Metabolic Relations of Inositol 3,4,5,6-Tetrakisphosphate Revealed by Cell Permeabilization. Identification of Inositol 3,4,5,6-Tetrakisphosphate 1-Kinase and Inositol 3,4,5,6-Tetrakisphosphate Phosphatase Activities in Mesophyll Cells

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Using a permeabilization strategy to introduce Ins(3,4,5,6)P₄ into mesophyll protoplasts of Commelina communis, we have identified Ins(3,4,5,6)P₄ 1-kinase activity in mesophyll cells. Multiple InsP₄ isomers were identified in Spirodela polyrhiza and Arabidopsis. Only two of these, Ins(1,2,3)P₃ and Ins(3,4,6)P₃, have previously been identified in plants and only in monocots. The isomers detected in S. polyrhiza included α- and/or l-Ins(3,4,5)P₄, α- and/or l-Ins(3,5,6)P₄, and α- and/or l-Ins(2,4,5)P₃, Ins(1,4,5), P₃ if present, was only a tiny fraction of total InsP₃ species. We have also identified inositol polyphosphate phosphatase activities, Ins(3,4,5,6)P₄ 6-phosphatase and Ins(3,4,5,6)P₄ 4-phosphatase, whose action on endogenous inositol polyphosphates explains the presence of α- and/or l-Ins(3,4,5)P₄ and α- and/or l-Ins(3,5,6)P₄ in mesophyll cells. Inositol trisphosphates identified in Arabidopsis include Ins(1,2,3)P₃ and α- and/or l-Ins(3,4,6)P₄, suggesting that dicots may share pathways of InsP₄ biosynthesis and breakdown in common with monocots.

Perhaps the single most distinctive feature of plant inositol phosphate metabolism is the accumulation of inositol hexakisphosphate (InsP₆)² to levels up to several percent of dry weight in seed or storage tissues (Raboy and Dickin-...
mented with 20 g/L Glc, 50 mg/L kinetin, 0.5 mg/L 2,4-
dichlorophenoxyacetic acid, and 0.5 g/L 2-(N-morpholino)-
ethanesulfonic acid (MES), pH 5.7 (22°C). Labeled cultures
were initiated by the transfer of a small spatula load of cells
from a 7-d-old stock culture to labeling medium comprising
Gamborg’s B5 basal salt mixture (Sigma G-5768) sup-
plemented as above but also containing 1 mg/L nicotinic
acid, 1 mg/L pyridoximine hydrochloride, and 10 mg/L
thiamine hydrochloride. Cells 0.26 to 0.34 g fresh weight
were removed from a subculture of cells labeled for 4 d in
10 mL of medium containing 20 µCi of myo-[2-3H]inositol.

Erythrocyte Ghost Treatment of Inositol Phosphates
and Hanke, 1996a).

Tissue Extraction

Labeled S. polyrhiza tissue and Arabidopsis suspension
cultures were extracted with perchloric acid, neutralized
and eluted from a strong anion-exchange HPLC column
(Partisphere SAX, Whatman International, Maidstone, UK)
with gradients of NaH2PO4. Peak fractions were desalted
on Dowex AG1 X8 resin (formate form) columns (Brearley
with gradients of NaH2PO4. Peak fractions were desalted
on Dowex AG1 X8 resin (formate form) columns (Brearley
and Downes, 1990). After reduction, approximately 50

Erythrocyte Ghost Treatment of Inositol Phosphates

Desalted inositol phosphates were treated with erythro-
cyte ghosts under ionic conditions, 12.5 mM-HEPES, pH 7,
10 mM-MgCl2, and 1 mM-EGTA, favoring the activity of
inositol polyphosphate 5-phosphatase (Brearley et al., 1997).

Periodate Oxidation, Reduction, and Dephosphorylation of
Inositol Phosphates to Polyols

We have used the periodate oxidation, reduction, and
dephosphorylation technique originally devised by Clinton
Ballou (Tomlinson and Ballou, 1961) and given a contem-
porary reading with myo-[3H]inositol labeled substrates (as
described by Stephens [1990]) to determine the stereoisom-
erism of inositol phosphates identified in this study. Perio-
date attack on the inositol ring cleaves C

RESULTS

Multiple Isomers of InsP3 in S. polyrhiza

Partisphere SAX HPLC of perchloric acid extracts from
[3H]inositol-labeled S. polyrhiza resolved three peaks of InsP3
(Fig. 1). Peak I eluted before [32P]Ins(3,4,5) P3, [32P]Ins(1,4,5)
P3, and [32P]Ins(2,4,5) P3; peak II eluted after [32P]Ins(3,4,5)
P3, with [32P]Ins(1,4,5) P3, and before [32P]Ins(2,4,5) P3. We have previously shown that peak III contains Ins(3,4,6) P3 (Brearley and Hanke, 1996a).

Standards

[32P]Ins(1,4,5) P3 and [32P]Ins(2,4,5) P3 were prepared by
alkaline hydrolysis of PtdIns(4,5)P2, the product of a
purified PtdIns4P 5-kinase. Alternatively, [32P]PtdIns(4,5) P2
was prepared from [32P]-labeled turkey erythrocyte
lipids. [32P]Ins(2,4,5) P3 and traces of [32P]Ins(3,4,5) P3 were
also obtained by mild acid treatment of [32P]Ins(1,4,5) P3.
[14C]Ins(3,4,6) P3 was obtained from [14C]inositol-labeled S.
polyrhiza (Brearley and Hanke, 1996a). [14C]Ins(1,2,3) P3
and d- and/or l-[14C]Ins(1,2,6) P3 were prepared by treat-
ment of [14C]InsP6 obtained from S. polyrhiza with a com-
mercial preparation (Sigma) of wheat bran phytase essen-
tially according to the method of Stephens (1990).

Permeabilization Experiments

Mesophyll protoplasts were prepared from C. communis
electroporated (Brearley et al., 1997) at a cell density of
approximately 2 × 10^7 cells/mL in 0.3 mL of medium
containing 4.5 × 10^5 dpm of [3H]Ins(3,4,5,6) P4 obtained
from S. polyrhiza (Brearley and Hanke, 1996a), sup-
plemented in some cases with 20 mM MgATP. Glc 6-P was
included at a 20 mM concentration to guard against the
potential breakdown of added inositol phosphates by un-
specified enzyme activities. Protoplasts were incubated for
7 min following electroporation and quenched with per-
chloric acid.

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Ins(1,2,3) P₃ Is Present in Vegetative Tissues of Monocots

The polyol products of periodate oxidation, reduction, and dephosphorylation of peak I were mixed with standards and resolved on Polypore Pb²⁺ HPLC (Fig. 2A). A single major peak of ³H-labeled material was detected and this eluted precisely with [¹⁴C]ribitol and before standards of arabitol, altitol, xylitol, glucitol, and iditol. However, because of the difficulty in obtaining separations of ribitol and inositol on Polypore Pb²⁺ columns, aliquots of peak fractions containing [¹⁴C]ribitol and ³H-label were pooled, freeze-dried, and re-run on an aminopropyl column (Brearley and Hanke, 1992). Under these conditions the ³H-label co-eluted precisely with [¹⁴C]ribitol and before [¹⁴C]inositol. This identifies the parent inositol phosphate as Ins(1,2,3) P₃.

Ins(1,4,5) P₃, if Present, Is Only a Minor Component

The identity of the inositol phosphate(s) in peak II was pursued in several ways. It was desalted and co-presented with an authentic standard of [³²P]Ins(1,4,5) P₃ to a preparation of human erythrocyte ghosts, and samples of the reaction products were withdrawn at intervals for HPLC. The results (not shown) revealed that the kinetics of metabolism of the ³H- and ³²P-labeled compounds to products with the chromatographic properties of InsP₂s, were very different. The principal [³H]InsP₂ product of dephosphorylation eluted after [³²P]Ins(1,4) P₂. Although this analysis cannot discount the possibility that the parent ³H peak contains some Ins(1,4,5) P₃, the indication is that the major component is not Ins(1,4,5) P₃. The analysis does suggest, however, that a component in the peak bears a phosphate in the five position.

Identification of Inositol Phosphates Novel to Plants: ³H- and/or ³H-Ins(3,4,5) P₃ and ³H- and/or ³H-Ins(3,5,6) P₃

Another preparation of [³H]InsP₃ peak II was resolved on Partisphere SAX HPLC, desalted, and applied to an Adsorbosphere SAX HPLC column (Brearley and Hanke, 1996b) on which a single peak of ³H label was resolved. Periodate oxidation, reduction, and dephosphorylation of this peak yielded a major product that co-eluted precisely with xylitol, but after inositol, arabitol, and altitol, and before glucitol and iditol (Fig. 2B). Smaller amounts of ³H label co-eluted precisely with inositol, glucitol, and iditol. We have not determined the enantiomeric identity of the polyols obtained.

Because the parent peak co-eluted with Ins(1,4,5) P₃ but before both Ins(3,4,6) P₃ and Ins(2,4,5) P₃ on Partisphere SAX HPLC [see Stephens and Downes (1990) and Stephens and Irvine (1990) for separation of Ins(1,4,5) P₃ from Ins(3,4,6) P₃, and Chilvers et al. (1991), for separation of Ins(1,4,5) P₃ from Ins(2,4,5) P₃], the production of xylitol identifies the major component of this peak as ³H- and/or ³H-Ins(1,5,6) P₃. The glucitol-yielding component is ³H- and/or ³H-Ins(1,2,5) P₃, since ³H- and/or ³H-Ins(2,4,5) P₃, the

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**Figure 1.** Inositol trisphosphates in *S. polyrhiza*. Inositol trisphosphates from [³H]inositol-labeled *S. polyrhiza* were separated by HPLC and radioactivity monitored with a flow detector (Radiomatic series A-500, Canberra Packard, Pargbourne, Berks, UK). Separations such as this have been observed in more than 10 experiments.

**Figure 2.** HPLC of polyols derived from InsP₃s. Polyols from [³H]InsP₃ peaks I, II, and III (Fig. 1) were mixed, respectively, with [¹⁴C]riboitol, xylitol, and glucitol (A); [¹⁴C]inositol, xylitol, and glucitol (B); or [¹⁴C]riboitol, xylitol, and glucitol (C), and resolved by HPLC. Fractions were collected and radioactivity in aliquots was determined. Unlabeled polyol standards, monitored at 190 nm, are indicated with arrows. ³H, ³²P; O, [¹⁴C]; ●, ins, inositol; □, rib, ribitol; ara, arabitol; alt, altitol; xyl, xylitol; glu, glucitol; idi, iditol. Repeat analysis of polyols in [³H]InsP₃ peaks II and III yielded similar results. Confirmation of the identity of the polyol derived from [³H]InsP₃ peak I was provided by subsequent analysis on an aminopropyl column (see text).
only other isomers that yield glucitol (Stephens, 1990), elute after Ins(1,4,5)P3. Because iditol, the third polyolobtained from the parent InsP3, peak, is obtained from both d- and/or L-Ins(1,4,5)P3 and d- and/or L-Ins(3,4,6)P3, the elution of InsP3 II before Ins(3,4,6)P3 (the major peak in Figure 1), leaves d- and/or L-Ins(1,4,5)P3 as the only possible parents for the iditol obtained. That this minor peak eluted before Ins(3,4,6)P3, which was present in excess over the others, means that the iditol obtained from the second peak could not be derived from peak III.

**d-** and/or l-Ins(2,4,5)P3 Is Present in Plants

We also performed an analysis of peak III in an attempt to identify any minor components in this peak. The peak was desalted, subjected to periodate oxidation, reduction, and dephosphorylation, and the products were resolved on HPLC (Fig. 2C). Among the potential products of oxidation and dephosphorylation, and the products were resolved on HPLC (Fig. 2C). Among the potential products of oxidation of authentic InsP3s, four peaks of 3H-polyols were detected. The first eluted just before [14C]ribitol and is likely to be [3H]inositol. Its presence is either the result of a failure in the oxidation of the InsP3s in the parent peak (peak III) or is indicative of the presence of Ins(1,3,5)P3 or Ins(2,4,6)P3, which are both resistant to periodate oxidation. We are not aware of the identification of Ins(1,3,5)P3 or Ins(2,4,6)P3 in either plants or animals.

The second polyol, a minor component, co-eluted precisely with [14C]xylitol. The third, also a minor component co-eluted precisely with d-[14C]glucitol. No attempt was made to determine the enantiomerism of the [3H]glucitol product, whereas xylitol and myo-inositol are both meso-compounds. The fourth and major peak co-eluted with iditol. The enantiomerism of this product was characterized previously (Brearley and Hanke, 1996a). The parent inositol phosphate is Ins(3,4,6)P3.

The presence of a trace of material that eluted with [14C]xylitol is perhaps more difficult to explain. The only possible parents for the iditol obtained from peak III are Ins(1,2,3)P3 and Ins(2,4,5)P3, which elutes before [32P]Ins(2,4,5)P3, while the third co-eluted with d-[14C]Ins(3,4,6)P3 after [14C]Ins(1,2,6)P3. In a separate HPLC run (Fig. 3B) the first peak eluted before [32P]Ins(1,4,5)P3, and before [32P]Ins(2,4,5)P3, and also before we assume to be a trace of [32P]Ins(3,4,5)P3, which elutes before Ins(1,4,5)P3 on SAX columns (Stephens and Downes, 1990). The second peak of 3H label co-eluted approximately with [32P]Ins(1,4,5)P3 and before [32P]Ins(2,4,5)P3, whereas the third peak eluted after [32P]Ins(2,4,5)P3.

At this level of analysis the indications are that root cell suspension cultures of Arabidopsis contain InsP3s with

**InsP3s in Arabidopsis: Identification of Ins(1,2,3)P3 and d-** and/or l-Ins(3,4,6)P3

When extracts from labeled Arabidopsis cultures were mixed with [14C]Ins(1,2,3)P3, d- and/or l-[14C]Ins(1,2,6)P3, and [14C]Ins(3,4,6)P3, three distinct peaks of [3H]InsP3 were resolved (Fig. 3A). The first 3H peak co-eluted precisely with [14C]Ins(1,2,3)P3 and, given the diagnostic very early-eluting nature of the latter compound on Partisphere SAX HPLC, is likely to be the same. The second peak of 3H label eluted after [14C]Ins(1,2,3)P3 and before [14C]Ins(1,2,6)P3, whereas the third co-eluted precisely with [14C]Ins(3,4,6)P3 after [14C]Ins(1,2,6)P3.

Figure 3. Inositol trisphosphates in Arabidopsis root suspension cultures. Extracts from [3H]inositol-labeled Arabidopsis were mixed with standards and resolved by HPLC. Radioactivity in column fractions was determined by dual-label scintillation counting (A: 3H, 32P; 14C, 32P; 32P, 32P). The data in B were five-point-smoothed by the flow-detector software. The peaks identified are the internal standards. Separation of three InsP3 peaks was confirmed in an independent experiment.

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the chromatographic properties of Ins(1,2,3) P₃ and Ins(3,4,6) P₃. The other peak was not identified, but we can exclude Ins(1,2,3) P₃, d/L-Ins(1,5,6) P₃, d/L-Ins(2,4,5) P₃, d/L-Ins(1,2,6) P₃, d/L-Ins(1,4,6) P₃, and probably also d/L-Ins(1,3,4) P₃, which elutes before d/L-Ins(1,5,6) P₃ on SAX columns (Stephens and Downes, 1990), as potential identities.

**Ins(3,4,5,6) P₄ and Ins(3,4,5,6) P₃**

The chromatographic properties of Ins(3,4,5,6) P₄ and Ins(3,4,5,6) P₃ were also studied. The chromatographic behavior of Ins(3,4,5,6) P₄, Ins(3,4,5,6) P₃, and d/L-Ins(3,4,5,6) P₃ was examined using Partisphere SAX HPLC (Stephens and Downes, 1990; Stephens and Irvine, 1990). The identification of the same InsP₃s, albeit as stereoisomers and not individual enantiomers, in S. polyr rhiza provides strong evidence that d- and/or l-Ins(1,5,6) P₃ and d- and/or l-Ins(1,4,5) P₃ are the products of Ins(3,4,5,6) P₄ metabolism. The chromatographic properties of InsP₃s on SAX columns, we identified the first two InsP₃s as Ins(3,4,5) P₃ and Ins(3,5,6) P₃, in order of increasing elution time, and the third as Ins(4,5,6) P₃. The identification of the same InsP₃s, albeit as stereoisomers and not individual enantiomers, in S. polyr rhiza provides strong evidence that d- and/or l-Ins(1,5,6) P₃ and d- and/or l-Ins(1,4,5) P₃ are the products of Ins(3,4,5,6) P₄ metabolism. The foregoing also explains the origins of d- and/or l-Ins(1,4,5) P₃ in S. polyr rhiza and suggests that the isomer might be the l-enantiomer [d-Ins(3,5,6) P₃].
Ins(3,4,5,6) P₄ 1-Kinase Activity in Mesophyll Protoplasts

The InsP₅ product of metabolism of [³H]Ins(3,4,5,6) P₄ was chromatographed (Fig. 4C) against internal standards of [¹⁴C]InsP₅, as described previously (Brearley and Hanke, 1996a, 1996b). The order of elution of InsP₅ on Partisphere SAX columns is Ins(1,3,4,5,6) P₅, d/l-Ins(1,2,3,4,5) P₅, Ins(1, 2, 3, 4, 6) P₅, d/l-Ins(1,2,4,5,6) P₅ (Stephens et al., 1991). Thus, the precise co-elution of the [³H]InsP₅ product with an internal standard of [¹⁴C]Ins(1,3,4,5,6) P₅ and before standards of d/l-[¹⁴C]Ins(1,2,3,4,5) P₅ and d/l-[¹⁴C]Ins(1,2,4,5,6) P₅ identifies the product as Ins(1,3,4,5,6) P₅ and so defines Ins(3,4,5,6) P₄ 1-kinase activity in mesophyll protoplasts. This provides a convincing explanation of the metabolic origin of Ins(1,3,4,5,6) P₅ in plants and S. polyrhiza in particular.

DISCUSSION
Inositol Trisphosphates in Plants

Detailed analysis of inositol trisphosphates in two plants has revealed a complex array of isomers. None of the isomers detected is unique to plants though d/l-Ins(2,4,5) P₃ and Ins(1,2,3) P₃ have only occasionally been reported in vivo in any kingdom (Brearley and Hanke, 1992, 1996b; Barker et al., 1993). Although Ins(1,4,5) P₃ has been only a few InsP₃s identified to date, it appears that this isomer is only a minor component of the inositol phosphate complement of higher plants.

A schematic diagram of the metabolic relationships of InsP₃ species identified in mesophyll cells is indicated in Figure 5. In this context, it is worth remembering that the storage tissue of the turion of S. polyrhiza is a specialized form of the mesophyll of the frond.

Metabolic Origins of Inositol Trisphosphates in Plants: InsP₃s as Products of Catabolism

The existing literature provides clues to the origins of InsP₃ species identified in mesophyll cells. The work of Johnson and Tate (1969), Lim and Tate (1971, 1973), and Tomlinson and Ballou (1961, 1962) reviewed by Cosgrove (1980), suggests that both Ins(1,2,3) P₃ and Ins(1,2,6) P₃ are products of InsP₆ breakdown in vivo. The identification of these two isomers in aleurone tissue and of Ins(1,2,3) P₃ in S. polyrhiza and Arabidopsis in the present study suggests that these isomers are products of InsP₆ metabolism (cytosolic or not) in vivo.

If d- and/or l-Ins(1,5,6) P₃ in S. polyrhiza turns out to be the l-enantiomeric form [d-Ins(3,4,5) P₃], then an explanation of the origin of this compound is provided by our demonstration of metabolism of Ins(3,4,5,6) P₄ to Ins(3,4,5) P₃ in mesophyll protoplasts. Other investigators (Radenberg et al., 1989) have speculated that the d- and/or l-Ins(1,5,6) P₃, which they identified in avian erythrocytes, may be a product(s) of dephosphorylation of Ins(3,4,5,6) P₄ and Ins(1,3,4,5) P₄. Our approach affords an experimental
text of this. Whereas Ins(3,4,5,6) P_4 is present in higher plants, there is no evidence yet for Ins(1,3,4,5) P_4.

Finally, in consideration of the range of Ins P_3 isomers in plants, it is apparent that Ins(1,2,3) P_3 is common to monocots (S. polyrhiza and barley) and dicots (Arabidopsis). It occurs in non-highly differentiated cells (mesophyll cells) in the fronds and turions of S. polyrhiza, in root cortex cells of Arabidopsis, and in terminally differentiated cells in barley aleurone. It is likely that the route of Ins(1,2,3) P_3 generation is shared in monocots and dicots and perhaps also in animal cells (Barker et al., 1995), where it has been shown that Ins(1,2,3) P_3 is a product of InsP_5 breakdown by cell homogenates. Clearly, Ins(1,2,3) P_3 is not restricted to specialized cells or storage tissues in plants, so we should consider a general “housekeeping” function for this isomer. One such suggestion is that Ins(1,2,3) P_3 is an inhibitor of hydroxyl free radical formation (Barker et al., 1995; Phillippy and Graf, 1997).

**InsP_3s as Intermediates in Synthetic Pathways**

Ins(3,4,6) P_3 is one of the few InsP_3s for which there is direct evidence in vivo of the identity of its metabolic neighbors. In avian erythrocytes, Ins(3,4,6) P_3 is the precursor of Ins(3,4,5,6) P_4, which in turn is the precursor of Ins(1,3,4,5,6) P_5 (Stephens and Downes, 1990). A caveat is necessitated by the possibility, however hypothetical, of substrate cycles involving these inositol phosphates, which could compromise the interpretation of non-equilibrium labeling studies. The operation of substrate cycles for these specific isomers has not been tested directly or indirectly. Ins(3,4,6) P_3 is also an intermediate in a pathway of InsP_6 biosynthesis in D. discoideum (Stephens and Irvine, 1990).

In plants (Brearley and Hanke, 1996b), Ins(3,4,6) P_3 is an intermediate in a biosynthetic sequence to InsP_6, which shares the partial sequence Ins(3,4,6) P_3 → Ins(3,4,5,6) P_4 → Ins(1,3,4,5,6) P_5, which has been described in avian erythrocytes (Stephens and Downes, 1990) and may represent steps in a route of synthesis of InsP_6 in the animal kingdom. An alternative possibility (Shears, 1996) places Ins(1,3,4) P_3 at a branch point in inositol phosphate metabolism leading either to inositol through the action of inositol phosphate phosphatases or to InsP_6 via Ins(1,3,4,5) P_4 and Ins(1,3,4,5,6) P_5. Thus, the recent cloning of human (Wilson and Majerus, 1996) and Arabidopsis (Wilson and Majerus, 1997) genes whose products, tested against a limited number of substrates, show Ins(1,3,4) P_3 5/6-kinase activity in vitro is particularly interesting.

Given the lack of consensus on the route(s) of InsP_6 synthesis in animals, plants and *D. discoideum*, our demonstration that Ins(3,4,5,6) P_4 is a substrate for Ins(3,4,5,6) P_4 1-kinase activity in mesophyll protoplasts of monocots is entirely consistent with the earlier proposal that in plants the 1-P of InsP_6 is added after the 4- and 5-Ps (Brearley and Hanke, 1996b). Because the 3-P and not the 1-P is added first, such observations discount the possibility that Ins(1,4,5) P_3 or Ins(1,3,4) P_3 are precursors of InsP_6 in *S. polyrhiza*. We found no evidence for the presence of Ins(1,4,5) P_3-kinase activity in mesophyll protoplasts (Brearley et al., 1997) under similar experimental conditions to those described here.

Moreover, an inositol polyphosphate 5/6-kinase has been cloned from Arabidopsis (H.W. Xue, C.A. Brearley, and B. Mueller-Roeber, unpublished data) that is identical to that previously reported in Arabidopsis (Wilson and Majerus, 1997). The product of our gene shows Ins(3,4,6) P_3 5-kinase activity, consistent with the precursor-product relationship of Ins(3,4,6) P_3 and Ins(3,4,5,6) P_4 in a pathway to InsP_6 (Brearley and Hanke, 1996a, 1996b), and which, considering the identification of D- and/or L-Ins(3,4,6) P_3 in Arabidopsis, might suggest a common pathway in monocots and dicots. It is, however, quite possible that there are multiple routes to InsP_6 in plants. Phillippy (1998) has recently identified separate inositol 1,3,4-trisphosphate 5-kinase and inositol 1,3,4,5-tetrakisphosphate 6-kinase activities in immature soybean plants.

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