ICS and fertilization

related signals, indicating that conventional indicators of flower maturation, such as opening of the petal whorl and anther dehiscence may not be accurate stage markers for ICS. In addition to identification of candidate genes that are dynamically expressed, the analysis could also reveal fine developmental stages, providing opportunities for a focused approach to single-cell RNA sequencing (scRNA-seq) and spatial transcriptomics to dissect ICS from an intricate temporal, spatial and cellular manner. These integrated, unbiased experimental designs would be highly informative and necessary to guide reverse genetics. Candidate genes that are dynamically expressed would then be extracted and curated for their known functions to refine the list, which can be further interrogated with CRISPR-Cas9 gene editing.

We also propose that the genetics dissection of ICS would greatly benefit from a critical mass of genomic data and new computational tools such as our "Conservatory" project (conservatorycns.com) [36]. Conservatory is an algorithm that has identified conserved non-coding sequences (CNSs) from hundreds of genomes across 72 plant families. These CNSs represent both family-specific and deep *cis*-regulatory conservation, which are predicted to control gene expression or phenotypes. This could be particularly informative where morphological novelties, as shown in many other cases, evolved from *cis*-regulatory modifications of key genes, resulting in changes of their temporal and spatial expression [35]. In the possible scenario where gain of a *cis*-regulatory control (space, time, level) of a key gene underlies the evolution of ICS, the corresponding non-coding sequence would show conservation within lineages displaying ICS, but not at deeper levels. Indeed, the power to compare CNSs at different evolutionary depths of conservation levels would facilitate the identification of key genes and associated *cis*-regulatory sequences within these possible mechanisms. The conservatory database could also aid in the sophisticated genetic dissection of candidate genes using CRISPR-Cas9 editing, especially when pleiotropy or developmental epistasis in knock-out mutations could complicate the interpretation of any particular gene's role in ICS. Moreover, it is now possible to perform targeted mutagenesis on candidate regulatory sequences within the context of other null mutations. This approach is particularly feasible in situations where the interpretation of higher order genetic interactions is complicated by extreme pleiotropic mutant defects.

Final thoughts

Rapid advancement of genomic resources and functional tools have reestablished *Physalis* as the pre-eminent system to elucidate the genetic and molecular complexities of ICS. A systematic and unbiased dissection is imperative to establish a foundational understanding and guide future research. Equally exciting is the opportunity to study this morphological novelty in the context of the Solanaceae family, where multiple species, each representing different forms of the evolutionary steps of ICS, can potentially be harnessed for genetic research. Similar approaches including the establishment of reference genomes,

gene editing, and single organ transcriptome profiling can be applied to the independently arisen appressed-accrescent and inflated species within the Solanaceae and eventually to other angiosperm species such as the aforementioned mint and mallow family species, to determine if the same, overlapping, or different genes are evolutionary drivers of ICS. Mechanistic insights from these parallel systems not only advance our understanding of ICS evolution, but also provide invaluable contribution to deciphering the genetic basis for convergent evolution.

In addition, the potential molecular and genetic dissection of ICS opens up possibilities to investigate ICS beyond its significance as an evolutionary novelty. The sudden and rapid growth of the calyx is an innately regulated developmental process, which differentiates it from many rapid morphological responses to changing environments, such as shade avoidance syndrome or the root hydropatterning. Moreover, the magnitude and dynamics of calyx inflation far exceed those in *Arabidopsis* and most other angiosperms where the sepal is a model to study organ morphogenesis. ICS as a secondary system to study sepals provides an exciting entry point to understanding the intricate processes and precise control of developmental switches that lead to cell division and cell size patterning, and to reveal how certain rules of growth control can be bent or broken.

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The first author acknowledges using generative AI tools (ChatGPT and Copilot) solely to improve grammar and readability of specific phrases and sentences in the first draft. After using these tools, all authors reviewed and edited the content in later drafts, as needed. All authors take full responsibility for the content of the publication.

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Declaration of competing interest

The authors declare that there is no conflict of interest.

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Figure 1

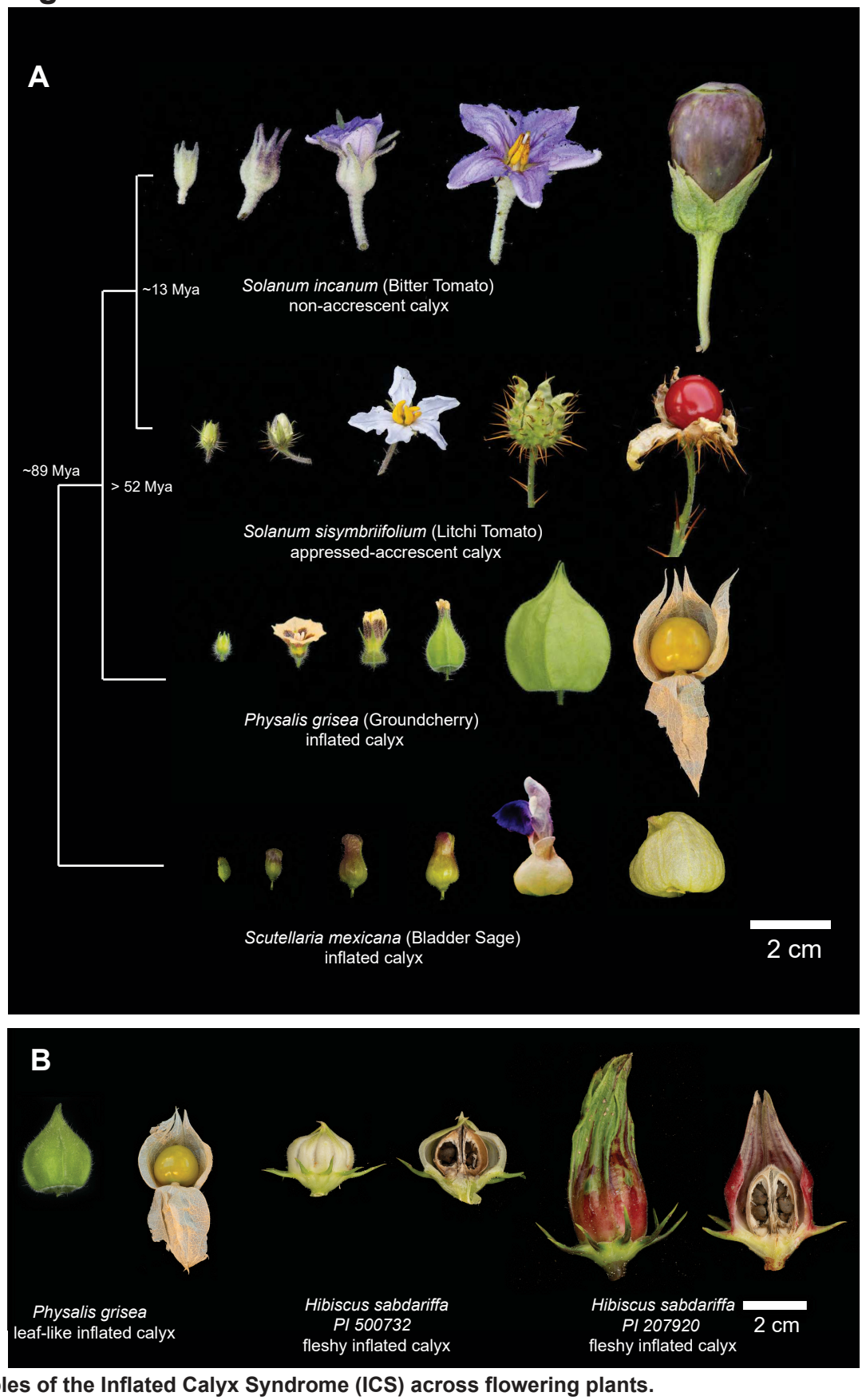

Figure 1. Examples of the Inflated Calyx Syndrome (ICS) across flowering plants.
A. Series of images showing sequential developing flowers from three Solanaceae (nightshade) species (*Solanum incanum*, *Solanum sysymbrifollium*, *Physalis grisea*), and a Lamiaceae (mint) species (*Scutellaria mexicana*) illustrating sepal whorls that develop into a non-accrescent calyx, an appressed-accrescent calyx, and an inflated calyx. Evolutionary distances are estimates from https://timetree.org/ [21] and from the fossil records of *Physalis infinemundi* [20].
B. Examples from angiosperms of different types of inflated calyx phenotypes. *P. grisea* produces a leaf-like texture of inflated calyces, which become desiccated paper-like husks as the fruit matures. *Hibiscus sabdariffa* accessions develop fleshy inflated calyces with varying colors, which unlike *P. grisea*, do not undergo programmed desiccation.

Figure 2

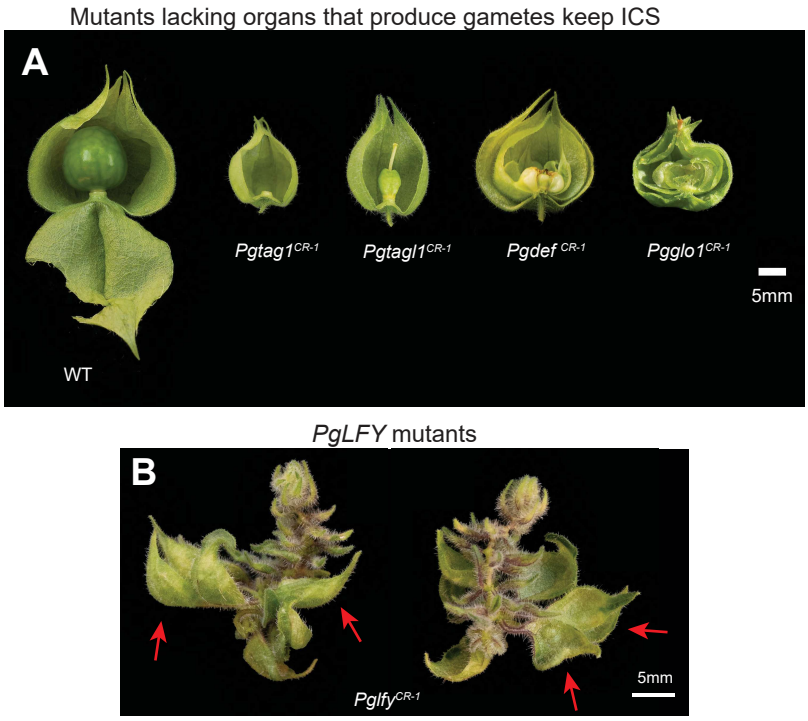

Figure 2. ICS persists in CRISPR-engineered mutants with defects in fertilization and floral identity.

A. Mutations in the *Physalis* MADS-box gene family members *PgTAG1*, *PgTAGL1*, *PgDEF*, and *PgGLO1* have floral organ homeotic defects, which results in flowers that fail to produce gametes and self-fertilize. Despite these defects, calyx inflation remains intact in these mutants, indicating ICS can be uncoupled from the canonical fertilization program. Modified with permission from Figure 4 in [12].

B. The CRISPR-engineered mutation in *PgLFY* results in loss of flower specification and severe defects in floral organ identity. Instead of the typical flower development program and associated floral whorl initiation and structures, reiterative leaf-like structures known as bracts develop in place of each single-flower inflorescence. The outermost whorls of these bracts, as indicated by red arrows, expand and curve towards the adaxial side, indicating that aspects of the inflated calyx program remain intact.