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A *CYCLOIDEA*-like gene mutation in sunflower determines an unusual floret type able to produce filled achenes at the periphery of the pseudanthium

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Abstract

The pseudanthium of sunflower (*Helianthus annuus* L.) consists of two floret types: zygomorphic sterile ray florets and actinomorphic hermaphrodite disc florets. In the *tubular ray flower* (*turf*) mutant, the loss-of-function of a *CYCLOIDEA* (*CYC*) gene generates hermaphrodite tubular-like ray florets that replace the normal sterile ray florets. We evaluated whether tubular-like ray florets have a multifaceted set of floral traits and the presence of heteromorphic seeds in the *turf* inflorescence. During early stages of floral ontogeny, primordia of both tubular-like ray florets and typical ray florets displayed a comparable shape. In contrast, during later stages of development, the form of tubular-like ray floret primordia was most similar to disc floret primordia. In mature tubular-like ray florets, corolla and ovary had both ray and disc floret characteristics but also displayed distinct identity traits. In open-pollinated tubular-like ray florets, the seed set was low but a noteworthy increase of filled achenes was obtained by hand pollination. Wild type ray achenes were always empty. Embryos of tubular-like ray florets were shorter and lighter than the embryos of disc florets but able to produce fertile plants. In conclusion, the different identity characteristics combined in tubular-like ray florets of the mutant evolved a capitulum type not described in the genus *Helianthus*.

Keywords: corolla symmetry, floral morphometrics, floral ontogeny, *Helianthus annuus*, heteromorphic achenes, papillose conical cells, zygotic embryo.

Introduction

Pseudanthia are inflorescences characterized by different floret types which mimic a large single flower with a strong visual impact. Evolution of pseudanthia has occurred many times in angiosperms, and inflorescence-blossoms have been reported in more than 40 families (Claßen-Bockhoff 1990). The sunflower head (capitulum) is a well-known example of this type of inflorescence. The capitulum is produced by an expanded and flattened meristem which develops into an array of sessile units (florets) arranged on a flat surface and surrounded by a protective wrap of involucral bracts (Palmer and Palmer 1982). The peripheral whorl of the capitulum is composed by large sterile ray florets with monosymmetric corolla, while the internal whorls are filled by smaller and polysymmetric hermaphrodite disc florets. The corolla of ray florets makes the capitulum very attractive to pollinators. However, the corolla of disc florets remains less conspicuous (with the exception of the distal lobes).

In the radiate capitulum of Asteraceae, the corolla of ray florets is usually strap-shaped with three or fewer teeth (ligule or perianth lamina) at the apex and a short tube at the base. The corolla shape of the disc floret is actinomorphic, pentamerous or sometimes tetramerous, and characterized essentially by an elongated tube (Harris 1995; Jeffrey 1997). However, in some species, the strap-shaped corolla where the ray is positioned is replaced by a tubular corolla with five prominent apical teeth (i.e. *Cyanus triumfettii*). In addition, pseudanthia with tubular-like ray florets have been described after interspecific hybridization (Ford and Gottlieb 1990; Carr et al.

1996; Sujath 2008), induction of polyploidy (Majdi et al. 2010; Oates et al. 2012), gamma radiation (Banerji and Datta 2003), and spontaneous mutations (Robbins 1906; Fick 1976; Berti et al. 2005; Bello et al. 2013; Nakagawa and Ito 2014). In sunflower, an atypical floret type at the periphery of the inflorescence has been observed in *tubular ray flower* (*turf*), and *tubular-rayed* (*tub*) mutants characterized by loss-of-function of a *CYCLOIDEA* (*CYC*) gene, *HaCYC2c* (Fambrini et al. 2011; Chapman et al. 2012). Interestingly, *CYC*-like genes encoding transcription factors of the TCP class are key players in the elaboration of floral symmetry in several eudicots (Uberti Manassero et al. 2013; Hileman 2014).

When compared to huge wild type ray florets, tubular-like florets of *turf* plants are clearly dissimilar because they have a smaller polysymmetric corolla, as well as reproductive organs and ovules (Berti et al. 2005). However, from this preliminary characterization, it is unclear whether some identity traits of ray florets are retained in the tubular-like ray florets, and whether filled achenes can be collected at the periphery of the *turf* inflorescence.

In this work, we assess the hypothesis that tubular-like ray florets combine identity traits of both ray and disc florets, as well as their own exclusive characteristics. We thus extensively describe floral ontogeny, corolla histology, and organ morphometry to compare the floral characteristics of disc and tubular-like ray florets of *turf* with ray and disc florets of wild type (WT) inflorescences. Furthermore, we also assess the hypothesis that tubular-like florets are able to produce filled achenes with heteromorphic

traits. Therefore, we evaluated floret fertility under different pollination methods and we analysed achene and embryo features.

Materials and methods

Plant material and growth conditions

Sunflower seedlings (*Helianthus annuus* L.) from wild type (WT, TURF inbred line), and *tubular ray flower* (*turf*) mutant (Berti et al. 2005) were grown in field conditions (experimental fields, San Piero a Grado; University of Pisa, Italy). Trials were carried out during the spring/summer of 2011 - 2014 in a medium fertility and high field capacity soil. Conventional management practices were used (Fambrini et al. 2006). Briefly, mutant and WT seeds were sown in rows with 50 cm inter-row spacing. After germination, the seedlings were hand-thinned, and the final distance between plants within each row was 25-30 cm. Fertilizers were applied at a rate of 100 Kg N ha^{-1}, 25 Kg P ha^{-1} and 30 Kg K ha^{-1} when plants developed three/four pairs of fully expanded leaves (V6-V8 stages according to Schneiter and Miller 1981)[1]. No irrigation was supplied. For weed control, cultivation machines and hand-weeding practices were adopted.

Imaging under UV light

Photographs of detached florets under UV-B rays were taken using a UV Transilluminator 2000 (Bio-Rad Laboratories, Hercules CA, USA) and the DigiDoc-it Darkroom apparatus (UVP, Upland, CA, USA). Detached

[1] See Supplementary Table 1

inflorescences of WT and *turf* were photographed using a digital camera (Canon PowerShot A590 IS; Canon, Tokyo, Japan) in a climatic chamber equipped with UV-B lamp tubes (Philips Ultraviolet B, TL 20W-12RS, Koninklijke Philips Electronics, Eindhoven, The Netherlands).

Morphological analyses

Morphological analyses were performed on both mutant and WT florets from randomly selected plants (n = 5) characterized by inflorescences with 50% of disc florets in flowering on the head area (R-5.5 stage according to Schneiter and Miller 1981)[2] from three replicate progenies grown in the field. From each floret type (ray, tubular-like ray and disc florets) 10 organ types (corollas, floral bracts and ovaries) were randomly collected at 9:00 a.m., and rapidly photographed with a digital camera (Canon PowerShot A590 IS; Canon, Tokyo, Japan). Disc florets were harvested from the periphery of the inflorescence adjacent to ray florets or tubular-like ray florets. Corolla length was measured in mature florets using image analysis software (ImageJ; http://rsbweb.nih.gov/ij/). After the acquisition of digital images, the number of petal primordia was evaluated using a binocular microscope (Wild Makroscop M420; Wild Heerbrugg Ltd, Heerbrugg, Switzerland).

In tubular-like ray florets, the corolla tube length was defined as the length from the bottom of the corolla to the deepest lacinium. Ovary and corolla were isolated from each floret and dried at 70°C to estimate dry weight (DW). In tubular-like ray florets, anthers and pistils were removed to exclude contamination, and only corolla explants were collected. To investigate ovary

[2] See Supplementary Table 1

pubescence, explants in the middle portion of ovaries from ray florets and tubular-like ray florets (0.5 cm) were detached and fixed in FAA solution [5% (v/v) acetic acid, 45% (v/v) ethanol, 5% (v/v) formaldehyde and 45% distilled water] under a vacuum. After 24 h, explants were cleared in 5% sodium hydroxide at 60°C for 2-3 d, and then placed in a saturated chloral hydrate solution until transparent. The cleared explants were rinsed in distilled water and observed with a dissecting microscope. Images were recorded with a digital camera. The morphology and/or defects of the style, stigma, and anthers in tubular-like ray florets were observed immediately after harvesting with an eyepiece micrometer, using a dissecting microscope. In these florets, corolla tubes of tubular-like florets were split open, and the morphology of each organ was evaluated.

Embryo length, embryo DW and pericarp DW were analysed from achenes of both mutant and WT plants (n = 5) selected randomly from three replicate progenies grown in the field when the color of the back of the head became yellow (R-7/R-8 stage according to Schneiter and Miller 1981)[3]. In both genotypes, achenes were harvested from the periphery of the inflorescence (ray and tubular-like ray florets) and at the first row of disc florets. From each floret type, 10 achenes were randomly collected. Images of embryos were recorded with a digital camera and their length was measured using ImageJ software. Dry weight of embryos and pericarp envelopes were determined after incubation at 70°C~~, when~~ a constant weight was achieved.

[3] See Supplementary Table 1

Achenes harvested from tubular-like ray florets after two different pollination processes

Frequencies of seed set (the proportion of filled achenes) from tubular-like ray florets following hand pollination were recorded for two different progenies (*turf 3* and *turf 5*) of the *turf* mutant (three plants for each progeny). The corolla of each tubular-like floret was cut with a lancet to expose the stigma. Pollen grains were collected from disc florets of the same plant in a Petri dish with a spatula between 3:00 and 4:00 p.m. Immediately after, pollen grains were distributed with a paint-brush on stigmas of tubular-like ray florets. Frequencies of seed set under open pollination were also evaluated in 5 - 10 plants randomly selected from four progenies (*turf* G15, *turf* G10, *turf* 10/1, *turf* 101/61). A random sample (n = 50) of filled achenes from tubular-like ray florets was tested to evaluate germination ability and seedling vigour (data not shown). The plants obtained were grown in the field until flowering (data not shown).

Histological analysis

Corolla samples of mature ray florets, tubular-like ray florets and disc florets were harvested from field-grown plants characterized by inflorescences with 10% of disc florets in flowering on the head area (R-5.1 stage according to Schneiter and Miller 1981)[4]. Ray florets and tubular-like ray florets at an immature stage of development (approximately 1 cm long) were isolated from inflorescences of WT and *turf* plants characterized by inflorescence buds extending more than 2.0 cm above the nearest leaf attached to the stem (R-3

[4] See Supplementary Table 1

stage according to Schneiter and Miller 1981)[5]. In addition, immature inflorescences of WT and *turf* were harvested from plants characterized by terminal buds shaped like a miniature floral head (R-1 stage according to Schneiter and Miller 1981)[6].

Plant material was fixed for 24 h in FAA solution [5% (v/v) acetic acid, 50% (v/v) ethanol, 10% (v/v) formaldehyde and 35% distilled water], dehydrated using a graded ethanol series, and then cleared in Noxil (Italscientifica S.p.A., Genova, Italy) in a five step-process according to Ruzin (1999). Samples were embedded in Paraplast Plus® (Sigma-Aldrich Co. LLC St. Louis, USA) and sectioned at 8 µm using a manual rotary microtome (Reichert, Vienna, Austria). The serial transverse sections were stained with a solution containing Alcian Blue 8GX, Bismarck Brown Y and Safranin O according to Graham and Trentham (1998) or with Delafield's haematoxylin, as previously reported (Fambrini et al. 1996; 2011). Sections were observed with a Leica DMRB light microscope (Leica Microsystems, Wetzlar, Germany) and images were recorded with a digital camera.

Scanning Electron Microscopy

Samples from immature inflorescences of WT and *turf* were collected at different stages of development according to Tähtiharju et al. (2012)[7]. In addition, corolla samples from ray florets of WT and from tubular-like ray florets of the *turf* mutant were collected from inflorescences ~~in plants~~ at stage R5-1 (Schneiter and Miller 1981)[8]. Samples were fixed overnight in FAA

[5] See Supplementary Table 1
[6] See Supplementary Table 1
[7] See Supplementary Table 2
[8] See Supplementary Table 1

solution [5% (v/v) acetic acid, 50% (v/v) ethanol, 10% (v/v) formaldehyde and 35% distilled water], dehydrated in an ethanol series (50, 70, 85, 95, and 100%), followed by critical point drying in liquid carbon dioxide (CO_2). Samples were then gold coated using a sputter coater (SEMPREP2, Nanotech Manchester, UK) and observed with a LEO 1430 scanning electron microscope (LEO Electron Microscopy ltd. Cambridge, UK). Conical cell density ~~on adaxial side, and~~ rib cell length and trichome density on abaxial side, were evaluated ~~in samples of corolla~~ using ImageJ software.

Statistical analysis

Differences between means were tested using Student's *t*-test (P = 0.05, 0.01 or 0.001).

Results

Inflorescence and floret features

The sunflower inflorescence (Fig. 1A) appeared modified in the *turf* mutant since tubular-like ray florets (Fig. 1C) developed at the periphery in place of ray florets (Fig. 1B). In the WT capitulum, the corolla of ray florets was made up of an elongated lamina and a very short tube (Table 1). In contrast, tubular-like ray florets showed only an elongated tube (Table 1). At maturity, the corolla growth in ray florets was significantly higher than in tubular-like ray florets; however, the corolla of tubular-like ray florets was three times longer than in disc florets (Table 1). Bracts subtending ray and tubular-ray florets were undistinguishable (Table 1); moreover, no differences

between WT and mutant were detected for floral bracts of disc florets (Table 1). We previously documented the distinctive development of reproductive organs in tubular-like ray florets (Berti et al. 2005); in addition, a closer view of the corolla in tubular-like ray florets showed a wavy ~~surface~~ corolla and a gibbous shape (Berti et al. 2005). However, in the present study we found further distinctive traits of tubular-like ray florets: the presence of nectaries and large ovaries with a dense pubescence (Table 1). ~~In disc florets~~ we did not observe differences between WT and *turf* (Fig. 1D; Table 1).

Floret ontogeny in WT and *turf* plants

To compare the corolla development in WT and *turf* inflorescences, we analysed the ontogeny of floret primordia using scanning electron microscopy (SEM). At stage 1 (according to Tähtiharju et al. 2012)[9], ray and disc primordia had undifferentiated structures. In WT inflorescences, the ray primordia had a flatter abaxial side (Fig. 2A, white arrowheads), whereas disc floret primordia were bigger and circular (Fig. 2A, black arrowheads). Later in development, WT ray florets grew two ventral petal primordia (Fig. 2B), which fuse to give rise to a hairless ligule (Figs. 2C, 2D). In contrast, *turf* tubular-like ray floret primordia were ellipsoid in shape (Fig. 2E, white arrows). Later in development, tubular-like ray florets developed a ring-shaped petal primordium that revealed a radially symmetrical corolla tube (Figs. 2F, 2G). At a subsequently stage, numerous trichomes were observed (Fig. 2H). In *turf* disc florets, no differences were detected in corolla ontogeny compared to WT (compare Figs. 2I-L and 2M-P).

[9] See Supplementary Table 2

Epidermal cell development and trichome density in corolla of mature ray and tubular-like ray florets

On the adaxial side of the corolla tube of both tubular-like ray floret and ray floret ligule of WT, papillose conical cells of the same shape, were observed (Figs. 3A, 3B). Additionally, in the mutant, we observed reflective terminal cones with typical patterns of striations (Wojtaszek and Maier 2014). ~~Nevertheless, differences were observed with respect to the~~ conical cell density ~~with a~~ higher ~~density~~ in the *turf* mutant (Table 2).

On the abaxial side of the corolla, flat epidermal cell surfaces were observed both in WT and *turf*. Differences were detected in rib cell length and trichome density: in corollas of tubular-like ray florets, the length of rib cells was reduced (Figs. 3C, 3D; Table 2), while the trichome density was higher (Figs. 3E, 3F; Table 2).

Response of WT ray floret ligules and *turf* tubular-like ray floret corolla tubes to UV-B light

The ligule of WT ray floret was reactive to UV-B light, but the corolla tube of a tubular-like ray floret was nonresponsive (Figs. 4A, 4B). However, after splitting, the open halves of the tubular-like ray floret corolla tube responded to UV-B light (Figs. 4A, 4B). Consequently, only the WT inflorescence was brilliant under this light (Fig. 4C).

Floral anatomy of WT and *turf* florets

In longitudinal sections of immature inflorescences, a very different architecture between ray florets and tubular-like ray florets was observed (Figs. 5A, 5D). This ~~differed from~~ cross sections of corollas isolated at stage R5-1 (Schneiter and Miller 1981)[10]. In cross sections, similar anatomy and cellular structure was observed in ray florets and tubular-like florets (Figs. 5B, 5E). In both WT and *turf*, adaxial epidermal cells were larger than abaxial epidermal cells (Figs. 5C, 5F). In addition, the mesophyll was organised into multilayered parenchyma cells but in the mutant intervein, constrictions (Figs. 5B, 5E) and smaller vascular bundles were detected (Figs. 5C, 5F).

Longitudinal sections in the centre of immature inflorescences showed actinomorphic and hermaphrodite disc florets (Fig. 5G). Longitudinal sections of single and mature florets revealed a heterogeneous structure in WT disc floret corollas (Figs. 5H-K). In the distal portion, WT corolla tips were characterized by multilayered parenchyma cells, while the adaxial epidermis had papillose conical cells (Fig. 5C). In contrast, in the middle portion of the WT corollas, there were only a few layers of parenchyma cells, and the papillose cells of the inner adaxial epidermis were not conical (Fig. 5D). The bulbous base of the WT corollas was the thickest portion (Figs. 5B, ~~5E~~).

In order to analyse ~~more in detail~~ floral anatomy at the periphery of the inflorescence at immature stages of development, we performed cross sections of single florets (1 cm long). Floral anatomical structure was analysed through serial cross-sections from the tip of the ovary to the distal portion of the corolla (Fig. 6). In WT, a characteristic folding of the ligule (c fold type) was detected in the distal zone of the corolla (Fig. 6A). In the

[10] See Supplementary Table 1

mutant, there was a prismatic ring of wavy corolla surrounded anthers and stigmas (Fig. 6B). In the basal portion of the WT corolla, the ligule was also folded, but cross-sections clearly showed a thicker corolla (Fig. 6C). In the mutant, the corolla was tubular (Fig. 6D). In the proximal zone of the corolla tube of tubular-like ray florets, a circular stylopodium nectary surrounding the base of style was also detected (Fig. 6F). In the WT, these structures were not observed (Fig. 6E).

Morphological characteristics of the gynoecium and androecium of tubular-like ray florets

An earlier, preliminary microscopic analysis of the style, stigma and stamens in tubular-like ray florets showed homeotic transformations and malformations (Berti et al. 2005). In the present work, we evaluated the frequency of these phenomena (Table 3). A high frequency of styles with a different number of stigmas (1 or 3) was observed, while nearly all the stamens were normal. However, low frequencies of malformed anthers and petaloid stamens were found (Table 3). In disc florets of *turf* and WT, the same analysis did not reveal significant numbers of abnormal transformations (data not shown). In WT ray florets, this analysis was not carried out due to the lack of reproductive organs.

Characteristics of embryos and achenes developed from tubular-like ray florets

In achenes from tubular-like ray florets of the *turf* mutant, embryo frequency under open pollination conditions was seven times lower than by

hand pollination (Table 4). In addition, embryo development was precociously coupled with phytomelanin deposits in pericarps (Fig. 7A). Three-sided prismatic achenes developed from tubular-like ray florets, while achenes from disc florets were two-sided (Fig. 7B). In achenes from tubular-like ray florets, zygotic embryos were shorter and lighter than embryos from disc florets (Fig. 7C; Table 1) but were able to produce fertile plants. By contrast, WT ray achenes were empty, thin and partially crumpled (Fig. 7B); in addition, in ray achenes, pericarp DW was lighter than in tubular-like ray achenes (Table 1). Seed set from disc florets of WT and *turf* mutant in open-pollinated progenies was comparable (Table 1).

Discussion

How distinct floret types develop in the same pseudanthium is a challenging question for plant biology (recently reviewed by Broholm et al. 2014). In the last few years, the molecular aspects of pseudanthium development have been studied in Asteraceae, Dipsacaceae and Myrtaceae, implicating *CYC2* clade genes in the regulation of floret type identity (Broholm et al. 2008; Chapman et al. 2008; Kim et al. 2008; Carlson et al. 2011; Tähtiharju et al. 2012; Claßen-Bockhoff et al. 2013; Juntheikki-Palovaara et al. 2014).

The huge radiate capitulum of the sunflower requires the *HaCYC2c* gene. Mutants with down-regulation of this gene showed the development of tubular-like ray florets at the periphery of the inflorescence (Fambrini et al. 2011; Chapman et al. 2012). To date, few traits of floret identity have been

defined in *turf* inflorescences (Berti et al. 2005). In this work, we show that tubular-like ray florets combine an unusual set of identity traits in the Heliantheae tribe, and that filled achenes develop at the periphery of the mutant capitulum.

SEM images of very early stages of floret bud development at the periphery of *turf* inflorescences showed initial morphogenic stages similar to those of WT ray florets. In particular, peripheral floral buds were subtended by involucral bracts, were not cylindrical, and developed later than the disc floret buds. Similar observations have been made in *Anacyclus valentinus* (Bello et al. 2013), suggesting that in different tribes, the ontogeny of tubular-like ray buds is a comparable process.

In sunflower, scanning electron microscopy analysis of ray floret development has been documented (Marc and Palmer 1981; Tähtiharju et al. 2012); however, our observations are the first to report an unusual pseudanthium of the genus *Helianthus*. We hypothesize that the three-cornered shape of the immature buds of both ray and tubular-like ray florets could be related to spatial constrictions. This may be caused by the earlier development of a large and flat involucral bract originating from a distinct primordium. The lack of paleae and the delay in development would seem to indicate that florets at the periphery are remnants of a different order of branches (Pozner et al. 2012; Bello et al. 2013).

A multifaceted corolla identity was observed in the *turf* inflorescence in peripheral florets. In tubular-like ray florets, conical cell distribution on the dorsal side of the corolla tube and the ~~corolla~~ anatomy showed clear similarities with WT ray floret ligules. In fact, a well-differentiated layer of

conical cells, conforming to the Chrysanthemum-type (on the basis of the nomenclature introduced by Barthlott and Ehnler, 1977), was detected in both tubular-like ray florets and WT ray florets. In contrast, in the corolla of disc florets, conical cells were only evident on petal tips. In addition, histological analysis revealed very different organization in proximal, medial and distal regions of disc floret corollas. On the other hand, the analysis of rib cell length and conical cell size of the corolla tube of tubular-like ray florets showed differences with respect to ray florets ligules. On the abaxial side of tubular-like ray florets, trichome density was higher than on the abaxial side of ray floret ligules, and more similar to the abaxial side of disc floret corollas. Lastly, corolla tubes were wavy only in tubular-like ray florets.

Development of conical papillose cells is an adaptive trait, and this cell type may increase pollinator visitation rates (Glover and Martin 2002). The presence of conical cells on the inner adaxial side of the corolla tubes in tubular-like ray florets would thus seem to be a non functional trait because only the petal tips are directly exposed ~~to sunlight~~.

Likewise, a mixed combination of ovarian traits was observed in the tubular-like ray florets, because the presence of ovules and pubescence density are typical of disc floret ovaries. However, the three-sided shape of the tubular-like ray florets is reminiscent of ray floret ovaries. Gibbous tubular-like ray florets with characteristic folds of corolla tissue and dense ovary pubescence have also been described in the tribe Heliantheae, subtribe Madiinae, during a breeding program involving *Layia glandulosa* (radiate inflorescence) and *L. discoidea* (discoid inflorescence): the origin of the gibbous florets was related to gene recombination (Ford and Gottlieb 1990).

Another distinctive trait of tubular-like ray florets is a collar-like discoidal nectary at the base of the style. In sunflower, floral nectaries are usually annular multicellular structures characterized by epidermal cells which are densely packed into uniform columns (where stomata are differentiated). Such columns are found at the top of the inferior ovary surrounding the base of style in disc florets (Sammataro et al. 1985). A high density of stomata and high sugar concentration are preferred traits for honeybees. In *turf* inflorescences, both tubular-like ray florets and disc florets produced discoidal nectaries and it would be worthwhile to investigate the nectary characteristics related to these floral types.

Interestingly, tubular-like ray florets of *turf* develop reproductive organs while ray florets of WT are sterile (Berti et al. 2005). In the present work, we demonstrated that under open pollination the fertility of tubular-like ray florets was very low. However, a substantial increase in filled achenes was obtained by hand pollination. In tubular-like ray florets, the reproductive organs may be affected by the length of the corolla tube, which is not the case in disc florets. The low seed set could also be related to homeotic conversions and malformations detected in the reproductive organs of tubular-like ray florets.

In sunflower, dry single-seeded two-sided achenes consisting of a fruit coat (pericarp) and large zygotic embryos are produced from disc florets, while ray florets have crumpled and unfilled achenes. However, in the *turf* mutant, three-sided filled achenes were produced from tubular-like ray florets. In Asteraceae, achene heteromorphism is frequent and linked to the anatomy of the heterogamous inflorescence. Differences ~~of~~ fruit and/or embryo morphology include size, color, shape, hairiness, and the presence of pappus

structures (Imbert 2002). In the *turf* mutant, achenes from peripheral tubular-like ray florets ~~showed~~ smaller zygotic embryos with respect to disc floret embryos. However, in some heterocarpic Asteraceae (e.g. *Picris amalecitana*) the weight of peripheral achenes is greater than that of central one (Ellner and Shmida 1984). The genetic regulation of heteromorphic fruits and seeds is poorly known (Imbert 2002). Thus, it is remarkable that in sunflower, the loss-of-function of *HaCYC2c* is sufficient to induce hermaphroditism in florets at the periphery of the inflorescence.

Taken together, we demonstrated that an atypical floret type at the periphery of the *turf* pseudanthium recapitulates both ray and disc identity traits, while acquiring a set of unique traits. Some features of tubular-like ray florets are similar to larger disc florets but simultaneously show corollar and ovarian traits typically present in ray florets. Moreover, they possess a wavy corolla and a gibbous shape not described in WT inflorescences. As highlighted by Ford and Gottlieb (1990), the differentiation of gibbous florets demonstrates that new developmental processes can be readily integrated without evident detrimental effects. Heterogamous inflorescences with tubular-like ray florets are not common in the Heliantheae tribe, but are frequent in the Cardueae-tribe, where marginal tubular-like ray florets have evolved as a means of attracting pollinators. It is interesting to note that, compared to *Centaurea cyanus* for example, tubular-like ray florets in the *turf* mutant are less showy, but are capable of developing seeds.

In conclusion, the loss-of-function of the specific gene *HaCYC2c* in the *turf* mutant redesigns the floral traits and fertility at the periphery of the capitulum in myriad ways. This gene controls corolla symmetry both in ray

and disc florets, as was demonstrated in sunflower mutants (Fambrini et al. 2011; 2014; Chapman et al. 2012). However, the relationship between the expression of this gene and floral fertility remains unclear. The negative control of the *HaCYC2c* gene in the development of male and female parts is evident only in ray florets, because the expression in disc florets, at later stages of development, has no effect on the differentiation of reproductive organs (Tähtiharju et al. 2012). We hypothesize that other *CYC/TB1*-like genes are important to determine the specific characteristics of tubular-like ray florets. In fact, 10 genes of the CYC/TB1-like gene family have been identified in gerbera as well in sunflower, and duplicated *CYC2* clade genes are associated with pseudanthium architecture and differentiation of floral reproductive organs (Broholm et al. 2008; Chapman et al. 2008; Tähtiharju et al. 2012; Juntheikki-Palovaara et al. 2014). *CYC2* clade genes show highly overlapping expression patterns, and functional gene specificity could be obtained through context-specific protein complexes that activate different downstream targets.

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Figure legends

Fig. 1. The *tubular ray flower* (*turf*) mutant of sunflower (*Helianthus annuus*). A) *turf* (left) and wild type (WT; right) plants. B) A ray floret of WT. C) A tubular-like ray floret of *turf*. D) Disc florets of *turf* (1) and WT (2). Scale bars = 8.6 mm (B), 7.4 mm (C), and 4.8 mm (D).

Fig. 2. Scanning electron micrographs of sunflower floret primordia at various developmental stages in wild type (WT) (A-D, I-L) and *tubular ray flower* (*turf*) mutant (E-H, M-P). (A) Stage 1 of WT ray floret buds (white arrows); at the periphery of the inflorescence, note stage 2 of disc floret primordia (black arrowheads). WT ray floret primordium development at stage 3 (B), stage 5 (C) and stage 6 (D). (E) Stage 1 of *turf* tubular-like ray floret buds (white arrows). *turf* tubular-like ray floret bud at stage 3 (F), stage 5 (G) and stage 6 (H). (I-L) Progression of WT disc floret primordia development from stage 1 to stage 4. (M-P) Progression of *turf* disc floret primordia development from stage 1 to stage 5. The stages of floret development are numbered according to Tähtiharju et al. (2012)[11]. Scale bars = 100 µm (A-E, G-P) and 50 µm (F).

Fig. 3. Corolla traits observed with a scanning electron microscope in ray florets of wild type (WT) and tubular-like ray florets of *tubular ray flower* (*turf*) mutant. A) Conical cells on the adaxial side of a WT ray floret ligule. B) Conical cells within the inner side (adaxial) of a corolla tube of a tubular-like ray floret. C) Vein on the abaxial side of a ray floret ligule. D) Vein on the

[11] See Supplementary Table 2

abaxial side of a corolla tube of a tubular-like ray floret. E) Trichomes on the abaxial side of a ray floret ligule. F) Trichomes on the abaxial side of a corolla tube of a tubular-like ray floret. Scale bars = 50 μm

Fig. 4. Appearance of florets and detached inflorescences of wild type (WT) and *tubular ray flower* (*turf*) mutant observed under UV-B light. A) A ray floret (1) and a tubular-like ray floret intact (2) or with corolla tube divided in two (3). B) Specimens as in A but under UV-B ~~rays~~ using an imaging device. C) *turf* (left) and WT (right) inflorescences were exposed in climate chamber equipped with UV-B lamp ~~tubes~~ and photographed with a digital camera (black and white photo). Note, some florets at periphery of the inflorescences were removed in both genotypes. Scale bars = 13.3 mm (A) and 10.5 mm (B).

Fig. 5. Floral anatomy in wild type (WT) and *tubular ray flower* (*turf*) mutant. A) Longitudinal section of a WT ray floret in an immature inflorescence. B) Cross section of a mature ray floret ligule. C) Detail of the ligule with a vein. D) Longitudinal section of a *turf* tubular-like ray floret in an immature inflorescence. E) Cross sections of a corolla of tubular-like ray floret. F) Detail of the corolla with a vein. G) Longitudinal section of a disc floret in an immature WT inflorescence. H) Longitudinal section of a WT disc floret corolla. I) Cross section of distal portion of corolla lobes. J) Cross section of medial portion of corolla tube. K) Cross section of proximal portion of a corolla tube in the bulged region. Scale bars = 0.88 mm (A), 0.11 mm (B), 0.05 mm (C), 1.00 mm (D), 0.16 mm (E), 0.04 mm (F), 0.50 mm (G), 0.70 mm (H), 0.10 mm (I), 0.06 mm (J), and 0.15 mm (K).

Fig. 6. Floral anatomy of immature WT ray florets (A, C, E; light microscopy micrographs) and *turf* tubular-like ray florets (B, D, F; light microscopy micrographs). (A) Cross section of the distal portion of a corolla (co) ligule of a ray floret. (B) Cross section of distal portion of a corolla tube (co) of a tubular-like ray floret. Note the presence of anthers (an) and stigma (s) inside the tube. (C) Cross section of a middle portion of a corolla (co) ligule of a ray floret. (D) Cross section of a middle portion of a corolla (co) tube of a tubular-like ray floret. Inside the tube note five anther filaments (f) and style (st). (E) Cross section of proximal corolla tube of a ray floret. (F) Cross section of a proximal corolla tube of a tubular-like ray floret. Inside the tube is the nectary (n). Scale bars = 0.22 mm (A, E), 0.25 mm (B, F), 0.38 mm (C), and 0.32 mm (D).

Fig. 7. Achenes and embryos of wild type (WT) and *tubular ray flower* (*turf*) mutant. (A) A *turf* inflorescence (side view) showing three fertilized achenes characterized by phytomelanin deposition. Involucral bracts were removed to reveal florets at the periphery of the inflorescence. (B) An empty achene from a WT ray floret (1). A fertile achene from a WT disc floret (3). A filled achene from a tubular-like ray floret (2). (C) Heteromorphic zygotic embryos isolated from a mature achene at the periphery (tubular-like ray florets) (1) or from a mature achene of the first row of disc florets (2) in a *turf* inflorescence. Scale bars = 0.48 cm (A), 0.40 cm (B), and 1.28 mm (C).

Supplementary Table 1. Description of sunflower growth stages, modified from Schneiter and Miller (1981).

Stage	Description	Note
VE	Seedling has emerged and the first leaf beyond the cotyledons is less than 4 cm long.	Vegetative emergence
V1, V2, V3 etc.	These are determined by counting the number of true leaves at least 4 cm in length beginning as V1, V2, V3, V4 etc.	Vegetative stages
R1	The terminal bud forms a miniature floral head rather than a cluster of leaves. When viewed from directly above the immature bracts form a many-pointed star-like appearance.	Reproductive stage
R2	The immature bud elongates 0.5 to 2.0 cm above the nearest leaf attached to the stem. Disregard leaves attached directly to the back of the bud.	Reproductive stage
R3	The immature bud elongates more than 2.0 cm above the nearest leaf.	Reproductive stage
R4	The inflorescence begins to open. When viewed from directly above immature ray flowers are visible.	Reproductive stage
R5 (decimal)	This stage is the beginning of flowering. The stage can be divided into substages dependent upon the percent of the head area (disc flowers) that has completed or is in flowering. Ex. R5.3 (30%).	Reproductive stages
R6	Flowering is complete and the ray flowers are wilting.	Reproductive stage
R7	The back of the head has started to turn a pale yellow color.	Reproductive stage
R8	The back of the head is yellow but the bracts remain green.	Reproductive stage
R9	The bracts become yellow and brown. This stage is regarded as physiological maturity.	Reproductive stage

Supplementary Table 2. Description of early stages (1-6) of floret primordia development in sunflower inflorescence after scanning electron microscopy analysis, according to Tahtiharju et al. (2012).

Stage	Ray floret primordia	Disc floret primordia
1	Primordia are small undifferentiated bumps with a flatter side.	Primordia are small undifferentiated bumps with a spherical ~~-like~~ shape.
2	Primordia are clearly flatter and only two petal primordia begin to grow.	Petal primordia characterized by a ring-shaped form develop and disc floret primordia are almost perfectly circular.
3	Two petal primordia grow.	The individual petal tips could be clearly distinguished and the anther primordia start~~ed~~ to become recognizable.
4	The petal primordia grow out further.	Petals beg~~an~~ to elongate, covering the developing stamen and carpel primordia.
5	The petals are clearly fused.	~~In primordia,~~ floral organs develop~~s~~ and on the surface of petals, small trichomes emerge.
6	The structure of ligule primordium is now ~~observed.~~	~~The~~ trichome density significantly increase on petal primordia.

Table 1. Evaluation of various characteristics in ray florets, tubular-like ray florets, disc florets, bracts and achenes of wild type (WT) and *tubular ray flower* (*turf*) inflorescences of sunflower (*Helianthus annuus*).

	WT	*turf*	WT	*turf*
Floral morphological trait	ray floret	tubular-like ray floret	disc floret	disc floret
Corolla				
Total corolla length (mm)	58.25 ± 5.67[a]	23.06 ± 1.42***[b]	8.67 ± 0.26	8.87 ± 0.18 ns[b]
Tube length (mm)	3.16 ± 0.06	17.74 ± 1.47***	7.62 ± 0.38	7.82 ± 0.38 ns
Ratio Tube/corolla	0.05 ± 0.002	0.80 ± 0.06***	0.88 ± 0.03	0.87 ± 0.04 ns
Corolla DW (mg)	52.57 ± 5.72	26.19 ± 1.95**	7.79 ± 0.34	8.53 ± 0.85 ns
Petal primordia (number)	2.25 ± 0.24	4.91 ± 0.11***	4.74 ± 0.13	4.80 ± 0.06 ns
Bract				
Floral bracts DW (mg)	12.07 ± 4.69	15.14 ± 4.91 ns	1.20 ± 0.50	1.59 ± 0.39 ns
Nectary				
Florets with nectary (%)	0	100***	100	100 ns
Ovary				
Ovary length (mm)	8.02 ± 0.49	8.54 ± 0.3 ns	9.21 ± 0.61	9.69 ± 0.53 ns
Ovary DW (mg)	7.96 ± 0.84	14.30 ± 2.60*	11.11 ± 0.65	12.28 ± 1.42 ns
DW ratio Corolla/ovary	6.82 ± 1.17	1.86 ± 0.22*	0.71 ± 0.05	0.66 ± 0.07 ns
Ovary pubescence	reduced	preeminent	preeminent	preeminent
Achene				
Embryo length (mm)	0	6.25 ± 0.44***	7.25 ± 0.31	7.07 ± 0.34 ns
Embryo DW (mg)	0	12.72 ± 4.46***	21.12 ± 6.39	18.46 ± 2.44 ns
Pericarp DW (mg)	3.82 ± 0.69	8.11 ± 1.93***	11.78 ± 2.58	9.12 ± 2.02 ns
Filled seed				
Seed set (%)	0	6.37 ± 7.16***	66.79 ± 0.14	71.78 ± 0.07 ns

[a] Values are mean ± SD.

[b] ns = not significant, $P > 0.05$. Asterisks indicate significant differences from wild type (WT): *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, respectively (Student *t* test).

Table 2. Data regarding corolla micromorphology of WT ray florets and tubular-like ray florets of *tubular ray flower* (*turf*) of sunflower (*Helianthus annuus*).

	WT	*turf*
Floral morphological trait	ray floret	tubular-like ray floret
Rib cell length (μm)	47.77 ± 18.58[a]	36.06 ± 5.8***[b]
Conical cell density (No./mm^2)	1722.75 ± 302.55[a]	2841.00 ± 106.07**[b]
Trichome density (No./mm^2)	30.82 ± 3.83[a]	104.24 ± 58.08*[b]

[a] Values are mean ± SD.

[b] ns = not significant, $P > 0.05$. Asterisks indicate significant differences from wild type (WT): *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, respectively (Student *t* test).

Table 3. Frequency of morphological characteristics revealed in gynoecium and androecium of tubular-like ray florets of the *tubular ray flower* (*turf*) mutant of sunflower (*Helianthus annuus*).

Character	Tubular-like ray floret
Two-branched stigmas (normal) (%)	60.34 ± 34.64 [a]
Non-branched stigmas (%)	24.80 ± 41.69
Three-branched stigmas (%)	13.73 ± 16.63
Petaloid styles (%)	0
Normal anthers (%)	96.11 ± 3.67
Malformed anthers (%)	2.1 ± 2.64
Petaloid stamens (%)	1.81 ± 2.1

[a] Values are mean ± SD.

Table 4. Development of zygotic embryos from achenes in different progenies of the *tubular ray flower* (*turf*) mutant of sunflower (*Helianthus annuus*) after hand or open pollination.

Progeny	Total achenes	Achenes with embryo	(%)
Hand pollination			
turf 3/1	31	23	74.19
turf 3/2	31	24	77.41
turf 3/3	33	19	57.57
turf 5/1	35	22	62.85
turf 5/2	32	14	43.75
turf 5/3	36	12	33.33
Total	198	114	57.60
Open pollination			
turf G15	270	3	1.12
turf G10	269	12	4.46
turf 10/1	267	8	2.99
turf 101/61	491	83	16.91
Total	1297	106	8.17