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MORPHACTIN SUBSTANTIALLY INDUCED THE FOURTH INTERNODE GROWTH IN DECAPITATED TULIPS: RELEVANCE TO ENDOGENOUS LEVELS OF INDOLE-3-ACETIC ACID

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A B S T R A C T

Important role of auxin polar transport inhibitors such as TIBA (2,3,5-triiodobenzoic acid), NPA (*N*-(1-naphthyl)phthalamic acid) and morphactin (methyl 2-chloro-9-hydroxyfluorene-9-carboxylate) in stem growth of tulips was intensively studied. After the application of morphactin above the 3rd node of decapitated tulips, the stimulated growth of the 4th internode was clearly observed. On the other hand, NPA and TIBA were slightly effective in stimulating the 4th internode growth of tulips. Endogenous levels of IAA in the 4th internode after the treatment of auxin polar transport inhibitors were determined using gas-liquid chromatography-mass spectrometry (GC-MS) and gas-liquid chromatography-mass spectrometry selected ion monitoring system (GC-SIM) with deuterium labelled IAA (Indole-2,4,5,6,7-d_5-3-acetic acid, d_5-IAA) as an internal standard, resulted in significant accumulation of IAA in the 4th internode of tulips after the treatment of morphactin. In the treatment of NPA and TIBA, there was almost no such an accumulation of IAA. These results strongly suggest that stimulated growth of the 4th internode of tulips induced by the application of morphactin is the consequence of accumulated endogenous levels of IAA after the treatment. The possible mode of action of auxin polar transport inhibitors in tulip stem growth is also discussed.

Abbreviations: GC-MS − gas-liquid chromatography-mass spectrometry, GC-SIM − gas-liquid chromatography-mass spectrometry selected ion monitoring system, IAA − indole-3-acetic acid, NPA − *N*-(1-naphthyl)phthalamic acid, TIBA − 2,3,5-triiodobenzoic acid

Key words: deuterium labelled IAA, endogenous levels of IAA, growth, internodes, stem, morphactin, NPA, TIBA, tulips

INTRODUCTION

Rapid elongation of the 4th (top) internode in tulips takes place after earlier elongation of the first, second and third internodes (Ranwala and Miller, 2008). Elongation of the 4th internode, after removal of the flower bud in growing tulips, with intact or removed leaves, at different stages of growth, was very weak (Saniewski et al., 2010). This suggests that the flower bud is always responsible for elongation of the 4th internode. Exogenously applied auxin (IAA 0.1%, w/w in lanolin paste) to the cut surface of tulip stem (in the place of removed tulip bud) with intact or removed leaves, resulted in greatly stimulated growth of the 4th internode and lower internodes of tulip shoot (Saniewski and De Munk, 1981; Saniewski et al., 2010).

The elongation growth of the excised 4th internode of tulip shoot with or without node, after removal of the flower bud was much higher, in comparison with that of the intact 4th internode in tulip shoot which was growing and with that the 4th internode which the flower bud had been cut (Saniewski et al., 2010). Elongation depended on the initial length of the 4th internode.

The main question is why the growth of the excised 4th internode, independently of the length – with or without node, is much higher than in tulips which are growing after removal of the flower bud? One possible explanation is that endogenous auxin in the 4th internode in growing tulip shoot after removal of the flower bud, is easily transported basipetally to lower internodes. This transport takes place because there is an interaction between the growth of each internode in intact tulip shoot. Lack of endogenous auxin would then cause a limitation on the growth of the 4th internode. The higher growth of the excised 4th internode, in comparison to that in growing tulips after removal of the flower bud, is possibly caused by weaker and/or slow flow of auxin into water. Consequently, relative higher levels of endogenous auxin are still present in the 4th internode, which accounts for the elongation. In addition, the growth rate of the excised 4th internode with flower bud with different initial lengths greatly elongated. The results suggest again that flower bud is a source of auxin to induce the growth of the excised 4th internode (Saniewski et al., 2010).

In the present study we report that morphactin substantially stimulated the growth of the 4th internode in growing tulips after removal of flower bud when it was applied above the 3rd node or on the top of the 4th internode. The possible role of accumulated endogenous IAA in the 4th internode of tulips after the treatment of auxin polar transport inhibitors is also discussed.

MATERIAL AND METHODS

Plant materials

Bulbs of tulip (*Tulipa gesneriana* L. 'Apeldoorn') with circumference of 10-11 cm, after lifting, were stored at 19-22 °C until transferred on October 15 to 5 °C for dry cooling or planted in field conditions. During February, after full cooling of bulbs in 5 °C, tunics were removed and the bulbs were individually planted in pots and cultivated at 18-20 °C in a greenhouse under natural light conditions. At different stage of tulips, when the length of the 4th internode ranged between 25 mm to 60 mm, flower buds were removed and lanolin only was applied on the top of the 4th internode, and inhibitors of auxin polar transport, NPA, TIBA and morphactin, at a concentration of 0.2% in lanolin paste and lanolin only (as a control) were applied above the 3rd node, below the 3rd node and on the top of the 4th internode, respectively. Detail initial length of the 4th internode and lower internodes at time of treatment in all experiments (Exp. 1-6) are presented in the Table 1. The length of all internodes was measured at different period of duration of experiments, but always all internodes after final growth. Ten plants were used per treatment in all experiments and experiments were repeated two to three times. The analysis of variance and Duncan's t-test were used to estimate the difference between means of the length of 4th internode at $p = 0.05$.

Determinations of endogenous levels of IAA after treatment above the 3rd node with NPA, TIBA and morphactin at a concentration of 0.2% in lanolin paste and lanolin only (as a control), applied over the 3rd node when the length of the 4th internode was 58 mm, were made on tulips planted in field conditions. Treatments were made on April 17, ten plants per treatments were used. Determinations of endogenous IAA in the 4th internode were made in the samples fixed on April 17 (initial sample) and in the samples treated with NPA, TIBA, morphactin and control (lanolin only) fixed on April 23. For fixation was used 80% methanol. The length of the 4th internode on April 23 was following: Control (lanolin only) – 59 mm, NPA – 61 mm, TIBA – 63 mm, morphactin – 83 mm.

Identification and determination of endogenous levels of IAA

Extraction and solvent fractionation

Plant material was extracted with methanol and ethanol two times at −20 °C for several weeks. Extracts were combined and evaporated *in vacuo* to give a small volume of aqueous solution. Indole-2,4,5,6,7-d_5-3-acetic acid (d_5-IAA) was added to the aqueous solution as an internal

standard at a rate of 40-50 ng/g dry weight. The aqueous solution containing d_5-IAA was adjusted to pH 3 with HCl and then partitioned two times against water-saturated diethyl ether in the usual way (Yokota et al., 1980, Ueda et al., 1991), giving the diethyl ether soluble-acidic materials including IAA. Acidic diethyl ether fraction was dried over anhydrous Na_2SO_4 and then concentrated to dryness *in vacuo*. Partial purification was performed with silica gel thin-layer chromatography (TLC) developed with the solvent of *n*-hexane-ethyl acetate-chloroform-acetic acid (40 : 40 : 16 : 1, v/v/v/v, multiple development). Zone corresponding to that of authentic IAA was scraped off and eluted with ethyl acetate for an appropriate time in low temperature. Eluate of ethyl acetate was evaporated to dryness *in vacuo*.

Identification and estimation of IAA using a gas-liquid chromatography-mass spectrometry (GC-MS)

Partially purified sample was dissolved in a small amount of ethyl acetate again and then methylated with ethereal diazomethane. Methylated samples were subjected to a Finnigan GCQ gas-liquid chromatography-mass spectrometry (GC-MS) and/or gas-liquid chromatography-mass spectrometry selected ion monitoring system (GC-SIM) as already reported (Ueda et al., 1991). In GC-SIM analyses, two prominent fragment ions of m/z 189 (molecular ion [M^+]), 130 (base peak) for d_0-IAA methyl ester and m/z 194 (molecular ion [M^+]) and 135 (base peak) for d_5-IAA one were monitored. Carrier gas was He (4 kg/cm^2) and ionizing voltage was 70 eV. Column temperature was 160 for 1 min and then was increased to 250 for 7.75 min at a rate of 8/min, and it was kept at 250 for 11.25 min. Total time of the program was 20 min.

All results were expressed as μg/total dry weight of the sample.

RESULTS AND DISCUSSION

Effects of auxin polar transport inhibitors on the growth of tulip stem

After application of morphactin above the 3rd node, the inhibitor is translocated both basipetally (to the 3rd, the 2nd, the 1st internodes) and acropetally (to the 4th internode) and finally the growth of the 4th internode is stimulated (Exp. 1: initial length of the 4th internode on Feb. 08 – 40 mm: Fig. 1, Table 1; Exp. 2: initial length of the 4th internode on Feb. 0.8 – 60 mm: Table 1). Thus, transport of auxin was inhibited by morphactin at the place of treatment and in entire the 4th internode.

When morphactin was applied in the place of removed flower bud (Exp. 3: initial length of the 4th internode on Feb. 08 – 50 mm: Table 1; Exp. 4: initial length of the 4th internode on April 08 – 46 mm: Fig. 2, Table 1) morphactin stimulated the growth of the 4th internode. In this case morphactin is transported only basipetally and probably the transport of morphactin thought to be faster than auxin in the 4th internode and finally accumulation of auxin takes place on the 4th internode.

Control paste | NPA 0.2% | TIBA 0.2% | Morph. 0.2%

Figure 1. The effect of Morphactin, NPA and TIBA at a concentration of 0.2%, applied above the 3rd node, after excision of flower bud, on the growth of the 4th internode in tulips (Exp. 1, see Table 1); leaves were removed before picture was made

Control Paste | TIBA 0.2% | NPA 0.2% | Morph. 0.2%

Figure 2. The effect of Morphactin, NPA and TIBA at a concentration of 0.2%, applied in the place of removed flower bud, on the growth of the 4th internode in tulips (Exp. 4, see Table 1)

Table 1. The effects of inhibitors of auxin polar transport (morphactin, TIBA, NPA) on the growth of the 4th internode in tulips

Treatments	Initial length of internodes (mm)				Increase the length of internodes [mm]				Increase the length of 4th internode over the control (minus control) [mm]
	I	II	III	IV	I	II	III	IV	
Exp. 1. Above 3rd node (Feb. 8)									
Control	74	58	48	38	4.6	7.0	6.6	3.2 a*	
Morphactin	70	63	50	38	7.0	6.2	9.1	70.3 c	67.1
TIBA	66	57	48	38	3.3	1.7	5.8	26.2 b	23.0
NPA	78	58	54	41	0.0	4.2	8.3	5.2 a	2.0
Exp. 2. Above 3rd node (Feb. 8)									
Control	66	56	69	57	6.2	4.0	9.2	5.0 a	
Morphactin	73	64	64	60	6.7	3.7	5.3	49.3 c	44.3
TIBA	74	64	70	63	1.4	1.5	6.7	13.0 b	8.0
NPA	68	64	67	63	4.8	1.5	5.8	9.3 b	4.3
Exp. 3. On the top of 4th internode (Feb. 8)									
Control	74	70	54	45	3.3	0.0	5.5	5.8a	
Morphactin	76	62	56	52	4.0	3.6	7.6	86.4b	80.6
TIBA	-	-	-	-					
NPA	-	-	-	-					
Exp. 4. On the top of 4th internode (April 8, field)									
Control	66	45	49	48	0.0	4.5	15.7	19.3 a	
Morphactin	52	39	48	46	0.0	4.2	14.0	79.0 b	59.7
TIBA	53	35	40	40	0.0	9.3	18.2	29.2 a	9.9
NPA	59	40	44	44	0.0	4.2	11.3	27.2 a	7.9
Exp. 5. Below 3rd node (Feb. 1)									
Control	52	32	19	24	1.7	3.9	10.9	3.0 a	
Morphactin	64	46	31	27	1.6	0.7	0.9	6.9 a	3.9
TIBA	69	45	25	26	0.7	1.9	2.1	3.4 a	0.4
NPA	46	32	21	23	1.4	1.7	0.6	1.7 a	-1.3
Exp. 6. On the top of 4th internode (Feb. 1)									
Control	72	47	34	29	1.3	3.8	7.6	3.3 a	
Morphactin	58	45	30	27	2.1	1.8	3.7	3.7 a	0.4
TIBA	63	47	33	28	2.0	2.7	5.4	5.3 a	2.0
NPA	73	47	31	27	1.6	2.1	6.7	3.3 a	0.0

Morphactin, TIBA and NPA were respectively applied above and below the 3rd node, and on the top of the 4th internode at different length of the internode after removal of flower bud. All leaves were remained in the shoot in Exp.1 to 6. *Means in the column followed by the same letter are not significantly different at P = 0.05 according to Duncan's t-test

When morphactin was applied in the place of removed flower bud in case of small length of the 4th internode (Exp. 6: initial length of the 4th internode on Feb. 01 – 26 mm: Table 1) we did not observe stimulation of growth of the 4th internode. It is possible that in this case endogenous levels of auxin was extremely low since the length of the 4th internode was too short and finally morphactin did not stimulate the growth of the 4th internode; almost no auxin and no action of morphactin.

When morphactin was applied below the 3rd node with short length of the 4th internode (Exp. 5: initial length of the 4th internode on Feb. 01 – 27 mm: Table 1), the stimulatory effect of morphactin was significant but the increase of growth was very low.

The effects of NPA and TIBA on the growth of 4th internode in all these experiments were very small, independently from place of the treatment.

Endogenous levels of auxin in tulip stems after the treatment of auxin polar transport inhibitors

Identification of IAA in the extract of tulip stem segments was made by GC-MS analysis. The prominent fragment ions of m/z 135 and 194 (d_5-IAA methyl ester), and m/z 130 and 189 (d_0-IAA methyl ester) were observed at the retention time of 7.18 min and 7.21 min, respectively, indicating that the retention time of d_5-IAA methyl ester is just only short compared to that of d_0-IAA one. From these results, IAA was substantially identified in the acidic acid diethyl ether fractions of tulip stem segments. To estimate endogenous levels of IAA in tulip stem segments, GC-SIM analyses were introduced. Two prominent peaks of m/z 194 (d_5-IAA methyl ester) and 189 (d_0-IAA methyl ester) were used to determine endogenous levels of IAA in tulip stem segments. As shown in Fig. 3, endogenous levels of IAA rapidly decreased after removal flower buds in tulips. Endogenous levels of IAA in the 4th internode in the treatment of morphactin applied at the 3rd node of tulips was highest among the samples after the treatment of auxin polar transport inhibitors (Fig. 3), suggesting strongly that polar transport of endogenous IAA produced in pistils (Xu et al., 2008) was almost completely inhibited by the application of morphactin compared to that of NPA and TIBA.

Saniewski and Węgrzynowicz-Lesiak (1993) have reported that a continuous supply of auxin is necessary for tulip stem growth. Xu et al. (2008) examined diffusible IAA, from various parts of tulips, during rapid elongation of the flower stalk using GC-MS. The amount of diffusible IAA from different organs followed the order of that of the internodes>flower organs>leaves at that time. The 4th internode exported higher quantity of IAA than did the flower during most of the rapid elongation period, except of the beginning of the rapid elongation stage and on one day after flowering. They also suggested, that the top (the 4th internode) was probably more important

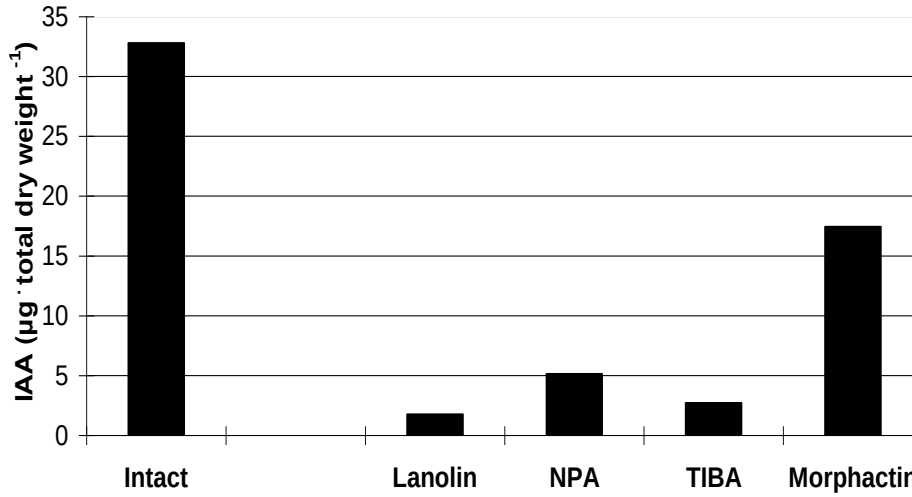

Figure 3. Endogenous levels of IAA in the 4th internodes in Intact (fixed on April 17) and decapitated tulips treated with Lanolin only (control), NPA, TIBA and Morphactin (fixed on April 23). Determination procedures of endogenous levels of IAA are described in MATERIAL AND METHODS

than the flower, in the production of IAA accountable for rapid elongation of the flower stalk. Xu et al. (2008) also described that it is possible that before the beginning of the rapid elongation stage, the flower exudes higher levels of IAA than does each internode, acting as the major source of auxins.

Indole-3-acetic acid moves out of plant cells thorough an efflux carrier apparatus that is sensitive to synthetic inhibitors of auxin transport, NPA, TIBA and morphactin, but the mechanism by which auxin transport inhibitors control auxin efflux is not fully known (Muday and DeLong, 2001). It is no doubt fact that NPA inhibits IAA transport by specific binding to so-called NPA receptor, thereby blocking the carrier-mediated efflux of IAA; IAA does not compete with NPA for binding sites and NPA-binding site is important for auxin transport (Lomax et al., 1995; Ruegger et al., 1997; Muday et al., 1993). The binding site of morphactin has been controversial. It has been also shown that morphactin binds to NPA receptor, suggesting that morphactin inhibits polar IAA transport by the same mechanism as NPA (Sussman and Goldsmith, 1981; Thomson and Leopold, 1974). Some authors suggest that NPA and TIBA have different binding sites and TIBA competes with IAA for the same binding sites (Thomson et al., 1973; Michalke et al., 1992). Moreover, it is suggested that TIBA is transported in a polar

basipetal manner when NPA is not (Thomson et al., 1973). Same explanation will be possible to morphactin. It is believed that morphactin is translocated in plants basipetally as well as acropetally through both sieve tubes and xylem elements (Neumann et al., 1977; Sundberg et al., 1994), suggesting that morphactin is faster to move in plant tissues than TIBA and NPA as described above. Further intensive studies to clarify the mode of movement of auxin polar transport inhibitors will be required in near future.

It is possible to indicate that NPA and TIBA are much less in action as inhibitors of auxin polar transport than morphactin in tulip stem. It should be mentioned that stimulated growth of tulip stems induced by the application of morphactin is not a cause but a consequence of accumulated endogenous levels of IAA after morphactin treatment. Some other properties of morphactin might have direct influence on the growth of the 4th internode as well. Further investigation relevance to this point will also be necessary.

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REFERENCES

Lomax T.L., Muday G.K., Rubery P.H. 1995. Auxin transport. In: Plant Hormones, ed. By P.J. Davis, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 509-530.

Michalke W., Katekar G.F., Geissler A.E. 1992. Phytotropin-binding sites and auxin transport in *Cucurbita pepo*: evidence for two recognition sites. PLANTA 181: 254-260.

Muday G.K., Brunn S.A., Haworth P., Subramanian M. 1993. Evidence for a single naphthylphthalamic acid binding site on the zucchini plasma membrane. PLANT PHYSIOL. 103: 449-456.

Muday G.K., DeLong A. 2001. Polar auxin transport: controlling where and how much. TRENDS PLANT SCI. 6: 535-542.

Neumann P.M., Doss R.P., Sachs R.M. 1977. A new laboratory method used for investigating the uptake, translocation and metabolism of bark banded morphactin by trees. PHYSIOL. PLANT. 39: 248-251.

Ranwala A.P., Miller W.B. 2008. Gibberellin-mediated changes in carbohydrate metabolism during flower stalk elongation in tulips. PLANT GROWTH REGUL. 55: 241-248.

Ruegger M., Dewey E., Hobbie L., Brown D., Bernasconi P., Turner J., Muday G., Estelle M. 1997. Reduced naphthylphthalamic acid binding in the tir3 mutant of Arabidopsis is associated with a reduction in polar transport and diverse morphological defects. PLANT CELL 9: 745-757.

Saniewski M., de Munk W.J. 1981. Hormonal control of shoot elongation in tulips. SCI. HORT., 5: 363-372.

Saniewski M., Góraj J., Węgrzynowicz-Lesiak E., Okubo H., Miyamoto K., Ueda J. 2010. Different growth of excised and intact fourth internode after removal of the flower bud in growing tulips: focus on auxin action. J. FRUIT ORNAM. PLANT RES. 18: 297-308.

Saniewski M., Węgrzynowicz-Lesiak E. 1993. Continuous supply of auxin is necessary for tulip stem growth. J. FRUIT ORNAM. PLANT RES. 1: 59-66.

Sundberg B., Tuominen H., Little C.H.A. 1994. Effects of the indole-3-acetic

acid (IAA) transport inhibitors N-1-naphtylphtalamic acid and morphactin on endogenous IAA dynamics in relation to compression wood formation in 1-year-old *Pinus sylvestris* (L.) shoots. PLANT PHYSIOL. 106: 469-476.

Sussman M.R., Goldsmith M.H.M. 1981. The action of specific inhibitors of auxin transport on uptake of auxin and binding of N-naphthylphthalamic acid to a membrane site in maize coleoptiles. PLANTA 152: 13-18.

Thomson K.S., Hertel R., Muller S., Tavares J.E. 1973. 1-N-Naphthylphthalamic acid and 2,3,5-triiodobenzoic acid. *In-vitro* binding to particulate cell fractions and action on auxin transport in corn coleoptiles. PLANTA 109: 337-352.

Thomson K.S., Leopold A.C., 1974. *In vitro* binding of morphactins and 1-N-naphthylphthalamic acid in corn coleoptiles and their effect on auxin transport. PLANTA 115: 259-270.

Xu R.-Y., Niimi Y., Kojami K., 2008. Changes in diffusible idole-3-acetic acid from various parts of tulip plant during rapid elongation of the flower stalk. PLANT GROWTH REGUL. 54: 81-88.

Ueda J., Komaki M.K., Okada K., Shimura Y., 1991. Identification and quantitative distribution of indole-3-acetic acid in *Brassica junco* Czern. J. PLANT PHYSIOL. 137: 628-630.

Yokota T., Murofushi N., Takahashi N. 1980. Extraction, Purification, and Identification. In: MacMillan J. (ed.), Hormonal Regulation of Development I, Springer-Verlag, Berlin, Encyc. PLANT PHYSIOL. 9: 113-201.

MORFAKTYNA INDUKUJE WZROST CZWARTEGO MIĘDZYWĘŹLA W DEKAPITOWANYCH TULIPANACH: ZWIĄZEK Z ENDOGENNYM POZIOMEM KWASU INDOLILO-3-OCTOWEGO

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S T R E S Z C Z E N I E

Rola inhibitorów polarnego transportu auksyny, takich jak kwas 2,3,5-trójjodobenzoesowy (TIBA), kwas naftyloftalamowy (NPA) i morfaktyna (kwas metylo 2-chloro-9-karboksylowy) była intensywnie badana na wzrost łodygi tulipana. Traktowanie morfaktyną łodygi tulipana powyżej 3. węzła, po odcięciu pąka kwiatowego, powodowało stymulację wzrostu 4. międzywęźla. Z drugiej strony, inhibitory

polarnego transportu, NPA i TIBA, przy takim samym traktowaniu, wywierały mały wpływ na wzrost 4. międzywęźla. Endogenny poziom IAA w 4. międzywęźlu, po traktowaniu inhibitorami polarnego transportu, morfaktyną, NPA i TIBA, określano stosując metodę GC-MS i GC-SIM ze znakowanym IAA (kwas indolo-2,4,5,6,7-d_5-3-octowy) jako standard wewnętrzny. Stwierdzono silną akumulację IAA w 4. międzywęźlu łodygi po traktowaniu morfaktyną. Traktowanie NPA i TIBA powodowało tylko niewielką akumulację IAA w 4. międzywęźlu. Otrzymane wyniki wskazują, że stymulacja wzrostu 4. międzywęźla pod wpływem morfaktyny jest konsekwencją akumulacji IAA w tym międzywęźlu. Możliwy sposób działania inhibitorów polarnego transportu auksyny we wzroście łodygi tulipana jest w pracy dyskutowany.

Słowa kluczowe: IAA znakowane deuterem**,** endogenny poziom IAA, wzrost, międzywęźla, łodyga, morfaktyna, IAA, NPA, TIBA, tulipan