Induction of Monozygotic Twinning by Ascorbic Acid in Tobacco

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Abstract

Embryo development in plants initiates following the transverse division of a zygote into an apical, proembryo cell and a basal cell that gives rise to the suspensor. Although mutants affected in embryo development through changes in cell division have been described, little is known about the control of the first zygotic division that gives rise to the proembryo. Ascorbic acid (Asc) promotes cell division by inducing G1 to S progression but its role in embryo development has not been examined. In this study, we show that the level of dehydroascorbate reductase (DHAR) expression, which recycles Asc and promotes the G1 to S phase progression of cells within the QC of maize roots and increases considerably during logarithmic growth of tobacco cell culture [21,22]. Asc also affects the rate of monozygotic twinning and polycotyly. DHAR-induced twinning resulted from altered cell polarity and longitudinal instead of transverse cell division that generated embryos of equal size. Direct injection of Asc into ova ries phenocopied DHAR-induced twinning. Twinning induced by Asc was developmentally limited to the first two days after pollination whereas polycotyly was induced when the level of Asc was elevated just prior to cotyledon initiation. This work describes the first example of gene-directed monozygotic twinning and shows that Asc regulates cell polarity during embryo development.

Introduction

Embryo development initiates following the transverse division of a zygote into an apical, proembryo cell and a basal cell that gives rise to the suspensor, to generate one embryo per seed. The presence of more than one embryo per seed can result from apomixis, which occurs in a wide range of flowering plant taxa, or by twinning [1–3,4]. Apomixis is the assexual formation of a seed from maternal ovule tissues not involving meiosis and fertilization which results in monoembryonic or polycotyly. In apomicts, embryos can arise spontaneously from ovule cells (i.e., sporophytic apomixis) or from cells of an unreduced embryo sac (i.e., gametophytic apomixis) depending on the species [4]. In either case, embryos resulting from apomixis are genetically maternally-derived. Twinning commonly refers to the generation of more than one zygotically-derived embryo following fertilization. Identical twins arise from the same zygote, either following the first zygotic division (i.e., monozygotic twinning) or ectopically from other embryonic tissues such as the suspensor [5–7].

In Arabidopsis, embryos of the twn2 mutant exhibit reduced valyl-tRNA synthetase expression resulting in growth arrest of the embryo proper and embryogenetic transformation of suspensor cells [6]. Embryogenetic development of suspensor cells also occurs in the twnl and amp1 mutants in the absence of embryo proper arrest, suggesting loss of embryo proper-suspensor communication [5–7]. In these examples, twinning resulted from embryo development from suspensor cells once suppression of the embryogenetic potential of the suspensor was released. In such mutants, the development of ectopic embryos requires changes in the plane of cell division within the suspensor (e.g., longitudinal instead of transverse) that enable the growth of embryos or embryoid tissue. These mutants also exhibited cotyledon fusion or polycotyly independent of suspensor transformation, suggesting that these genes can affect cotyledon development as well as embryo development. Despite the isolation of several mutants affecting embryo development, no mutant affecting the first zygotic division in a way that results in monozygotic twinning has been reported and therefore those factors controlling the correct formation of the apical, proembryo cell and basal cell are unknown.

Ascorbic acid (Asc) is a major antioxidant that serves several functions in plants. Asc is involved in the detoxification of reactive oxygen species, e.g., superoxide, singlet oxygen, ozone, and H2O2, which are produced during aerobic metabolic processes such as photosynthesis or respiration. Asc is required for the re [8] generation of α-tocopherol (vitamin E) from the tocopheroxyl radical [9]. Asc serves as a co-factor for enzymes such as violaxanthin de-epoxidase (VDE) in the xanthophyll cycle, ACC oxidase which generates ethylene, and prolyl and lysyl hydrolases [10–12] as well as for 2-oxoacid-dependent dioxygenases required for the synthesis of asbicyclic acid and gibberellic acid [13–15].

Asc may also be involved in regulating cell growth where it promotes the G1 to S phase progression of cells within the quiescent center (QC) of onion roots [16–20]. Asc is present in low amounts in the QC of maize roots and increases considerably during logarithmic growth of tobacco cell culture [21,22]. Asc also reversed the inhibition of cell division caused by hyacin treatment which reduces Asc content [19]. Once used, Asc is oxidized to the monodehydroascorbate radical (MDHA) that can be reduced to Asc in the chloroplast...
Ascorbic Acid Controls Twinning

Results

DHAR expression regulates polyembryony

In previous work, we reported the generation of tobacco (i.e., Nicotiana tabacum) in which DHAR was overexpressed or suppressed [25–28]. To overexpress DHAR, tobacco was transformed with a wheat (i.e., Triticum aestivum) DHAR cDNA (DTa) under the control of the cauliflower mosaic virus (CaMV) 35S promoter [25]. As DTa has diverged sufficiently from the tobacco DHAR ortholog at the nucleotide level, this approach avoided RNA silencing of DHAR expression. To suppress DHAR, tobacco was transformed with a tobacco DHAR cDNA (DNt) under the control of the 35S promoter, resulting in the generation of lines in which DHAR expression was suppressed [26]. An increase in DHAR expression results in a larger Asc pool size [27,28]. Measurements of Asc revealed that its level in DHARSt ovules was lower (i.e., 0.861 μg/g FW) than in WT ovules (i.e., 1.25 μg/g FW) whereas the level of Asc in DHARTa ovules was higher (i.e., 1.48 μg/g FW). Because of this, it was possible to separate maternal effects of Asc from Asc produced in the zygote specifically. The frequency of polyembryony was lower in a cross between a WT-male (WT-m) and a DTa-female (DTa-f) than between a WT-male and a DNt-female (DNt-f) (0.28%) than between a WT-male and a DTa-female (1.70%), demonstrating that increasing DHAR expression throughout the plant increases polyembryony. As Asc can be transported to sink tissues including floral organs [29], these results indicate that the frequency of polyembryony is likely determined by contributions made by maternal tissues in addition to embryo-derived Asc. The frequency of polyembryony in the cross between a WT-male and a DTa-female was also lower than it was in a cross between a DNt male (DNt-male) and a WT-female (0.68%), the reverse of the parental influence observed in reciprocal crosses between WT and DTa plants (Fig. 2). Therefore, the frequency of polyembryony was highest in crosses using a DTa female, followed by crosses using a WT female (when the DHAR transgene was provided by the male), which in turn was higher than in crosses using a DNt female. Because the Asc pool size is higher in DTa plants than it is in WT plants which in turn is higher than it is in DNt plants [Chen and Gallie, 2006], these data support the
conclusion that the maternal contribution of Asc influences the frequency of polyembryony. The frequency of polyembryony in the cross between a WT-m and a DNt female (DNt-f), however, was higher (0.28%) than in WT plants (0.034%) despite the fact that the level of Asc is higher in WT plants than in DNt plants. As it is the level of Asc in the fertilized egg specifically that should determine the level of twinning, this observation suggests that the DNt transgene in the embryo is sufficient to modestly increase polyembryony even when the level of Asc in surrounding maternal tissues is low.

Figure 1. Ascorbate-induced twinning and polycotyly. A–F, Twin progeny emerging from DHAR-overexpressing tobacco seed. G–H, Triplet progeny emerging from DHAR-overexpressing tobacco seed. I–J, Twin progeny emerging from WT seed that developed from ovaries injected with 10 mM Asc on the day of pollination. K–N, Tricocyledon progeny from DHAR-overexpressing tobacco seed in various states of fusion. O–P, Tetracocyledon progeny from DHAR-overexpressing tobacco seed. Q–T, Monococyledon progeny from DHAR-overexpressing tobacco seed where (T) the fused cotyledon was cleared of chlorophyll to reveal two main veins and plane of fusion. U, Wild-type seedling cleared of chlorophyll.
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Ascorbic Acid Controls Twinning

Figure 2. Frequency of ascorbate-induced twinning and polycotyly. Frequency of twinning and polycotyly resulting from crosses between WT and DHAR-overexpressing tobacco. Crosses between wild-type (WT) and tobacco transformed with wheat DHAR (DTa) or tobacco DHAR (DNt) were performed. The histograms represent the frequency of twinning (black bars, left scale) and cotyledon abnormalities (i.e., polycotyly or single fused cotyledon)(white bars, right scale) measured in the resulting seed.
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In reciprocal crosses between DTa and DNt plants, progeny from a cross between a DNt-m and a DNt-f exhibited a higher level of polyembryony (3.36%) than progeny resulting from a cross between a DTa-m and a DNt-f (0.43%) (Fig. 2), consistent with the previous conclusion that increasing the maternal Asc pool size substantially increases polyembryony. Interestingly, transmission of the DNt transgene through male gametes in the cross between a DNt-m and a DTa-f increased polyembryony approximately 2-fold more (3.36%) relative to a cross between a WT-m and a DTa-f (1.70%), suggesting that the DNt and DTa transgenes each contribute to polyembryony and that the DNt transgene does so following its transmission by the male gametes. Compared to the level of polyembryony observed in the cross between a DNt-m and a DTa-f, the level of polyembryony was reduced almost 2-fold in a cross between a hemizygous DNt-m (WT/DNt-m) and a DTa-f (1.9%) (Fig. 2), supporting the notion that transmission of the DNt transgene through male gametes contributes to polyembryony and reducing its presence in half of the male gametes results in a corresponding reduction in polyembryony. An increase in polyembryony was also observed in a cross between a DTa-m and a DNt-f (0.43%) relative to a cross between a WT-m and a DNt-f (0.28%), supporting the conclusion that both transgenes contribute to polyembryony regardless of their transmission through male or female gametes. A reduction in polyembryony was observed in a cross between a DTa-m and a hemizygous DNt-f (WT/DNt-f) (0.25%) relative to the cross between a DTa-m and a homozygous DNt-f (0.43%), additional evidence indicating that reducing the presence of the DNt transgene in half of the ovules results in a corresponding reduction in polyembryony.

Although the level of polycotyly also increased, this was largely limited to those crosses in which the DTa transgene was expressed in the female (Fig. 2). For example, relative to the level of polycotyly in wild-type plants (0.41%), a nearly 6-fold increase in polycotyly was observed in progeny from a cross between a WT-m and a DTa-f (2.4%) but only a slight increase was observed in progeny from the reciprocal cross (0.5%) (Fig. 2). No increase in polycotyly was observed in progeny from a cross between a WT-m and a DNt-f (0.41%) (where the female has a reduced Asc pool size in vegetative tissues) and only a slight increase in the reciprocal cross (0.68%) (Fig. 2). A substantial increase in polycotyly was observed in progeny from a cross between a DTa-m and a DTa-f (4.73%) but no increase in progeny from the reciprocal cross (0.32%) (Fig. 2). These results suggest that it is not so much the presence of the DHAR transgene in the developing embryo but rather the maternal Asc pool size that most influences the frequency of polycotyly. However, transmission of a DHAR transgene through male gametes can contribute moderately to polycotyly especially in females expressing the DTa transgene.

Activity from the CaMV 35S promoter in developing embryos is not detected until the early heart stage [30–32]. Significantly higher expression of DHAR was observed in the reproductive organs of DTa and DNt plants, including in pollen, ovules, and style (Fig. 3). If twinning results from an increase in Asc, the observed twinning in DTa plants can be understood by the increase of DHAR expression in maternal tissues and transport of Asc to developing embryos [29]. Maternally-generated Asc can not explain, however, the twinning observed following fertilization of WT ovules by DNt pollen (Fig. 2). Although some studies have reported that the CaMV 35S promoter is silent in pollen, others have observed substantial activity, e.g., in strawberry, cotton, and pine [32–34]. Differences in the regulatory elements included in the CaMV 35S promoter used as well as the species examined may account for differences in the level of activity observed [35–37]. In tobacco, expression in pollen from the CaMV 35S promoter has been reported, albeit at a lower level than other promoters, e.g., from the nos or lat52 genes [36,38]. The higher level of DHAR activity in pollen of DTa and DNt plants indicates that the CaMV 35S promoter used is active during pollen development. To follow the fate of expression from the CaMV 35S promoter in pollen during its subsequent growth through the style and in ovules following fertilization, luciferase expression from tobacco plants containing a 35S::Luc transgene was used as a proxy for DHAR expression. Significant luciferase expression was detected in mature pollen of DTa plants whereas wild-type pollen, style, or ovules had no detectable luciferase expression (Table 1). Two days following pollination, luciferase expression was also detected in the style (excluding the stigma where the pollen grains were present) and in ovules as well (Table 1). Similar results were obtained when pollen from 35S::Luc tobacco was used in crosses with DTa or DNt plants (Table 1). These results indicate that the CaMV 35S promoter used is active during pollen development in tobacco. These results also indicate that, like luciferase, DHAR accom-
Inherited the pollen-transmitted DNt transgene (Fig. 4A). These DTa transgene as transmitted through the male gametes (Fig. 4A), twins until after fertilization by DNt pollen. In some cases, m and a hemizygous DTa/DNt-f or between a hemizygous DTa/DNt-m and a WT-f was determined. The uniform inheritance of either the DNt transgene or the DTa transgene from a hemizygous DTa/DNt-m parent in the polyembryonic progeny of a seed would rule out dizygotic twinning. Progeny from a cross between a WT-m and a hemizygous DTa/DNt-f were identical in their transgene inheritance as were progeny from a cross between a hemizygous DTa/DNt-m and a WT-f (Fig. 4B), i.e., the inheritance or lack of inheritance of either the DTa, or DNt transgene was uniform for all progeny within a given polyembryonic seed. Progeny were also identical in their inheritance of the DNt transgene from male gametes hemizygous for this transgene in a cross with a DTa female (Fig. 4B). These results ruled out the possibility of fusion of independently fertilized ovules or dizygotic twinning, supporting the notion that the progeny arose from a single zygote.

Monozygotic twinning, i.e., twinning that occurs during the first zygotic division, delays embryo development by the time required for the first zygotic division. However, as the twin embryos would be delayed equally, they would exhibit a similar rate of development. To examine whether this may account for the DHAR-induced polyembryony, the development of twin embryos was compared to that of WT embryos. Two or more zygotes were observed in developing seed from DHAR-overexpressing plants (Fig. 5F–H) compared to only one zygote in WT seed (Fig. 5A–D). At 3 days after pollination (DAP), two proembryos were observed in DHAR-overexpressing seed (Fig. 5j). Each proembryo was composed of an apical to two-celled proembryo and a basal cell, indicating that twinning occurred prior to the transverse division of the zygote. Both proembryos were at a similar developmental stage, arguing against the possibility that the twin arose from the embryo-proper or suspensor of the primary embryo. Both embryos were developmentally delayed relative to WT embryos which were at quadrant to octant (Fig. 5i). Similarly, when WT embryos were at mid-globular (Fig. 5k) or transition (Fig. 5m) stages, twin DHAR-overexpressing embryos, each with a distinct suspensor, were at early-globular (Fig. 5l) or mid-globular (Fig. 5n) stages, respectively, supporting the conclusion that embryo development was delayed as a consequence of the twinning event during the first zygotic division. Analysis at subsequent stages revealed the continued development of double and triple progeny within a seed (Fig. 5p–q, s–t) relative to a single embryo in WT seed (Fig. 5r).

Seedlings from polyembryonic seeds were of similar size (Fig. 1a–h), supporting the notion that their development had initiated simultaneously.

### Table 1. Transmission of pollen cytoplasm to fertilized eggs.

| Female       | Male     | Organ assayed | Days after pollination (DAP) | Luciferase activity (Light units/mg protein/min) |
|--------------|----------|---------------|-------------------------------|-------------------------------------------------|
| Wild-type    | pollen   | 0             | 0                             |                                                  |
|              | style    | 0             | 0                             |                                                  |
|              | ovules   | 0             | 0                             |                                                  |
| 35S::Luc     | pollen   | 0             | $1.15 \times 10^6 \pm 1.21 \times 10^5$ |                                                  |
| Wild-type    | 35S::Luc | style         | 2                             | $9.53 \times 10^6 \pm 6.44 \times 10^5$          |
|              | 35S::Luc | ovules        | 2                             | $2.21 \times 10^7 \pm 3.96 \times 10^6$          |
| DTa          | 35S::Luc | style         | 2                             | $3.60 \times 10^5 \pm 2.10 \times 10^5$          |
|              | 35S::Luc | ovules        | 2                             | $3.30 \times 10^5 \pm 1.92 \times 10^5$          |
| DNt          | 35S::Luc | style         | 2                             | $4.82 \times 10^5 \pm 1.85 \times 10^5$          |
|              | 35S::Luc | ovules        | 2                             | $2.35 \times 10^7 \pm 3.66 \times 10^6$          |

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Determination of the embryo cell number (estimated from the number of cells in median, longitudinal sections) demonstrated that WT embryos contained an average of 75 cells (representing 6.2 cell divisions) at 6.5 DAP (Table 2) whereas twin DHAR-overexpressing embryos contained an average of 26.5 cells (4.7 cell divisions), a delay of 1.5 cell divisions. At 8 DAP, WT embryos contained 212 cells (7.7 cell divisions) whereas twin embryos contained 73 cells (6.2 cell divisions). These data suggest that twin embryos had undergone 1.5 fewer cell divisions than WT embryos, consistent with the delayed development associated with monozygotic twinning.

Ascorbic acid phenocopies DHAR-induced twinning

Overexpression of DHAR results in an increase in the Asc pool size [28]. To test directly whether an increase in Asc was responsible for the DHAR-induced twinning, Asc, at concentrations similar to those present in plants [42], was injected into WT ovaries on the day of pollination. The frequency of twinning in progeny of WT plants increased substantially following the injection of Asc (Figs. 1I–J and 6) or its immediate precursor, L-galactono-1,4-lactone (GL) (Fig. 6) which is known to increase the Asc pool size [43,44]. Injection of Asc or GL also further increased the frequency of twinning following injection into ovaries of DTa plants (Fig. 6). Injection of dehydroascorbate (DHA), which increases the Asc pool size [44] following its reduction to Asc by DHAR in a glutathione-requiring reaction, induced twinning as did injection with glutathione (Fig. 6). Injection of 5 mM Asc resulted in more polyembryony than did higher levels of Asc, perhaps due to a threshold effect beyond which further increases in Asc have no additional effect. It is also possible that injecting high concentrations ofAsc limits its ability to promote polyembryony through its effect on the Asc redox state of the apoplast. Asc is normally present in the apoplast at only very low concentrations as DHA is the dominant form [27]. Injection of Asc at higher concentrations than 50 mM caused death of the ovaries indicating that it has toxic effects not seen with GL or DHA at similar concentrations. Injection of Asc on the day of pollination induced only a slight increase in cotyledon defects (Fig. 6), demonstrating that the effect of Asc on twinning can be temporally separated from its effect on polyembryony. Injection of Asc later in embryo development, however, did increase polyembryony substantially (see below). No increase in the frequency of twinning

![Diagram](image-url)
Ascorbic acid-induced twinning is developmentally limited to the first two days after pollination

A study of the timing of fertilization in tobacco reported no evidence of fertilization by 48 hours after pollination (HAP) and only 50% egg cells showing various stages of fusion of the egg and sperm nuclei at 54 HAP [50]. Zygotic division did not occur prior to 84 HAP [50]. If Asc induces twinning, its effect should be limited to the period prior to the first zygotic division following fertilization. Twinning was induced substantially when GL or Asc was injected within 0–2 DAP but lower rates were observed when either was injected after 2 DAP (Fig. 8A), consistent with its role in regulating the first zygotic division but not subsequent cell divisions until cotyledon development. GL or Asc injected on the day of pollination did lead to a small increase in cotyledon defects (Fig. 8B) as was also observed in Fig. 6 but the highest frequency of polycotyly was observed when GL or Asc was injected at 7 DAP (Fig. 8B), the developmental stage representing the transition from globular to heart stage during which cotyledon formation initiates (Fig. 5K and M, respectively). These data demonstrate that early embryo development is sensitive to Asc-induced twinning and polycotyly, consistent with the notion that Asc alters zygotic division and apical patterning during cotyledon initiation, respectively. In contrast to the sos or rcp3 mutants of Arabidopsis in which the suspensor undergoes inappropriate cell divisions and takes on some characteristics of embryo-proper cells [51,52], suspensor development was unaffected by Asc [Fig. 3J, L, N], indicating that the signaling between the embryo-proper and suspensor cells was maintained.

### Cytoskeleton altering agents phenocopy ascorbic acid-induced polyembryony

The changes in cell polarity, and thus cell division, caused by Asc during embryo development suggested possible alterations to...
the elements of the cytoskeleton, i.e., microtubules or microfilaments, which determine the orientation or perhaps rate of cell division. Pharmacologically perturbing the cytoskeleton during zygotic cell division might also be expected result in polyembryony. To investigate this, WT ovaries were injected at 48 hours after pollination (HAP) with taxol, which stabilizes microtubules and thus perturbs the bundling of microtubules involved in formation of the spindle poles and the preprophase band (PPB) that marks the division site [53]. Progeny from taxol-treated ovaries exhibited a 15-fold increase in polyembryony (0.74%; 13/1747) relative to that observed in untreated ovaries (0.05%; 1/1846). Taxol injected at 54 HAP, i.e., following fertilization but prior to the first zygotic division [50], resulted in an 11-fold increase in polyembryony (0.56%; 7/1255). Cytochalasin B, which inhibits actin polymerization resulting in misorientation of new cell walls [54], increased polyembryony nearly 5-fold over the basal frequency (0.24%; 3/1248) when injected at 48 HAP or 3-fold (0.14%; 2/1461) when injected at 54 HAP.

Consistent with notion that Asc may affect aspects of cytokinesis was the central position of the nucleus (with transverse nucleoli) surrounded by vacuoles in a non-elongated, DHAR-overexpressing zygote (Fig. 5E). An increase from one nucleolus in the egg cell to two vertically positioned nucleoli in the fertilized zygote has been described and has been suggested to represent karyogamy [55]. Such a position of the nucleus is in contrast to WT zygotes in which the nucleus (with vertically positioned nucleoli) is typically present in the apical region of an elongated cell above a vacuolated basal region (Fig. 5A–D). Subsequent divisions of horizontally-positioned, twin zygotes show a wild-type asymmetric position of the nucleus (with vertically positioned nucleoli) above a vacuolated basal region in elongated cells (Fig. 5F–G).

Figure 6. Twinning is induced by Asc or compounds involved in Asc biosynthesis or recycling. Frequency of twinning and polycotyly in progeny of WT ovaries injected with (A) Asc, GL, dehydroascorbate (DHA), glutathione (GSH), H2O2, or malate at the concentrations indicated on the day of pollination. The histograms represent the frequency of twinning (black bars, left scale) and cotyledon abnormalities (i.e., polycotyly or single fused cotyledon)(white bars, right scale) observed following germination.
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Figure 7. Hormonal control of twinning. Frequency of twinning and polycotyly in progeny of WT ovaries injected with gibberellic acid (GA3), indole acetic acid (IAA), aminoethoxyvinylglycine (AVG), 2-chloroethylphosphonic acid (CEPA) that produces ethylene, or the cytokinin, benzyl adenine (BA) at the concentrations indicated on the day of pollination. The histograms represent the frequency of twinning (black bars, left scale) and cotyledon abnormalities (i.e., polycotyly or single fused cotyledon)(white bars, right scale) observed following germination.
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its pool size had on polyembryony strongly implicate ascorbic acid as responsible for the twinning.

Changes to the auxin gradient within the female gametophyte can alter the number of egg cells per embryo sac and consequently, the number of fertilization events by independent pollen tubes entering the ovule [41]. However, as the auxin gradient functions by regulating cell fate specification within the embryo sac at cellularization [41], the polyembryony resulting from transmission of a DHAR transgene by male gametes or following delivery of ascorbic acid to an ovary the same day as fertilization is inconsistent with an ascorbic acid-mediated change in the auxin gradient generating multiple egg cells as is the observation that twin progeny in DHAR-induced polyembryonic seed were genetically identical.

Although increasing the expression of DHAR in the male or female parent revealed that polyembryony was induced by paternal or maternal inheritance of a DHAR transgene, increasing DHAR expression throughout the female parent resulted in a greater induction of polyembryony than did inheritance of the same transgene through male gametes. Suppression of DHAR expression in tissues other than the style and ovules also resulted in a substantially lower level of polyembryony than when its expression was elevated throughout the plant. These observations suggest that the elevated expression of DHAR in maternal tissues likely contributes to the increase in the Asc pool size in the ovule. Nevertheless, the observation that inheritance of a DHAR transgene through male gametes can increase polyembryony demonstrates that confining expression of the transgene to embryonic tissues is sufficient to influence the rate of twinning.

Embryogenesis begins with an asymmetric transverse division of the zygote, resulting in an elongated basal cell that initiates acquisition of suspensor identity and a smaller nonvacuolated apical cell that specifies into the embryo proper. The asymmetric division resulting in daughter cells of different sizes or cytoplasmic determinants may be required for the subsequent specification of the apical and basal cells [56,57]. Cell specification may also require positional information, e.g., contact of the basal cell with the maternal tissue [7,58,59]. Asc-induced twinning suggests that the plane of cytokinesis during early embryo development is altered resulting in symmetric division, a loss of positional information following the first zygotic division, and a lack of cell specification of the daughter cells that then develop into genetically identical twins. This possibility is supported by the ability of cytoskeleton-perturbing agents to phenocopy Asc-induced polyembryony. Treatment with taxol is known to perturb formation of the preprophase band that marks the division site and treatment with cytochalasin B results in misorientation of new cell walls [54], consistent with the notion that the control of the plane or rate of cell division during the first zygotic division may be important to avoid twinning. Of the phytohormones tested, only cytokinin was able to induce polyembryony, suggesting that this hormone may influence the positioning of the plane or rate of cell division during the first zygotic division, consistent with its role in regulating cell division [60,61]. Kinetic has been shown to cause the longitudinal orientation of cortical microtubules resulting in the deposition of cellulose microfibrils in the same orientation [62,63] and demonstrating a potential role for this hormone in the orientation of cell division during embryogenesis.

Asc has been implicated in promoting G1 to S phase progression as was shown in cells within the QC of roots where cells remain in G1 longer than cells surrounding the QC [16–20,64,65]. The observation that the development of twin embryos is temporally delayed relative to WT embryos suggests that Asc does not induce a premature initiation of the first zygotic division
prior to the correct positioning of the plane of cell division. Our observations do suggest, however, that one or more steps involved in cytokinesis or the rate of cell division are affected by Asc which are revealed during critical developmental stages including the first zygotic division and specification of cotyledon-forming fields. Although these two stages during embryo development may be particularly sensitive to Asc, it is equally plausible that they simply represent developmental stages in which perturbations in cell division are most easily observed. The maximum rate of twinning resulting from increased DHAR expression was just over 3% and most stages of embryo development may be able to tolerate this level of alterations to cell division without resulting in a readily apparent phenotype. The identification of the step(s) during cell division affected by Asc will be necessary in future work in order to understand fully the role that Asc plays in cell division.

Materials and Methods

Plant growth conditions

Full-length wheat DHAR (DwTa) cDNA (Accession number: AY074784) and tobacco DHAR (Dn) cDNA (Accession number: AY074787) under the control of the CaMV 35S promoter in the binary vector, pBI101, were used to generate DTa-overexpressing AY074784) and tobacco DHAR (DNt) cDNA (Accession number: K2HPO4/KH2PO4 pH 6.5, 0.5 mM DHA, and 1 mM GSH and centrifuging twice at 13,000 rpm for 5 min to remove cell debris. DHAR activity was followed by an increase in absorbance at 265 nm.

Statistics

Soluble protein was extracted from tobacco ground in liquid nitrogen before grinding in extraction buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 2 mM EDTA, 1 mM MgCl2) and protein concentration was determined as described [67]. DHAR activity was assayed essentially as described [66]. DHAR enzyme assay

DHAR activity was assayed as described [66]. Soluble protein was extracted from tobacco ground in liquid nitrogen before grinding in extraction buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 2 mM EDTA, 1 mM MgCl2) and centrifuging twice at 13,000 rpm for 5 min to remove cell debris. Protein concentration was determined as described [67]. DHAR activity was assayed from an equal amount of protein in 50 mM K2HPO4/KH2PO4 pH 6.5, 0.5 mM DHA, and 1 mM GSH and activity was followed by an increase in absorbance at 265 nm.

DNA isolation

Leaf tissue was ground in liquid nitrogen to fine powder, then, mixed with extraction buffer (containing 100 mM Tris-HCl pH 9.0, 200 mM NaCl, 1% sarcosyl, 20 mM EDTA, and 10 μl/ml β-mercaptoethanol). After extracted with equal volume of phenol/chloroform, RNA was removed from the supernatant by precipitating with 2 M LiCl. Equal volume of isopropanol was added to the RNA-depleted supernatant to pellet the DNA.

Polymerase chain reaction

Amplification of transgenes was performed in 25 μl reactions containing 1× PCR buffer, 1 u HotStarTag DNA polymerase (Qiagen Inc, Valencia CA, U.S.A.), 250 μM dNTP, 10 μM forward and reverse primers, and 50 ng genomic DNA. Reactions were carried out using the following conditions: 95°C/15 min (1 cycle); 95°C/30 sec, 56°C/30 sec for the tobacco DHAR gene (62°C/30 sec for the wheat DHAR gene), 72°C/1.5 min (35 cycles); and extension at 72°C for 1.5 min; and a final extension at 72°C/5 min (1 cycle). The upstream primer is 5’-ACTGACGTAAGGGATGACGCA-3’ (wheat DHAR gene), and 5’GGATCCAGGGCTTACGGGTTCACTTTC-3’ (tobacco DHAR gene) respectively.

Statistical analysis of PCR results

Paternal or maternal inheritance using PCR was performed on multiple twin/triplet embryos. All progeny within a seed exhibited similar paternal inheritance, ruling out apomixis. The possibility of multiple fertilization events of a polyembryro-containing ovule or the post-fertilization fusion of ova was considered highly unlikely as Mendelian inheritance of paternal genes would be expected. The probability that twin progeny resulting from independent fertilization events exhibit similar paternal inheritance is extremely unlikely as Mendelian inheritance of paternal genes would be expected. The probability that twin progeny resulting from independent fertilization events exhibit similar paternal inheritance (i.e., either both inherit or both fail to inherit) of a pollen-transmitted transgene from a hemizygous parent is one out of two, i.e., a 50% chance that the twin seedlings are genetically identical. For triplet embryos, there is a 25% chance that they are genetically identical if from independent fertilization events. The probability that independent fertilization was responsible for twin embryos in 4 seed and triplet embryos in 3 seed and resulted in uniform inheritance of a pollen-transmitted transgene from a hemizygous parent is (1/2)2×(1/4)3 = 1/1024. Therefore, there is a 99.9% probability that the uniform inheritance resulted from twinning from a single zygote and not from multiple fertilization event.

Luciferase assay

Tobacco tissue was frozen in liquid nitrogen and ground in luciferase assay buffer (25 mM Tricine pH 8, 5 mM MgCl2, 0.1 mM EDTA supplemented with 33.5 mM DTT, 270 μM coenzyme A, 500 μM ATP). Following centrifugation, the extract was assayed for luciferase activity following injection of 0.5 mM luciferin using a Monolight 2010 Luminometer (Analytical Luminescence Laboratory). Triplicate samples were assayed and the average value is reported.

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Author Contributions

Conceived and designed the experiments: DRG ZC. Performed the experiments: DRG ZC. Analyzed the data: DRG ZC. Contributed reagents/materials/analysis tools: DRG ZC. Wrote the paper: DRG ZC.
References

1. Coulter JM, Chamberlain CJ (1903) In Morphology of Angiosperms. D. Appleton and Company, New York, New York. pp. 212–226.
2. Tisserat B, Exan BR, Murashige T (1979) Somatic embryogenesis in angiosperms. Hotr Rev 1: 1876.
3. Carman JG (1997) Asynchronous expression of duplicate genes in angiosperms may cause apomixis, heterony, tetrapody, and polyploidy. Biol J Linn Soc 61: 51–94.
4. Reddell RA, Kolotunov AM (2004) Understanding apomixis: recent advances and remaining conundrums. Plant Cell 16: S268–S285.
5. Vernon DM, Meinke DW (1994) Embryogenic transformation of the suspensor in turn, a polyembryonic mutant of Arabidopsis. Dev Biol 165: 566–575.
6. Zheng HJ, Sommer-CZ (1997) Suspensor-derived polyploidy caused by altered expression of vault-mRNA syntheses in the turn2 mutant of Arabidopsis. Proc Natl Acad Sci U S A 94: 7349–7355.
7. Vernon DM, Hannon MJ, Le M, Forsthofel NR (2001) An expanded role for the T/H31 gene in embryogenic defects in cotyledon pattern and morphology in the turn2 mutant of Arabidopsis (Brassicaceae). Ann J Bot 88: 570–582.
8. Asada K (1994) Production and scavenging of active oxygen in photosynthesis. In Kyle DJ, Osmond CB, Arntzen CJ, editors. Photoinhibition, Elsevier, Amsterdam. pp. 227–287.
9. Asada K (1994) Mechanisms for scavenging reactive molecules generated in chloroplasts under light stress. In Baker NR, Bowyer JR, editors. Photoinhibition: The role of gene-directed functions and unpredictable chemical reactions. J Plant Physiol 157: 481–488.
10. Davies MB, Austin J, Partridge DA (1991) In Vitamin C: Its chemistry and function. Norwood, Ross & Wyman, Norwood, Massachusetts. pp. 212–226.
11. Smith JJ, Ververidis P, John P (1992) Characterization of the ethylene-forming enzyme gene in Allium cepa L. cv Gerbel) leaves. Plant Physiol 106: 187–196.
12. Horemans N, Asard H, Caubergs RJ (1997) The ascorbate carrier of higher plants. Plant J 10: 351–360.
13. Horemans N, Foyer CH, Asard H (2000) Transport and action of ascorbate at different subcellular membranes. Plant Cell 12: 231–243.
14. Paciolla C, De Tullio MC, Chiapetta A, Innocenti AM, Bitonti MB, et al. (2001) Short- and long-term effects of dehydroascorbate in Lupinus albus and Allium cepa roots. Plant Cell Physiol 42: 857–863.
15. Yoder NA, Paiva G, Luís T, Kolotunov AM, Apuña N, et al. (1994) Transport of ascorbic and dehydroascorbic acids across protoplast and vacuole membranes isolated from barley (Hordeum vulgare L. cv Gerbel) leaves. Plant Physiol 106: 187–193.
16. Horemans N, Azadi H, Caulaberg RJ (1997) The ascorbate carrier of higher plant plasma membranes preferentially translocates the fully oxidized (dehydro-)ascorbate molecule. Plant Physiol 114: 1247–1253.
17. Yoder NA, Foyen CH, Azadi H (2000) Transport and action of ascorbate at the plant plasma membrane. Trends Plant Sci 5: 263–267.
18. Faure JE, Rotman N, Fortune P, Dumas C (2002) Fertilization in Arabidopsis thaliana: developmental stages and time course. Philos Trans R Soc Lond B Biol Sci 355: 1455–1464.
19. Schwartz BW, Yeung EC, Meinke DW (1994) Disruption of morphogenesis and transformation of the suspensor in Arabidopsis thaliana. Plant J 5: 265–275.
20. Schwartz BW, Yeung EC, Meinke DW (1994) Disruption of morphogenesis and transformation of the suspensor in Arabidopsis thaliana. Plant J 5: 265–275.
21. Twell D, Klein TM, Fromm ME, Cormick S (1990) Tangential pattern induction of cotyledon leaf morphology in tobacco. Plant Physiol 91: 1270–1274.
22. Young TE, Giede-Lee J, Gallie DR (2004) Senescence-induced expression of cytokinin reverses pistil abortion during maize flower development. Plant J 38: 910–922.
23. Paguasus GC, Yu HJ, Sundaresan V (2007) Cell-fate switch of synergid to egg cell in Arabidopsis must embryo sacs arises from misexpression of the BEL1-like homodomain gene BLH1. Plant Cell 19: 3578–3592.
24. Paguasus GC, Alandate-Saez M, Bowman JL, Sundaresan V (2009) Auxin-dependent patterning and gamete specification in the Arabidopsis female gametophyte. Science 324: 1648–1650.
25. Smirnoff N (2000) Ascorbate biosynthesis and function in photo protection. Philos Trans R Soc Lond B Biol Sci 355: 1435–1446.
26. Wheeler GL, Jones MA, Smirnoff N (1998) The biosynthetic pathway of vitamin C in higher plants. Nature 395: 365–369.
27. Chen Z, Gallie DR (2005) Increasing tolerance to ozone by elevating foliar ascorbic acid confers greater protection against ozone than increasing vitamin C and remaining conundrums. Plant Cell 16: S228–245.
28. Chen Z, Gallie DR (2006) Dehydroascorbate reductase affects leaf growth, development, and function. Plant Physiol 142: 775–787.
29. Asada K (1999) The water-water cycle in chloroplasts: scavenging of active oxygen and dissipation of excess photons. Annu Rev Plant Physiol Plant Mol Biol 50: 601–630.
30. Liu C, Xu Z, Chua NH (1993) Auxin polar transport is essential for the induction of root primordia in tobacco pollen tubes following particle bombardment. Plant Cell Rep 19:224–228.
31. Horemans N, Foyer CH, Asard H (2000) Transport and action of ascorbate at the plant plasma membrane. Trends Plant Sci 5: 263–267.
32. Faure JE, Rotman N, Fortune P, Dumas C (2002) Fertilization in Arabidopsis thaliana: developmental stages and time course. Plant J 30: 481–488.
33. Kirk MM, Ransick A, McRae SE, Kirk DL (1993) The relationship between cell size and cell fate in Volvox carteri. J Cell Biol 123: 191–208.
34. Paciolla C, De Tullio MC, Chiapetta A, Innocenti AM, Bitonti MB, et al. (2001) Short- and long-term effects of dehydroascorbate in Lupinus albus and Allium cepa roots. Plant Cell Physiol 42: 857–863.
35. Yoder NA, Paiva G, Luís T, Kolotunov AM, Apuña N, et al. (1994) Cell differentiation and morphogenesis are uncoupled in Arabidopsis thaliana embryos. Plant Cell 6: 1713–1729.
36. Weisbenburg G, Eikerman MM, Setterfield G, Seagull RW (1986) Effects of taxol on microtubule arrays in cultured higher plant cells. Cell Mot Cytosk 6: 469–478.
37. Wick SM (1991) Spatial aspects of cytokinesis in plant cells. Curr Opin Cell Biol 3: 253–260.
38. Faure JE, Rotman N, Fortune P, Dumas C (2002) Fertilization in Arabidopsis thaliana wild type: developmental stages and time course. Plant J 30: 481–488.
39. Kirk MM, Ransick A, McRae SE, Kirk DL (1993) The relationship between cell size and cell fate in Volvox carteri. J Cell Biol 123: 191–208.
40. Garrod JB (1999) The generation of diversity and pattern in the Arabidopsis female gametophyte. Science 284: 1648–1650.
41. Smirnoff N (2000) Ascorbate biosynthesis and function in photo protection. Philos Trans R Soc Lond B Biol Sci 355: 1435–1446.
42. Wheeler GL, Jones MA, Smirnoff N (1998) The biosynthetic pathway of vitamin C in higher plants. Nature 395: 365–369.
43. Yoder NA, Paiva G, Luís T, Kolotunov AM, Apuña N, et al. (1994) Cell differentiation and morphogenesis are uncoupled in Arabidopsis thaliana embryos. Plant Cell 6: 1713–1729.
44. Weisbenburg G, Eikerman MM, Setterfield G, Seagull RW (1986) Effects of taxol on microtubule arrays in cultured higher plant cells. Cell Mot Cytosk 6: 469–478.
45. Wick SM (1991) Spatial aspects of cytokinesis in plant cells. Curr Opin Cell Biol 3: 253–260.
62. Shibaoka H (1974) Involvement of wall microtubules in gibberellin promotion and kinetin inhibition of stem elongation. Plant Cell Physiol 15: 255–263.

63. Shibaoka H, Hogetsu T (1977) Effects of ethyl N-phenylcarbamate on wall microtubules and on gibberellin- and kinetin-controlled cell expansion. Bot Mag Tokyo 90: 317–321.

64. Liso R, Innocenti AM, Bitonti MB, Arrigoni O (1988) Ascorbic acid-induced progression of quiescent centre cells from G1 to S phase. New Phytol 110: 469–471.

65. Clowes FAL (1975) The quiescent centre. In Torrey JG, Clarkson DT, editors. The Development and Function of Roots. New York: Academic Press. pp. 3–19.

66. Hossain MA, Asada K (1984) Purification of dehydroascorbate reductase from spinach and its characterization as a thiol enzyme. Plant Cell Physiol 25: 185–92.

67. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.