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Transient efflux inhibition improves plant regeneration by natural auxins

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SUMMARY

Plant genome editing and propagation are important tools in crop breeding and production. Both rely heavily on the development of efficient *in vitro* plant regeneration systems. Two prominent regeneration systems that are widely employed in crop production are somatic embryogenesis (SE) and *de novo* shoot regeneration. In many of the protocols for SE or shoot regeneration, explants are treated with the synthetic auxin analog 2,4-dichlorophenoxyacetic acid (2,4-D), since natural auxins, such as indole-3-acetic acid (IAA) or 4-chloroindole-3-acetic acid (4-Cl-IAA), are less effective or even fail to induce regeneration. Based on previous reports that 2,4-D, compared to endogenous auxins, is not effectively exported from plant cells, we investigated whether efflux inhibition of endogenous auxins could convert these auxins into efficient inducers of SE in Arabidopsis immature zygotic embryos (IZEs). We show that natural auxins and synthetic analogs thereof become efficient inducers of SE when their efflux is transiently inhibited by co-application of the auxin transport inhibitor naphthylphthalamic acid (NPA). Moreover, IZEs of auxin efflux mutants *pin2* or *abcb1 abcb19* show enhanced SE efficiency when treated with IAA or efflux-inhibited IAA, confirming that auxin efflux reduces the efficiency of Arabidopsis SE. Importantly, in contrast to the 2,4-D system, where only 50–60% of the embryos converted to seedlings, all SEs induced by transport-inhibited natural auxins converted to seedlings. Efflux-inhibited IAA, like 2,4-D, also efficiently induced SE from carrot suspension cells, whereas IAA alone could not, and efflux-inhibited 4-Cl-IAA significantly improved *de novo* shoot regeneration in *Brassica napus*. Our data provides new insights into the action of 2,4-D as an efficient inducer of plant regeneration but also shows that replacing this synthetic auxin for efflux-inhibited natural auxin significantly improves different types of plant regeneration, leading to a more synchronized and homogenous development of the regenerated plants.

Keywords: 2,4-dichlorophenoxyacetic acid, Arabidopsis, auxin transport inhibition, Brassica, carrot, indole-3-acetic acid (IAA), shoot regeneration, somatic embryogenesis.

INTRODUCTION

The plant hormone auxin, or indole-3-acetic acid (IAA), plays a key role in plant development and growth by regulating basic cellular functions like cell division, elongation, and differentiation. The effect of auxin on cellular processes is concentration dependent, which is determined by the sum total of auxin biosynthesis, metabolism, and transport (Paque & Weijers, 2016; Vanneste & Friml, 2009). Following its biosynthesis in specific cells or tissues, auxin is transported to sink tissues either through phloem transport or via polar cell-to-cell transport. This polar auxin transport (PAT) involves different classes of auxin transporters, including PIN-FORMED (PIN) auxin efflux carriers (Adamowski & Friml, 2015), ATP-binding cassette-B (ABCB)/P-glycoprotein (PGP) transporters (Geisler et al., 2017), and AUXIN1/LIKE-AUX1 (AUX/LAX) auxin influx carriers (Péret et al., 2012). Of these transporters, the PIN proteins determine the direction of transport, through their asymmetric (polar) localization at the plasma membrane (Adamowski & Friml, 2015; Rakusová et al., 2016;

Robert et al., 2013). In contrast, ABCB/PGP transporters are mostly localized symmetrically at the plasma membrane and facilitate either influx or efflux of auxin, thereby regulating the amount of auxin in the cell available for PIN-mediated PAT (Geisler et al., 2017). At the same time, AUX/LAX proteins are localized at the plasma membrane either in a non-polar or polar fashion and enhance intracellular auxin concentrations by promoting auxin uptake (Péret et al., 2012; Swarup & Bhosale, 2019).

Apart from its importance in normal plant development, auxin also holds the key to the regenerative capacity of plant cells and tissues. The classical tissue culture experiments by Skoog and Miller showed that it is possible to obtain regeneration of either roots, callus, or shoots from plant tissues depending on the ratio of root-inducing auxin and the shoot-inducing hormone cytokinin in the medium (Skoog & Miller, 1957). Together with the identification of synthetic auxins that are more stable in tissue culture, like 1-naphthaleneacetic acid (1-NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) (Dunlap et al., 1986), this early finding supported the development of plant regeneration protocols for a wide range of plant species. Currently, plant regeneration holds significant importance in biotechnological applications like clonal propagation of elite cultivars and CRISPR-Cas genome editing.

A common method for clonal plant propagation is somatic embryogenesis (SE), where embryos are induced on somatic plant tissues by the exogenous application of plant hormones. The vast majority of SE protocols rely on treatment of tissues with the synthetic auxin 2,4-D for efficient regeneration, whereas treatment with endogenous IAA or the synthetic 1-NAA does not or only very inefficiently lead to SE induction (Gaj, 2004). Why 2,4-D is more effective than IAA or other auxin analogs remains to be addressed. While 2,4-D mimics the activity of IAA at the molecular level (Pufky et al., 2003; Tan et al., 2007), it differs in terms of its cellular stability and is a less efficient substrate for auxin efflux, resulting in a notable accumulation of 2,4-D within plant cells compared to IAA (Delbarre et al., 1996; Seifertová et al., 2014).

Previously we have shown that efficient SE induction on immature zygotic embryos of *Arabidopsis thaliana* (Arabidopsis) by 2,4-D initially relies on the activation of the canonical auxin signaling pathway by 2,4-D, but later requires the induction of endogenous auxin biosynthesis to maintain embryogenic cell identity (Karami et al., 2023a,b). Here we used the same experimental system to investigate the reason for the higher effectiveness of 2,4-D as an SE inducer compared to natural auxins or other synthetic analogs. We hypothesized that the ability of 2,4-D to accumulate to higher levels in plant cells forms the basis for its enhanced regeneration properties. To test this, we reduced the efflux of natural auxins, including IAA, 4-Cl-IAA and indole-3-butyric acid (IBA), and some synthetic auxins, by applying them together with the the auxin efflux inhibitor naphthylphthalamic acid (NPA). Recently, NPA was shown to specifically act on PIN-mediated auxin efflux by its high affinity binding to the auxin binding site in PIN proteins, but other reports indicate that it also acts on ABCB-mediated auxin efflux by interfering with the interaction of ABCBs with its chaperone TWISTED DWARF1 (Abas et al., 2020; Geisler et al., 2017; Ung et al., 2022; Yang et al., 2022).

In line with our hypothesis, both NPA and mutants for auxin efflux proteins significantly enhanced the SE regenerative capability of natural auxins and synthetic analogs thereof, not only in Arabidopsis, but also in carrot cell suspensions, and also enhanced shoot regeneration from *Brassica napus* cotyledon or hypocotyl explants. More importantly, the regenerants generated by the auxin efflux-inhibited approach showed a faster and more homogeneous development in plants. Our findings provide a mechanistic explanation for why 2,4-D is an effective inducer of plant regeneration approaches, but also why this synthetic auxin interferes with proper plant development in later stages of the regeneration process. They serve as a foundation for the development of new effective plant regeneration protocols.

RESULTS AND DISCUSSION

Reduced auxin efflux promotes the strong auxin response required for efficient SE

We used the efficient SE system of 2,4-D-treated Arabidopsis immature zygotic embryos (IZEs) (Gaj, 2001; Karami et al., 2023a,b) to investigate why 2,4-D is a much more effective inducer of SE than natural auxins or other synthetic auxins. In this system, somatic embryos are derived from epidermal cells at the adaxial side of IZE cotyledons. Based on previous observations that 2,4-D is a much less efficient substrate for efflux transporters than IAA (Delbarre et al., 1996), we hypothesized that the relatively high accumulation of 2,4-D in plant cells is causal for effective SE induction. To this end, we determined the efficiency of SE induced by natural and synthetic auxins in Arabidopsis IZE explants in the absence or presence of the auxin transport inhibitor NPA. Only a low number of somatic embryos were obtained when IZEs were treated with IAA or NPA alone, while the addition of 10 μM NPA enabled 5 μM IAA to induce similar numbers of somatic embryos as those obtained with 5 μM 2,4-D alone (Figure 1A,B). Addition of 10 μM NPA in combination with 5 μM IAA was optimal for somatic embryo induction, as higher concentrations of NPA or IAA resulted in reduced SE frequencies (Figure S1A,B). Treatment with 5 μM 2,4-D was the optimal concentration for SE induction (Figure S1C). Treatment with both 10 μM NPA and 5 μM 2,4-D significantly decreased the SE efficiency (Figure 1B), but could be increased back to the efficiency in the absence of NPA by lowering the 2,4-D concentration to

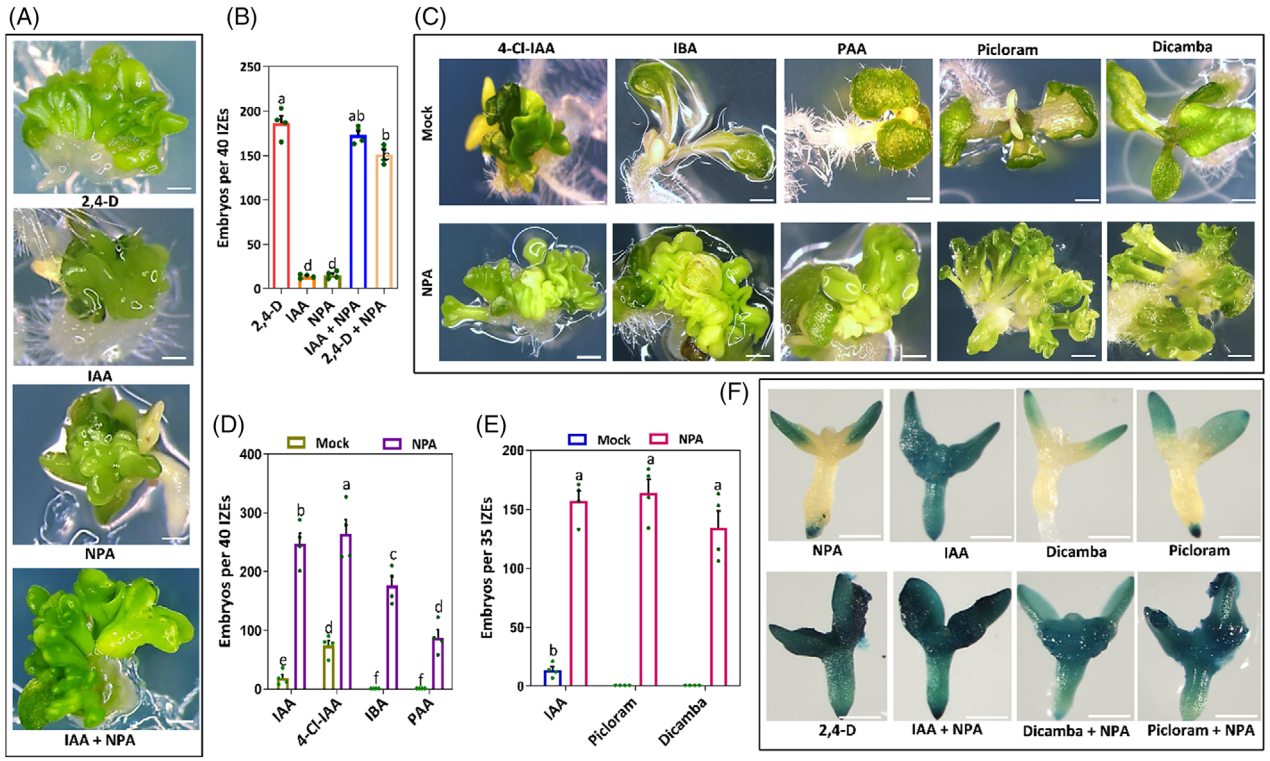

Figure 1. Reducing auxin efflux enhances the SE inducing capacity of natural and synthetic auxins. (A–E) The phenotype (A, C) and the number of somatic embryos produced from Arabidopsis immature zygotic embryos (IZEs) (B, D, and E). IZEs were initially grown for 2 weeks on medium supplemented with 5 μM 2,4-D, 5 μM IAA, 10 μM NPA, 5 μM IAA + 10 μM NPA or 5 μM 2,4-D + 10 μM NPA (A, B) or on medium supplemented with 5 μM 4-Cl-IAA, 5 μM IBA, 5 μM 2 PAA, 5 μM picloram, or 5 μM dicamba without or with the addition of 10 μM NPA (C–E). Subsequently, explants were transferred to hormone-free medium for 1 week before counting the somatic embryos. The dots in B, D, and E indicate the number of somatic embryos produced per 40 (B, D) or 35 (E) IZEs ($n = 4$ biological replicates); colored bars indicate the mean; error bars indicate the s.e.m.; different letters indicate statistically significant differences ($P < 0.001$) as determined by one-way analysis of variance with Tukey's honest significant difference post hoc test. (F) The expression pattern of the *pDR5:GUS* auxin response reporter in Arabidopsis IZEs after 7 days of culture on medium supplemented with 10 μM NPA or 5 μM 2,4-D or with 5 μM IAA, 5 μM dicamba or 5 μM picloram without or with the addition of 10 μM NPA. Scale bars in A, C and F indicate 0.5 mm.

1 μM 2,4-D (Figure S1C). Together, these results indicate that not only 2,4-D but also the natural auxin IAA can efficiently induce SE, provided that an intracellular accumulation of auxin reaches optimal levels. For IAA, its efflux needs to be inhibited to reach these optimal levels. For 2,4-D, inhibition of auxin efflux leads to too high auxin levels, resulting in reduced SE efficiencies, and therefore lower levels of the synthetic hormone are required to obtain optimal intracellular accumulation levels.

In addition to IAA and 2,4-D, we also tested other natural (4-chloroindole-3-acetic acid [4-Cl-IAA], indole-3-butyric acid [IBA], or phenylacetic acid [PAA]) or synthetic (picloram and dicamba) auxins for their capacity to induce SE in the absence or presence of 10 μM NPA. Whereas 5 μM IBA, PAA, picloram, or dicamba did not induce SE by themselves, these auxins did so in the presence of NPA (Figure 1D,E). Interestingly, 5 μM 4-Cl-IAA appeared to be more effective in inducing SE compared to IAA, and 4-Cl-IAA was also significantly more effective than IAA in the presence of NPA (Figure 1D).

These results are in line with our recent observation that SE induction by 2,4-D primarily requires a strong auxin response that is dependent on the canonical auxin signaling pathway (Karami et al., 2023a). Analysis of the auxin-responsive reporters *pDR5:GUS* and *pDR5:GFP* (Benková et al., 2003; Ulmasov et al., 1997) showed that incubation of IZEs for 7 days on 2,4-D medium induces a strong auxin response (Figure 1F; Figure S2), especially at the adaxial side of cotyledons on positions where embryonic cell clusters form (Karami et al., 2023b). In comparison, NPA, dicamba, and picloram only induced a weak auxin response, and although the response induced by IAA was significantly stronger, the strong response in the cotyledons was lacking (Figure 1F). Addition of NPA significantly increased *pDR5:GUS* expression in IZE explants that were cultured with IAA, picloram, or dicamba to a level similar to what was observed for 2,4-D (Figure 1F). These results confirm our earlier observation that the effectiveness of 2,4-D in inducing SE relies on its relatively poor efflux, which allows sufficient intracellular accumulation to induce the strong auxin response required for SE. For other auxins, natural or synthetic, the efflux machinery is too efficient, and inhibition by NPA is required to reach

the intracellular auxin concentrations optimal for SE induction.

We determined whether efflux-inhibition of natural auxins is also effective in other regeneration systems. Somatic embryos can be efficiently obtained from 2,4-D-induced, carrot hypocotyl-derived cell suspensions (Zimmerman, 1993) (Figure S3), while treatment with IAA fails to induce SE. By contrast, treating hypocotyl-derived cell suspensions with IAA + NPA, induced comparable somatic embryo formation as with 2,4-D (Figure S3). These results suggest that sufficient auxin accumulation in plant cells is a common requirement in auxin-dependent SE systems.

Pin and *abcb* loss-of-function mutations enhance SE

Auxin efflux at the plasma membrane is mediated by PIN auxin efflux carriers and ABCB transporters. To assess which PIN transporters contribute to auxin efflux during Arabidopsis IZE culture, we analyzed the expression of the plasma membrane-localized PIN reporters *PIN1:PIN1-GFP*, *PIN2:PIN2-VENUS*, *PIN3:PIN3-GFP*, *PIN4:PIN4-GFP*, and *PIN7:PIN7-GFP*. IZEs were cultured on medium with 5 μM IAA alone or with IAA and 10 μM NPA and then screened for expression after 2, 4, and 7 days of culture. The *PIN1:PIN1-GFP* reporter was expressed in epidermis cells of cotyledons of IAA or IAA + NPA treated IZEs after 7 days of culture. No expression was observed in mock-treated IZEs, confirming previous observations that PIN1 expression marks the appearance of embryonic cells at day 7 (Figure 2A,C). In fact, we observed a significant reduction in the efficiency of SE when *pin1* mutant IZEs were used (Karami et al., 2023b). Interestingly, the *PIN2:PIN2-VENUS* reporter showed expression in epidermis cells at the

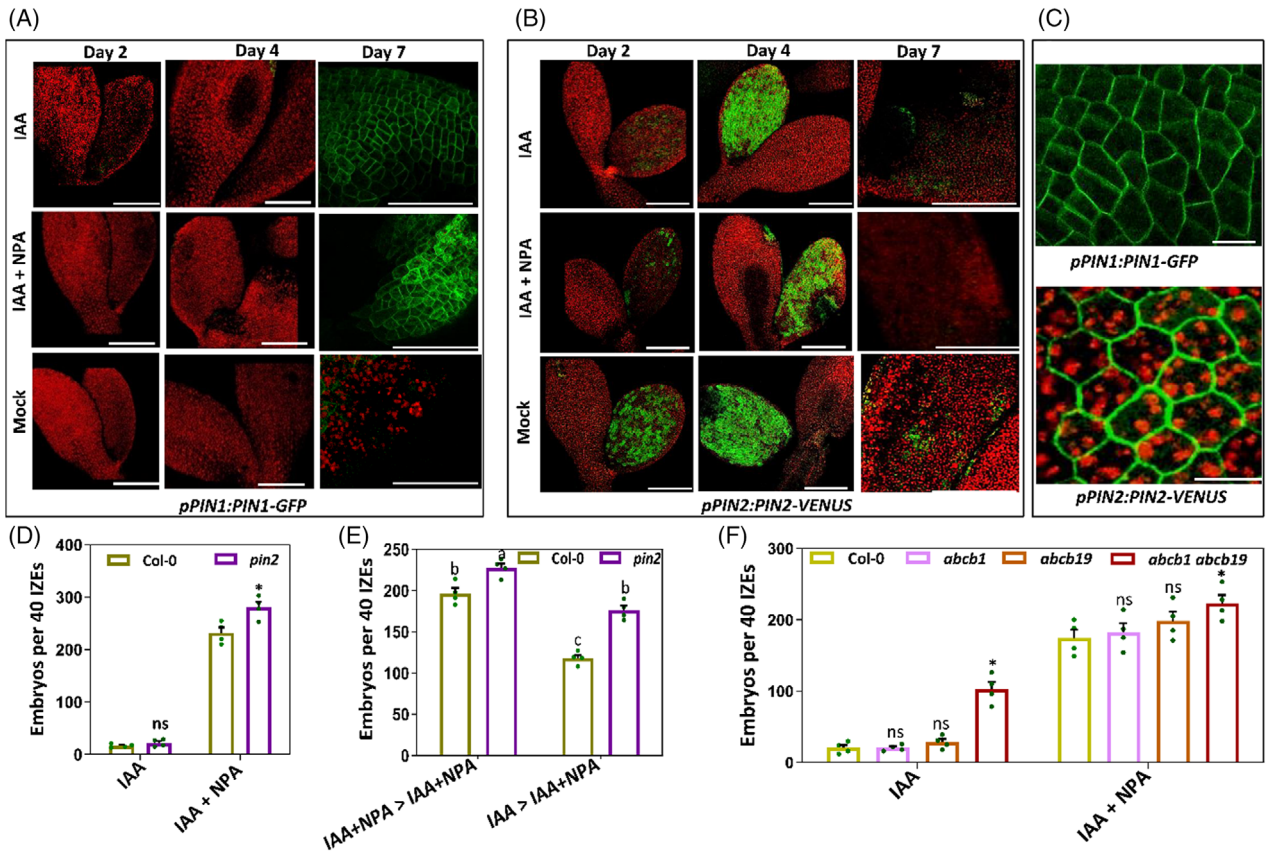

Figure 2. PIN and ABCB transporter-mediated auxin efflux reduces the capacity of IAA to induce SE. (A, B) The expression patterns of *pPIN1:PIN1-GFP* (A) and *pPIN2:PIN2-VENUS* (B) in epidermis cells at the adaxial side of cotyledons of Arabidopsis IZEs after 2, 4, or 7 days of culture on medium supplemented with 5 μM IAA, (upper panel), 5 μM IAA + 10 μM NPA (middle panel), or without any additional substances (mock, lower panel). N.b.: images in B show the adaxial side of one cotyledon and the abaxial side of the other one. (C) The non-polar localization of PIN1-GFP (upper panel) and PIN2-VENUS (lower panel) in IZE cotyledon cells after, respectively, 7 and 2 days of culture. (D, E) The number of somatic embryos produced from wild-type (Col-0) or *pin2* mutant IZEs either grown for 2 weeks on medium supplemented with 5 μM IAA or 5 μM IAA + 10 μM NPA (D), or grown for 4 days on medium supplemented with 5 μM IAA or with 5 μM IAA + 10 μM NPA and then transferred into medium supplemented with 5 μM IAA + 10 μM NPA for an additional 10 days (E). Explants were subsequently cultured for 1 week on hormone-free medium before counting the somatic embryos. (F) The number of SEs produced from wild-type (Col-0) or *abcb1*, *abcb19*, or *abcb1 abcb19* mutant IZEs that were grown for 2 weeks on medium supplemented with 5 μM IAA or 5 μM IAA + 10 μM NPA, and subsequently for 1 week on hormone-free medium. The dots in (D–F) indicate the number of somatic embryos produced per 40 IZEs (n = 4 biological replicates); bars indicate the mean; error bars indicate the s.e.m.; asterisks indicate a significant difference with wild-type ($P < 0.01$) as determined by a two-sided Student's t-test; scale bars in (A, B) indicate 0.5 mm; scale bar in (C) indicates 10 μm.

adaxial side of cotyledons of 2-day mock treated IZEs, cells from which somatic embryos are derived (Karami et al., 2023b), and this signal became stronger in both mock and IAA or IAA + NPA treated IZEs at day 4 (Figure 2B,C). At day 7, only a very weak signal could be observed (Figure 2B). As none of the other PIN reporters was expressed at this early time point, we hypothesized that PIN2 mediates auxin efflux during the early IZE culture. To test this, we incubated *pin2* mutant IZEs on medium with IAA or IAA + NPA and compared the SE efficiency with that of wild-type IZEs. Although the IAA-incubated wild-type and *pin2* IZEs did not show a significant difference in SE efficiency, the SE efficiency of *pin2* IZEs was significantly higher in the IAA + NPA treatment (Figure 2D). These results indicate that the contribution of PIN2 to auxin efflux during SE induction is relatively small. To investigate whether the increase in the number of embryos on *pin2* mutant IZEs was related to auxin efflux by PIN2 in cotyledon cells, we first cultured both *pin2* and wild-type IZEs for 4 days on medium containing IAA without NPA, after which they were transferred to medium containing IAA + NPA. The incubation of IZEs with only IAA for 4 days resulted in a significant decrease in the number of embryos in both *pin2* and wild-type IZEs (Figure 2E), however, *pin2* IZEs displayed a lower degree of sensitivity to this treatment compared to the wild type (Figure 2E). This finding suggests that the increase in the number of somatic embryos on *pin2* mutant IZEs is likely associated with the accumulation of IAA in the cotyledon cells during the early stages of IZE culture.

The *pin2* mutant data suggested that NPA-sensitive ABCB transporters might be more important in mediating IAA efflux during SE induction. In fact, when we compared *abcb1* and *abcb19* single and double mutant with wild-type IZEs on medium containing IAA or IAA + NPA, the *abcb1 abcb19* double mutant IZEs produced a significantly higher number of embryos, especially on medium containing IAA alone, compared to wild-type or single mutant IZEs (Figure 2F). This indicates that the ABCB transporters have a more prominent role, compared to the PIN2 auxin efflux carrier, in suppression of IAA accumulation in IZE cotyledon cells during SE induction.

Natural auxin-induced somatic embryos show improved embryo-to-seedling conversion

An important step in SE-mediated propagation is the conversion of somatic embryos to seedlings, as shown by proper shoot and root development. Although 2,4-D is used in many SE protocols because of its efficient somatic embryo induction, these protocols produce many embryos that do not develop into normal plants. In our Arabidopsis SE protocol, IZE explants are cultured for 2 weeks on hormone-containing medium to induce proembryonic masses (PEMS) and subsequently transferred to hormone-free medium for 1 week to allow proper patterning and development of somatic embryos (Karami et al., 2023a,b). When we left the explants for an additional 2 weeks on hormone-free medium, shoot development was observed, which lagged behind for 2,4-D treated explants compared to those treated with natural auxins + NPA (Figure S4). We suspected that this might reflect the efficiency of embryo to seedling conversion. To test this, individual embryos were isolated from the IZE explants after 1 week on hormone-free medium and placed separately again on hormone-free medium to allow embryo to seedling conversion (Figure 3A). In contrast to the 50–60% conversion rate observed in somatic embryos induced by 2,4-D (Figure 3B,D), nearly 100% of somatic embryos induced by the natural auxins + NPA developed into seedlings (Figure 3C,D). Furthermore, seedlings derived from natural auxin + NPA-induced somatic embryos showed homogenous shoot development (Figure 3B) and plants bolted between 30 and 40 days (Figure 3E). In contrast, seedlings derived from somatic embryos induced by 2,4-D showed delayed and variable shoot development and the bolting time of plants showed a larger variation (30–50 days). Based on these data, we conclude that the combined treatment of explants with efflux-inhibited natural auxins holds a great promise to significantly improve SE-based plant propagation methods, especially for protocols where it replaces 2,4-D treatment.

Efflux-inhibited natural auxin improves *de novo* shoot regeneration in *Brassica napus*

De novo shoot regeneration protocols commonly involve two steps. First callus, a mass of proliferating cells, is induced from somatic tissues on an auxin-rich callus-inducing medium (CIM), and subsequently shoot apical meristems are induced from this callus using a cytokinin-rich shoot-inducing medium (SIM). Here we investigated the *de novo* shoot regeneration capacity of callus induced on hypocotyls and cotyledons of the crop plant *Brassica napus* by 2,4-D, 2,4-D + NPA, 4-Cl-IAA, or 4-Cl-IAA + NPA. The analysis revealed that adding NPA to 2,4-D in CIM only had a two-fold negative or positive effect on the shoot regeneration capacity of the induced calli from hypocotyls or cotyledons, respectively (Figure 4A–C). For cotyledons, this result is in line with what was reported by Ohbayashi et al. (2022), as they observed a twofold enhancement in the shoot inducing efficiency on Arabidopsis root segments when NPA was added to CIM (Ohbayashi et al., 2022). The negative effect on shoot regeneration in Brassica hypocotyls suggests, however, that it is tissue dependent. Using 4-Cl-IAA in CIM instead of 2,4-D resulted in the same shoot regeneration efficiencies. However, when NPA was added to the 4-Cl-IAA-containing CIM, this led to a two- to threefold increase in the shoot regeneration efficiency from the induced calli (Figure 4A–C). Moreover,

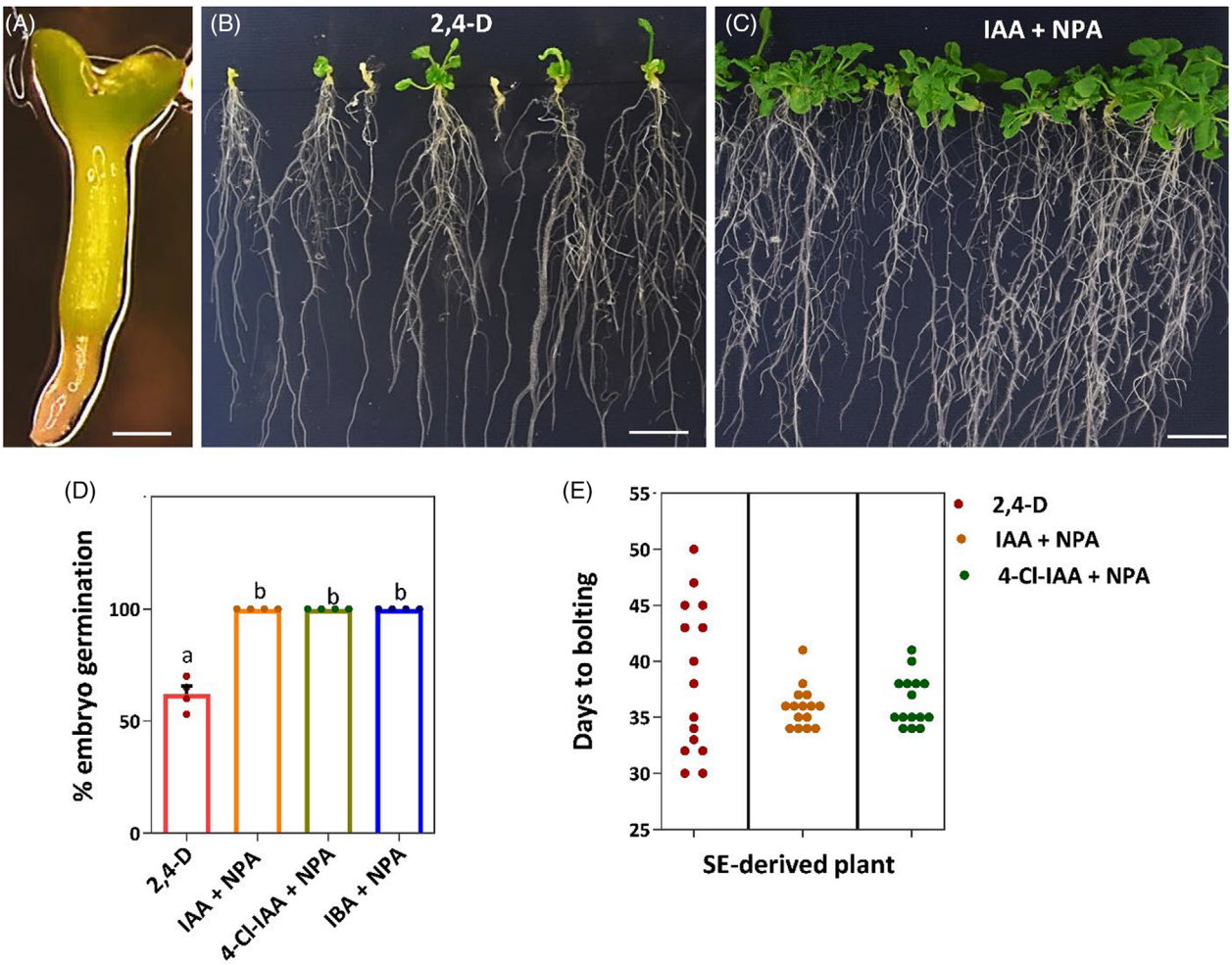

Figure 3. Somatic embryos induced by efflux-inhibited IAA show improved germination and subsequent plant development. (A) The phenotype of a single somatic embryos cultured on hormone-free medium for germination (embryo to seedling conversion = shoot and root formation). (B, C) Germination of somatic embryos that were induced by culturing IZEs either on 5 μM 2,4-D (B) or 5 μM IAA + 10 μM NPA (C) followed by 1 week culture on hormone-free medium. Embryos were subsequently separated from the explants and cultured for 3 weeks on hormone-free medium. (D) The embryo to seedling conversion (germination) rate of somatic embryos induced by 5 μM 2,4-D, 5 μM IAA + 10 μM NPA, 5 μM 4-Cl-IAA + 10 μM NPA, or 5 μM IBA + 10 μM NPA on hormone-free medium. The dots indicate the percentage of 20 somatic embryos showing complete embryo to seedling conversion ($n = 4$ biological replicates); colored bars indicate the mean; error bars the s.e.m.; different letters statistically significant differences ($P < 0.01$) as determined by one-way analysis of variance with Tukey's honest significant difference post hoc test. (E) The variation in flowering time among individual plants derived from somatic embryos induced by 5 μM 2,4-D (left), 5 μM IAA + 10 μM NPA (middle), or 5 μM 4-Cl-IAA + 10 μM NPA (right). Dots indicate the flowering time of individual plants; size bars indicate 0.5 mm in A and 1 cm in B and C.

shoot buds from calli induced by 4-Cl-IAA + NPA appeared early and shoots developed faster (Figure S5) and more synchronously (as shown by the reduced size variation) than those from 2,4-D-induced calli (Figure S6). Based on this data, we conclude that both SE protocols and *de novo* shoot regeneration protocols can be improved over standard 2,4-D-based protocols by implementing reduced efflux of natural auxins.

Conclusions

Many SE and *de novo* shoot regeneration protocols make use of the synthetic auxin 2,4-D to induce SE because of its effectiveness in inducing plant regeneration. Our previous results and the results presented here show that 2,4-D, as with natural auxins, acts through the canonical auxin response pathway (Karami et al., 2023a) and that the 'secret' of 2,4-D's efficiency lies in it being an inefficient substrate for the auxin efflux machinery (Delbarre eta al., 1996; this work). Due to these properties, 2,4-D is able to accumulate in the target cells to much higher levels than natural auxins and thus trigger a stronger (optimal) auxin response. The use of the auxin efflux inhibitor NPA in combination with natural auxins or synthetic auxins for which the auxin efflux machinery is not rate limiting, allows plant cells to accumulate these auxins to a similarly effective level as 2,4-D. The reason that this leads to improved

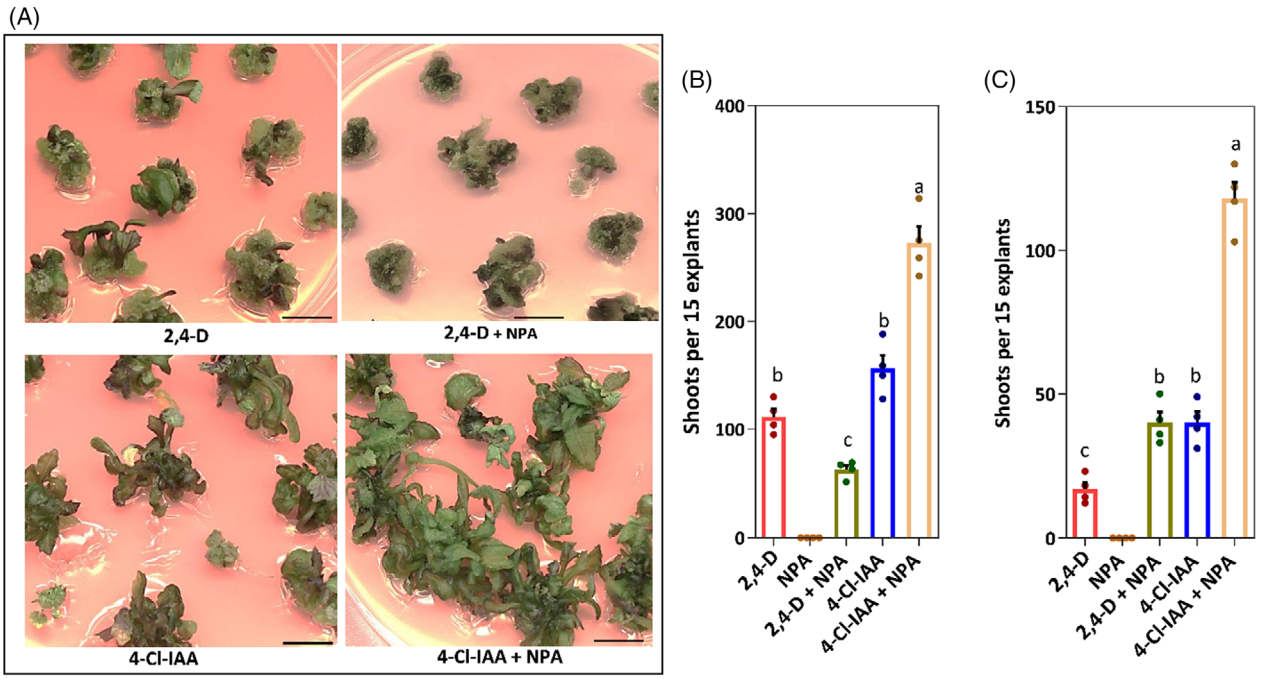

Figure 4. *Brassica napus* explants treated with efflux-inhibited natural auxin show enhanced *de novo* shoot regeneration. (A) The phenotype of shoots formed on callus induced from hypocotyls. (B, C) The number of shoots formed on callus induced on hypocotyls (B) or cotyledons (C). Explants were first cultivated for 2 weeks on media supplemented with 5 μM 2,4-D, 5 μM 4-Cl-IAA, 10 μM NPA, 5 μM 2,4-D + 10 μM NPA, or 5 μM 4-Cl-IAA + 10 μM NPA. Subsequently, they were transferred to a medium supplemented with 10 μM 6-benzylaminopurine for 3 weeks to induce shoot regeneration. The dots in B and C indicate the number of shoots produced per 15 explants ($n = 4$ biological replicates); bars indicate the mean; error bars indicate the s.e.m.; different letters indicate statistically significant differences ($P < 0.001$) as determined by one-way analysis of variance with Tukey's honest significant difference post hoc test; scale bars in (A) indicate 1 cm.

regeneration is that after SE or pluripotent callus is induced, the explants are transferred to medium without NPA, which releases the inhibitory effect on auxin efflux and allows the accumulated auxin to be efficiently exported from the PEMS or pluripotent cells, thereby promoting respectively homogenous patterning and development of somatic embryos or shoot apical meristem initiation and development. In protocols that make use of 2,4-D, the high auxin levels might be maintained for too long and this probably leads to inhibition of embryo tissue differentiation or the shoot developmental program, thus resulting in no, delayed or unsynchronized embryo-to-seedling conversion or shoot development. Based on our results, we suspect that many of the phenotypic variation and developmental defects observed during regeneration-based propagation of crop plants, which are generally classified under somaclonal variation (Larkin & Scowcroft, 1981), are the result of the inhibitory effect of prolonged high auxin accumulation. In fact, of all auxins used to induce somaclones for the purpose of plant genetic improvement, 2,4-D is the most commonly used (Ferreira et al., 2023). Irrespective of whether these defects are caused by a prolonged auxin response, epigenetic changes, DNA mutations, or chromosomal rearrangements, our results show clearly that they can at least be partially prevented by combining treatment with (natural) auxins that are efficiently metabolized and exported from plant cells with the transient inhibition of the auxin efflux machinery.

MATERIALS AND METHODS

Plant material and growth conditions

All *Arabidopsis thaliana* lines used in this study were in the Columbia (Col-0) background. The transgenic lines *pDR5:GUS* (Benjamins et al., 2001), *pDR5:GFP* (Benková et al., 2003), *pPIN1:PIN1-GFP* (Benková et al., 2003), *pPIN2:PIN2-VENUS* (Dhonukshe et al., 2010), *pPIN3:PIN3-GFP* (Ding et al., 2011), *pPIN4:PIN4-GFP* (Vieten et al., 2005), and *pPIN7:PIN7-GFP* (Blilou et al., 2005) have been described previously. The *abcb1* (AT2G36910), *abcb19* (AT3G28860), and *abcb1abcb19* mutant alleles were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Seeds were sterilized in 10% (w/v) NaOCl for 7 min and then washed four times in sterile water. Sterilized seeds were plated on half MS medium (Murashige & Skoog, 1962) containing 1% (w/v) sucrose and 0.7% agar. Seedlings, plants, and explants were grown at 21°C, 70% relative humidity, and 16 h photoperiod. Hormones and NPA were dissolved in DMSO at a concentration so that the appropriate concentration in the medium was obtained by a 1:1000 dilution. Mock treatments received the same volume of DMSO.

Somatic embryogenesis in Arabidopsis

To isolate Arabidopsis IZEs at the bent cotyledon stage of development, siliques were harvested 10–12 days after pollination, sterilized in 10% (w/v) NaOCl for 7 min, and washed four times

in sterile water. IZEs were dissected from the siliques in a laminar flow cabinet (Gaj, 2001). To induce SE, IZEs were cultured on solid B5 (Gamborg et al., 1968) media supplemented with natural and synthetic auxins with or without NPA, 2% (w/v) sucrose, and 0.7% agar (Sigma-Aldrich, St. Louis, MO, USA) for 2 weeks. Subsequently, the embryonic structures were allowed to develop further by transferring the explants to half MS medium with 1% (w/v) sucrose and 0.7% agar without hormones. One week after the subculture, the capacity to induce SE was scored under a stereomicroscope as the number of somatic embryos produced from IZEs per plate. Four plates were scored for each experiment.

Somatic embryogenesis in carrot

In this study, the carrot variety Lobbericher was used, and somatic embryos were induced as described previously (Ducos et al., 1993). Seeds were sterilized with 70% ethanol for 30 sec and subsequently with household bleach diluted with 0.5% (w/v) NaOCl for 7 min, and washed four times in sterile water. Sterilized seeds were plated on MS medium containing 2% (w/v) sucrose and 0.7% agar without plant growth regulators at 27°C and dark condition. Hypocotyl explants, approximately 0.5 mm in size, were prepared from 10-day-old seedlings and cultured on 50 ml MS liquid medium with 2% (w/v) sucrose in 250 ml Erlenmeyer flasks, supplemented with 0,5 μM 2,4-D or 2 μM IAA or a combination of 2 μM IAA and 10 μM NPA. The flasks were placed on a rotatory shaker at 90 rpm at 27°C in the dark. After 3 weeks, the obtained suspension cultures were centrifuged at 600 rpm for 6 min. The pellet was resuspended in 50 ml of MS liquid medium containing 2% (w/v) sucrose, 0.1 μM mannitol, and 1 μM zeatin. The suspension cultures were again placed on a rotatory shaker at 90 rpm for 2 weeks at 27°C in the dark. The capacity to induce SE was scored under a stereomicroscope as the number of somatic embryos produced per ml. Three replicates were scored for each experiment and three independent experiments were carried out.

Shoot regeneration

In this study, the *Brassica napus* cultivar Westar was used and shoots were regenerated as described previously (Moloney et al., 1989) with the following modifications. Seeds were sterilized in 10% (w/v) NaOCl for 12 min and then washed four times in sterile water. Sterilized seeds were plated on half MS medium containing 1% (w/v) sucrose and 0.7% agar without plant growth regulators at 24°C, 70% relative humidity, and 16 h photoperiod. Hypocotyl (1–2 mm) or cotyledon explants were prepared from 7-day-old seedlings and cultured on MS solid medium supplemented with 1% sucrose and 5 μM 2,4-D or 5 μM 4-Cl-IAA with or without 10 μM NPA at 24°C, 70% relative humidity and 16 h photoperiod. After 2 weeks, the explants were transferred to solid MS medium supplemented with 10 μM 6-benzylaminopurine (BA) for shoot regeneration at 24°C, 70% relative humidity, and 16 h photoperiod. Three weeks after transferring explants, the shoot regeneration capacity was scored under a stereomicroscope as the number of shoots produced per plate containing 15 explants. Four plates were scored for each experiment.

GUS staining

Histochemical staining of transgenic lines expressing the β-glucuronidase (GUS) reporter for GUS activity was performed as described previously (Anandalakshmi et al., 1998) for 4 h at 37°C, followed by rehydration in a graded ethanol series (75, 50, and 25%) for 10 min each.

Microscopy

GUS-stained tissues and cultured IZE explants were observed and photographed using a LEICA MZ12 microscope (Switzerland) equipped with a LEICA DC500 camera.

Confocal Laser Scanning Microscopy (CSLM) was performed with a ZEISS-003-18 533. GFP and VENUS signals were detected using a 534 nm laser, a 488 nm LP excitation filter, and a 500–525 nm bandpass emission filter. Simultaneously, background fluorescence (e.g., chlorophyll) was detected with a 650 nm long pass emission filter. Images were captured with ZEISS ZEN2009 software. Unmodified images were cropped (if needed) and used for assembly into figures in MS PowerPoint. Assembled figures were saved as pdf files and converted to TIF files in Adobe Photoshop.

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AUTHOR CONTRIBUTIONS

OK and RO conceived the project, RO acquired funding, OK and AK performed the Arabidopsis experiments, AR performed the *Brassica napus* experiments, NZ and SA performed the carrot SE experiments, OK and RO wrote the manuscript, AK, AR, NZ, and SA read and commented on the manuscript.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Optimizing the effect of auxin efflux inhibition on SE from Arabidopsis IZEs. (A–C) The number of somatic embryos produced from Arabidopsis IZEs that were grown for 2 weeks on medium supplemented with 5 μM IAA combined with varying concentrations of NPA (A), or on medium with 10 μM NPA in combination with different concentrations of IAA (B), or on medium with different concentrations of 2,4-D, either without (mock) or with 10 μM NPA (C). Explants were subsequently transferred to hormone-free medium for 1 week before the somatic embryos were counted. The dots in (A–C) indicate the number of somatic embryos produced per 35 (A and B) or 40 (C) IZEs ($n = 4$ biological replicates); bars indicate the mean; error bars indicate the s.e.m.; different letters indicate statistically significant differences ($P < 0.01$) as determined by one-way analysis of variance with Tukey's honest significant difference post hoc test.

Figure S2. The expression pattern of the *pDR5:GFP* auxin response reporter in epidermis cells at the adaxial and abaxial sides of cotyledons of Arabidopsis IZEs after 5 days of culture on medium supplemented without any additional substances (mock), with 5 μM IAA, and 5 μM IAA + 10 μM NPA. scale bars indicate 0.5 mm.

Figure S3. Efflux-inhibited IAA works as effectively as 2,4-D in producing embryonic carrot suspension cultures. Embryonic carrot suspension cultures were obtained by culturing hypocotyl explants for 3 weeks in liquid medium supplemented with 0.5 μM 2,4-D, 2 μM IAA, or 2 μM IAA + 10 μM NPA. Somatic embryos were induced by subsequently culturing the suspension cells in medium with 1 μM zeatin for 2 weeks. The dots indicate the

number of somatic embryos produced per ml suspension cultures ($n = 4$ biological replicates); bars indicate the mean; error bars indicate the s.e.m.; different letters statistically significant differences ($P < 0.01$) as determined by one-way analysis of variance with Tukey's honest significant difference post hoc test.

Figure S4. Somatic embryos induced by efflux-inhibited natural auxins show improved shoot development. Arabidopsis IZEs were cultured for 2 weeks on medium containing 5 μM 2,4-D, 5 μM IAA + 10 μM NPA, 5 μM 4-Cl-IAA + 10 μM NPA or 5 μM IBA + 10 μM NPA. Explants were subsequently transferred to hormone-free medium and photographed after 3 weeks. Scale bar indicates 1 cm.

Figure S5. *Brassica napus* explants treated with efflux-inhibited natural auxin show early *de novo* shoot regeneration. Hypocotyl explants cultured for 2 weeks on 5 μM 2,4-D or 5 μM 2,4-D + 10 μM NPA (upper panel) or 5 μM 4-Cl-IAA or 5 μM 4-Cl-IAA + 10 μM NPA (lower panel) and subsequently for 1 week om medium supplemented with 10 μM BA. Scale bars indicate 1 mm.

Figure S6. Shoots regenerated from *Brassica napus* explants treated with efflux-inhibited natural auxin show synchronized growth. Hypocotyl explants were cultured for 2 weeks on 5 μM 2,4-D, 5 μM 2,4-D + 10 μM NPA, 5 μM 4-Cl-IAA or 5 μM 4-Cl-IAA + 10 μM NPA for callus induction followed by 2 weeks on medium supplemented with 10 μM BA for shoot induction, and then 2 weeks on hormone-free medium. The obtained shoots were separated from explants and photographed. Scale bars indicate 1 cm.

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