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# Constitutive co-suppression of the *GA 20-oxidase1* gene in tomato leads to severe defects in vegetative and reproductive development

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ABSTRACT

To dissect the role of gibberellins in tomato development, we have constitutively down-regulated the gene *GA 20-oxidase1* (*GA20ox1*). Plants co-suppressed for *GA20ox1* (referred to as CO-6 plants) showed vegetative defects typical of GA deficiency such as darker and mis-shaped leaves and dwarfism. CO-6 plants flowered as the controls, although their flowers had subtle defects in the pedicel and in organ insertion. Analysis of male development revealed defects before, during and after meiosis, and a final pollen viability of 22%. The development of female organs and gametes appeared normal. Pollination experiments indicated that the pollen produced by CO-6 plants was able to fertilize control ovaries, but the analysis of the progeny showed that the construct was not transmitted. Ovaries of CO-6 plants showed high fruit set and normal fruit development when pollinated with control pollen. However these fruits were completely seedless due to a stenospermocarpic behaviour that was evidenced by callose layering in the endothelium between 7 and 15 days after pollination.

We conclude that *GA20ox1* in tomato exerts specific developmental roles that are not redundantly shared with other members of this gene family. For reproductive male development, silencing of this gene is detrimental for pollen production and either gametophytically lethal or severely hampering seed germination. In the pistil, the co-suppression construct does not affect the progamic phase, nor fruit set and growth, but it interferes with seed development after fertilization leading to seed abortion.
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## 1. Introduction

Gibberellins (GAs) are central to many aspects of plant development, both vegetative (seed germination, trichome development, stem and leaf elongation) and reproductive (flower induction, anther, seed and fruit development). In the reproductive process, a primary role of GA is exerted in inducing flowering by positively regulating the activity of key genes such as *LEAFY* in Arabidopsis [1]. During flower ontogenesis, GAs are important to promote male organ formation and function and flower organ size in general [2–5]. Recently, many researches have shown that GAs positively regulate the growth and development of pollen tubes [6–8]. Finally, GAs are important to stimulate fruit and seed development as GA deficient mutants hardly set normal fruit and seed [9–11].

The extensive knowledge on the role of hormones in plant growth and development has conventionally resulted from the measurement of endogenous hormone contents and the study of the effect of hormone application to wild-type and mutant genotypes [12,13]. The identification of several genes encoding enzymes involved in GA metabolism [14,15] and response [16,17] has opened the doors to the study of hormone physiology and function by altering hormone levels in plants where hormone-related genes are silenced or overexpressed. Such tailored experiments may unravel the complexity of functional redundancy or specificity of different members of gene families [6,18]. In addition, genetic engineering of genes involved in the metabolism and transport of or response to hormones entails significant applied perspectives in order to control plant stature, flowering or fertility [18–21].

Along the GA biosynthesis pathway, genes involved in the final part of the metabolic chain have been reported as being tightly modulated. In particular, the transcription of the *GA 20-oxidase* (*GA20ox*) gene family is recognized as a key step for the control of active GA levels in several organs and it has been demonstrated that GA levels may be manipulated by altering the expression of *GA20ox* genes [19,22–24].

In tomato, *GA20ox* genes form a small family composed of three members, that are differentially transcribed in different organs, tissues and developmental stages [25]. From expression studies, *SlGA20ox1* appears particularly important in anther [25] and ovary development [26]. In this last organ, the gene is a candidate to play

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a specific role in fruit set because it positively responds to the stimulus of pollination and fertilization [26–28]. However, no detailed study has been aimed to unravel the functional role of the *GA20ox1* gene during tomato reproduction.

Here, we set out to down-regulate the transcription of *SlGA20ox1* through a co-suppression approach in order to dissect its role in the vegetative and reproductive development of the tomato plant. The phenotypes described in co-suppressed plants indicate that *GA20ox1* plays specific roles in stem elongation and leaf morphogenesis during vegetative growth and in pollen formation and seed development during reproduction.

## 2. Materials and methods

### 2.1. Plant materials, construct preparation and genetic transformation

Tomato (*Solanum lycopersicum* L.) cv Chico III untransformed (hereafter referred to as Control) and transformed plants were grown at Viterbo, Italy (42°26′N, 12°04′E) in an unheated tunnel with standard agronomic practices.

For silencing the *SlGA20ox1* gene (GenBank Accession No. AF049898; [25]), a 622 bp truncated 5′ portion of the coding sequence starting at position 16 downstream from the transcription initiation codon was amplified from a tomato ovary cDNA sample using the primers SlGA20ox1TR-F and SlGA20ox1TR-R (Table S1). Oligos included the *XbaI* and *SstI* restriction sites used for cloning. The amplified region was selected because of its relatively low sequence match to the other two tomato *GA20ox* genes. The amplification protocol was as described [26]. The PCR product was inserted in the pGEM-T Easy (Promega) vector according to the manufacturer's instructions and the plasmid cloned in competent *E. coli* DH5α cells. The insert was then excised from the vector by restriction with *Xba*I and *Sst*I and ligated in sense orientation downstream to the CaMV 35S promoter into the *pBI*121 vector (Clontech, Palo Alto, CA) to generate *pBI-COox1*. The correct incorporation was confirmed by sequencing. *pBI-COox1* was inserted into the *Agrobacterium tumefaciens* strain EHA105.

Transgenic tomato plants expressing the *pBI-COox1* construct were generated by co-cultivation of cotyledon explants from cv Chico III tomato seedlings and genetic engineered *A. tumefaciens* according to established protocols [29]. Transformed shoots were regenerated and selected on MS [30] agar medium containing 100 mg $L^{-1}$ kanamicin, 0.7 mg $L^{-1}$ zeatin, 0.35 mg $L^{-1}$ IAA and 500 mg $L^{-1}$ carbencillin. Rooting medium for regenerated shoots contained 50 mg $L^{-1}$ kanamicin 0.35 mg $L^{-1}$ IAA, 0.1 mg $L^{-1}$ IBA and 200 mg $L^{-1}$ cefotaxime.

Plants rooting on kanamicin were acclimatized to *in vivo* conditions in Giffy-pots. Genomic DNA was extracted from leaflets by the CTAB method [31] and checked by PCR with the SlGA20ox1TR-F and SlGA20ox1TR-R primers and with primers amplifying the CaMV 35S promoter and the *NptII* gene (Table S1).

One PCR-positive primary transformant showing a severely dwarf phenotype was selected and *in vitro* micropropagated in order to have a clone of 25 individuals (hereafter referred to as the CO-6 clone). Control plants from untransformed Chico III were also micropropagated to obtain a comparable reference.

### 2.2. Semi-quantitative gene expression analysis

For gene expression analysis, total RNA was prepared from ovaries or anthers dissected from stage 3 flowers (opening flower, according to Ref. [32]) from Control and CO-6 plants using Trizol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed with M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA) as reported [33]. Amounts of cDNA were normalized by PCR using degenerate primers amplifying members of the actin gene family, which produce a 500-bp fragment of the coding sequence (Table S1).

After normalization, cDNA samples were analysed for the genes *GA20ox1* and *GA20ox2* with the primer pairs SlGA20ox1-1/SlGA20ox1-2 and SlGA20ox1-1/SlGA20ox2-2 (the same forward primer was used for the two copies of the gene, Table S1). To assess that GA levels were altered in co-suppressed plants, cDNAs were also analysed for the GA-sensitive *GAD2* gene [34] using primers GAD2-1 and GAD2-2 (Table S1). Reverse transcription (RT)-PCR conditions are described in Table S1. The number of cycles was calibrated to avoid saturation for all the primer pairs. RT-PCR amplification products were electrophoresed through a 1% (w/v) agarose gel and stained with ethidium bromide.

### 2.3. Analysis of vegetative and reproductive development of the CO-6 clone

CO-6 plants were inspected during vegetative development for growth habit and morphology and size of the leaflets. At flowering, inflorescence and flower morphology was observed in comparison with that of control plants. Flowers at known developmental stages [32] were fixed in FAA (10% commercial formaldehyde, 50% ethanol, 5% acetic acid) for 12 h and then stored in 70% ethanol at 4 °C until used. With reference to male development, stages corresponded to pollen mother cells (PMCs, early stage 0, hereafter referred to as stage 0a, flower bud 4 mm long), pollen tetrads (late stage 0, hereafter referred to as stage 0b, flower bud 5 mm long), pollen callose dissolution (early stage 1, hereafter referred to as stage 1, flower bud 6 mm long), mature pollen (stage 4, open flower).

Observations on the occurrence of meiosis on both the male and female side were carried out using hand-dissected specimens stained for callose with a drop of 0.005% (w/v) aniline blue in 0.15 M sodium phosphate buffer, pH 9.5, and observed with a Zeiss (Oberkochen, Germany) Axioscop II microscope equipped with an epifluorescence illuminator (HBO50/AC BP 390-420 exciter filter and LP 450 barrier filter). Micrographs were taken using a Canon (Tokyo, Japan) PowerShot G6 digital camera.

To determine the percentage of stainable pollen, flowers collected at full anthesis were used. Two anthers per flower from ten flowers of control and CO-6 plants were dissected, squashed on a microscope slide and the released pollen grains stained in a drop of 1% acetic orcein and 50% glacial acetic acid solution. Stainable pollen was calculated as the percentage of plump, purple pollen grains appearing in a sample of at least 100 grains.

For estimating seed set, flowers of control and CO-6 plants were emasculated before anthesis, tagged and pollinated with pollen from either control (average 90% stainable pollen) or CO-6 (average 22% stainable pollen) plants. Ten flowers were pollinated for each of the four combinations (Control × Control, Control × CO-6, CO-6 × Control, CO-6 × CO-6). Fruit set was calculated as the number of ovaries enlarging after pollination and the polar diameter (cm) was measured with a calibre at 15 and 55 days after pollination (DAP). The seed set was estimated at maturity by extracting and counting the seed from all fruits.

To investigate the histology of ovary development after setting, six mature flowers each from the control and the CO-6 genotype were emasculated and hand pollinated with control pollen. Three mature flowers for each genotype were collected and fixed in FAA at 7 and 15 DAP, together with three unpollinated flowers at anthesis (0 DAP). These samples were embedded and sectioned as described [32] with the difference that Paraplast (Sigma) was used for embedding instead of Technovit. Histological slides were stained either with 0.1% toluidine blue in 2.5% sodium carbonate buffer, pH 11.0 and mounted with Eukit (Kindler, Freiburg, Germany) or with ani-

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