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Alteration of Hormone Levels in Transgenic Tobacco Plants Overexpressing the Rice Homeobox Gene *OSH1*

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The rice (*Oryza sativa* L.) homeobox gene *OSH1* causes morphological alterations when ectopically expressed in transgenic rice, *Arabidopsis thaliana*, and tobacco (*Nicotiana tabacum* L.) and is therefore believed to function as a morphological regulator gene. To determine the relationship between *OSH1* expression and morphological alterations, we analyzed the changes in hormone levels in transgenic tobacco plants exhibiting abnormal morphology. Levels of the plant hormones indole-3-acetic acid, abscisic acid, gibberellin (GA), and cytokinin (zeatin and *trans*-zeatin [Z]) were measured in leaves of *OSH1*-transformed and wild-type tobacco. Altered plant morphology was found to correlate with changes in hormone levels. The more severe the alteration in phenotype of transgenic tobacco, the greater were the changes in endogenous hormone levels. Overall, GA_1 and GA_4 levels decreased and abscisic acid levels increased compared with wild-type plants. Moreover, in the transformants, Z (active form of cytokinin) levels were higher and the ratio of Z to Z riboside (inactive form) also increased. When GA_3 was supplied to the shoot apex of transformants, internode extension was restored and normal leaf morphology was also partially restored. However, such GA_3-treated plants still exhibited some morphological abnormalities compared with wild-type plants. Based on these data, we propose the hypothesis that *OSH1* affects plant hormone metabolism either directly or indirectly and thereby causes changes in plant development.

The molecular mechanisms underlying organ morphogenesis from undifferentiated cells represent one of the most important biological questions. Genes involved in eukaryotic development were first isolated from *Drosophila* (Garber et al., 1983) and later from several other animal species. The products of these genes share a unique and homologous structure, the homeobox (Gehring, 1987).

In animals cellular differentiation occurs only in the early stages of development, whereas in higher plants undifferentiated cells are maintained as meristems throughout the life of the plant and successively give rise to leaves and floral organs. Recently, the possibility that homeobox genes also play a part in the development and morphogenesis of higher plants has been suggested.

The maize (*Zea mays* L.) gene *KN1* (*knotted-1*) was the first plant gene shown to encode a homeodomain-containing protein (Hake et al., 1989). Ectopic expression of *KN1* in maize or tobacco (*Nicotiana tabacum* L.) causes altered morphology in the transformed plants (Smith et al., 1992; Sinha et al., 1993). We have also observed that ectopic expression of the rice (*Oryza sativa* L.) homeobox gene *OSH1* causes altered morphology in rice, Arabidopsis (Matsuoka et al., 1993, 1995), and tobacco (Kano-Murakami et al., 1993). For example, *OSH1*-transformed tobacco plants exhibit abnormally shaped leaves, flowers, and loss of apical dominance. These observations suggest that the *OSH1* gene product may regulate the expression of genes related to morphogenesis in plants.

Tobacco plants overexpressing *OSH1* exhibit a variety of specific morphological abnormalities. These include wrinkled, slender, or tiny leaves, dwarfing, and pale-colored flowers with dissected margins. The fact that *OSH1* overexpression causes pleiotropic morphological alterations in transgenic plants indicates that the activities of plant hormones may also be changed in vivo.

The relationship between plant hormones and development has been the subject of considerable discussion, and it is now widely accepted that plant hormones regulate growth and development of plants by controlling the expression of genes involved in these processes. Phenotypic modifications have been described in transgenic plants overexpressing the *Agrobacterium tumefaciens* T-DNA genes *tms* or *ipt*, which are involved in auxin and cytokinin biosynthesis, respectively (Gaudin et al., 1994). However, little is known about the genes that regulate the biosynthesis or metabolism of plant hormones. In this study, we analyzed the levels of several hormones in *OSH1*-transformed tobacco plants in an effort to determine whether *OSH1*-mediated morphological changes may involve alterations in plant hormone levels.

MATERIALS AND METHODS

Plant Material

The preparation of *OSH1*-transformed tobacco (*Nicotiana tabacum* cv Samsun NN) plants was as described by Kano-

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Abbreviations: AcOH, acetic acid; BuOH, 1-butanol; EtOAc, ethyl acetate; MeOH, methanol; NOS, nopaline synthase; Z, *trans*-zeatin; ZR, *trans*-zeatin riboside.

Murakami et al. (1993). T_2 seedlings of NOS-*OSH1* and 35S-*OSH1* transformants were grown under greenhouse conditions at 25°C. Tobacco leaves were frozen in liquid N_2 immediately after harvest and stored at −80°C until analysis.

Extraction and Purification of Plant Hormones

Plant material (10–20 g) was homogenized in 80% aqueous acetone (4:1, v/v) supplemented with 10 mg L^{-1} butylated hydroxytoluene, and then [4,5,6,7,8,9-^{13}C]IAA (Cambridge Isotope Laboratories, Andover, MA) and [6,6,6-^{2}H]ABA were added as internal standards. The homogenate was filtered and solid residue was further extracted twice with the same solvent. Extracts were combined and mixed and then divided into two equal samples. One sample was used for IAA, ABA, and GA analyses and the remaining sample was used for cytokinin analysis.

For analysis of IAA, ABA, and GAs, the extract was concentrated to an aqueous residue (30 mL) in vacuo, adjusted to pH 2.5 with 6 N H_2SO_4, and extracted with EtOAc (3 × 10 mL). The aqueous residue was discarded, and the EtOAc extracts were combined and extracted with saturated $NaHCO_3$ (3 × 10 mL). The EtOAc residue was discarded and the $NaHCO_3$ extracts were combined, adjusted to pH 2.5 with 6 N H_2SO_4, and extracted with EtOAc (3 × 10 mL). The aqueous residue was discarded, and the EtOAc extracts were combined and passed through a column of anhydrous Na_2SO_4 to remove water. The resulting eluate was evaporated to dryness, dissolved in 80% aqueous MeOH (1 mL), and loaded onto a Sep-Pak C_{18} cartridge (Millipore). The cartridge was eluted with 80% aqueous MeOH (2 × 5 mL) and the eluate was evaporated to dryness. The residue was dissolved in MeOH (1 mL), transferred to a small vial, and dried under a stream of N_2. The residue was subjected to HPLC on a column of PEGASIL ODS (6 mm i.d. x 150 mm, Senshu Kagaku, Tokyo, Japan) and eluted with 0.5% AcOH in 5% aqueous acetonitrile (solvent A) and 0.5% AcOH in 80% aqueous acetonitrile (solvent B) at 40°C as follows: 0 to 5 min, isocratic elution with solvent A; 5 to 50 min, linear gradient of 0 to 33% solvent B; and 50 to 70 min, linear gradient of 33 to 100% solvent B. The flow rate of the solvent was 1.5 mL min^{-1} and fractions were collected every 1 min. The retention times of IAA, ABA, GA_1, and GA_4 were 34.4, 42.5, 29 to 30, and 58 to 59 min, respectively. Fractions were evaporated to dryness and methylated using diazomethane, and the fraction that contained IAA was further trimethylsilylated in undiluted *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide for 20 min at 70°C. Fractions containing IAA or ABA were subjected to GC-MS. Fractions containing GA_1 or GA_4 (retention time ± 3 min) were assayed by ELISA.

For cytokinin analysis, the extract was concentrated to an aqueous residue (20 mL) in vacuo, 5 mL of AcOH:H_2O (1:2, v/v) was added, and the resulting solution was extracted with CH_2Cl_2 (2 × 10 mL). The aqueous residue was saved and the CH_2Cl_2 extracts were combined and extracted with AcOH:H_2O (1:2, v/v, 3 × 10 mL). The CH_2Cl_2 extracts were discarded and the AcOH:H_2O (1:2, v/v) extracts were combined and added to the former aqueous residue. This aqueous solution was adjusted to pH 9.0 with ammonia solution, and extracted with H_2O-saturated BuOH (4 × 25 mL). The aqueous residue was discarded and the BuOH extracts were combined. H_2O was added to the BuOH extract and the mixture was evaporated to an aqueous residue, which was loaded onto a Sep-Pak C_{18} cartridge. The cartridge was washed with 5 mL of H_2O and then eluted with 80% MeOH (20 mL). The eluate was evaporated to dryness and purified by HPLC under the conditions described above. The retention times of Z and ZR were 8.4 and 17.7 min, respectively. Each fraction that included a retention time of ± 3 min was assayed by ELISA.

GC-MS

GC-MS was performed with a mass spectrometer (model JMS DX303, Jeol) gas chromatograph. A bonded-phase capillary column (OV-1, 0.53 mm i.d. × 15 m, Gasukuro Kogyo, Tokyo, Japan) was used in a temperature-gradient mode for GC-selected ion monitoring analysis of IAA and ABA.

ELISA Procedure

ELISA was performed according to a modification of the procedure of Atzorn and Weiler (1983). Dynatech 96 immunoplates were coated first with 100 μL of 50 μg mL^{-1} goat anti-rabbit γ-globulin (in 50 mM $NaHCO_3$ and 0.9% NaCl, pH 7.8) and then with 100 μL of antibodies raised in rabbits against GA_1 methyl ester (5 μg mL^{-1}) or with 100 μL of antibodies raised in rabbits against ZR (2 μg mL^{-1}). To each antibody-coated well was added 50 μL of TBS buffer (50 mM Tris-HCl, 1 mM $MgCl_2$, and 0.01% NaN_3, pH 9.6) plus 25 μL of a standard in 5% aqueous MeOH or sample solution, and samples were allowed to incubate for 1 h at 4°C. Following this incubation, 25 μL of diluted tracer was added and samples were incubated for a further 3 h. The enzyme activity bound to the immunoplate-adsorbed antibodies was then determined using *p*-nitrophenyl phosphate as a substrate. The cross-reactivity of antibodies raised against GA_1 methyl ester to GA_4 methyl ester was 36%, and that of antibodies raised against ZR to Z was 73% under the analytical conditions described above.

Test for GA Sensitivity

The sensitivity of transgenic tobacco to GA was tested by applying 10 μL of a 3 mM aqueous solution of GA_3 (Kyowa Hakkokogyo, Tokyo, Japan) to the shoot apex. Treatments were started 40 d after sowing and repeated every 4th d until flowering. Eleven to 13 plants from each phenotype were analyzed.

RESULTS

Transgenic tobacco plants containing *OSH1* under the control of the NOS or the 35S promoter were divided into three categories that ranged from mild to severe phenotype (Kano-Murakami et al., 1993). In this study transformants containing the NOS-*OSH1* construct were used as mild-

phenotype plants and transformants containing the 35S-*OSH1* construct were used as severe-phenotype plants. Because the changes in plant hormone levels could be due to differences in developmental stage, mature leaves of the plants that we used in plant hormone analysis were harvested at the stage of vegetative growth.

Levels of Immunoreactive GA in *OSH1*-Transformed Tobacco Plants

Many GA derivatives are found in plants, and the activities of these GAs in plant growth and development differ. Because GA_1 and GA_4 are known to be highly active in causing GA-specific responses of plants (Graebe, 1987), we chose these GAs for analysis in this work. Levels of GA_1 and GA_4 were determined by ELISA, because this method was the most convenient for quantifying large numbers of samples.

The expression of *OSH1* was accompanied by a dramatic decrease of immunoreactive GA in fractions containing GA_1 in leaves of transgenic tobacco plants (Fig. 1a; Table I). The immunoreactive GA content in the GA_1 fraction of transformants exhibiting a severe or mild phenotype decreased to 0.4 and 3.5% of that seen in wild-type tobacco plants, respectively. In contrast, immunoreactive GA levels in GA_4-containing fractions were less severely affected, being reduced by approximately one-half in plants with either phenotype.

IAA and ABA Levels in *OSH1*-Transformed Tobacco Plants

Analysis of IAA and ABA was performed by GC-MS using stable isotope-labeled internal standards. This technique is believed to be the most quantitative method currently available for analysis of these hormones.

The IAA content of transgenic tobacco exhibiting a mild phenotype decreased to 21% of that seen in wild-type plants (Fig. 1b; Table I). However, the IAA content of severe-phenotype plants was almost the same as that of wild-type tobacco. This could be due to the presence of numerous abnormal shoots on leaves of severe-phenotype plants that may act as a source of IAA.

Although the role of ABA in plant development is not clearly defined, this hormone is involved in many physiological responses, including stomatal control, dormancy, and adaptation to stress (Zeevaart, 1988). The ABA contents of transgenic tobacco plants with the mild or severe phenotype were approximately 7 and 10 times higher than that of wild-type plants, respectively (Fig. 1b; Table I).

Cytokinin Levels in *OSH1*-Transformed Tobacco Plants

Cytokinins were first characterized as compounds that promote cell division and are now known to evoke a diversity of responses in plants. Cytokinin derivatives have a wide range of activity. In these derivatives ribosides are an important translocation form, and conversion of cytokinin ribosides to bases is necessary for activity because the latter may be the active form (Letham and Palni, 1983). The cytokinin contents of tobacco plants were separately analyzed as the active form, Z, and its inactive form, ZR, by ELISA after HPLC fractionation. The severity of morphological alteration of *OSH1*-transformed tobacco plants correlated with an increase in Z levels (Fig. 1c; Table I). In addition, this increase in Z level was accompanied by an increase in the ratio of Z to ZR.

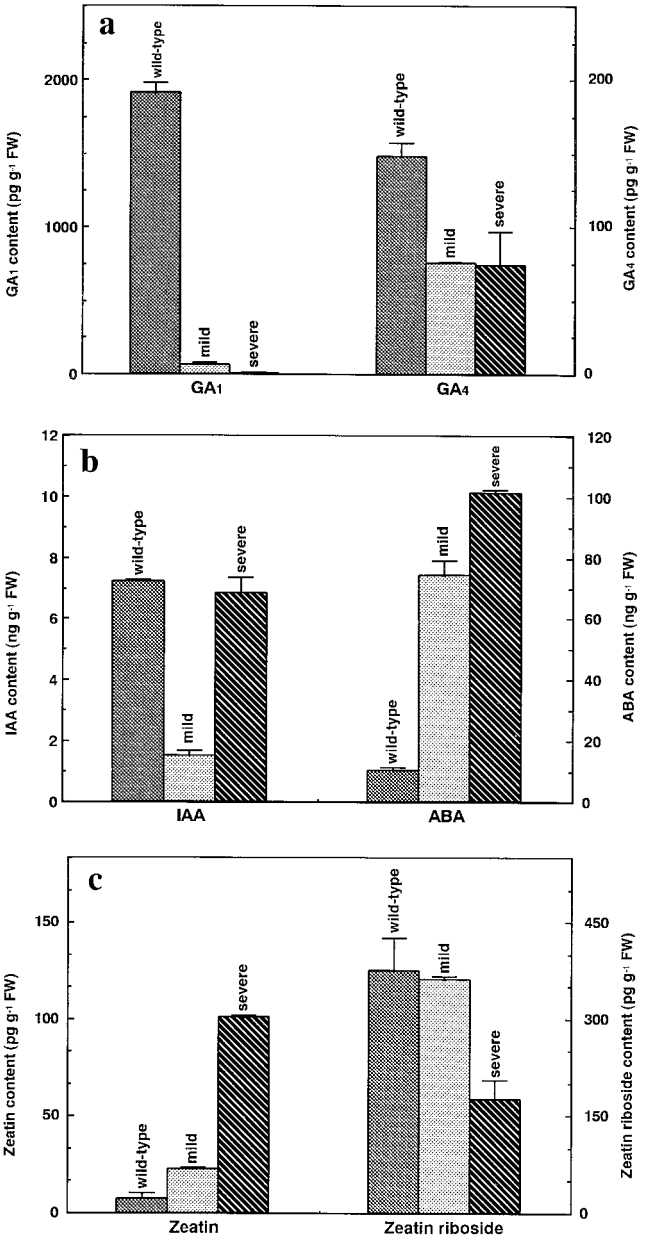

Figure 1. Hormonal contents in transgenic tobacco. a, GA_1 and GA_4; b, IAA and ABA; and c, Z and ZR. Each column represents the mean ± SE of two to four replicates of independently harvested plant material. FW, Fresh weight.

Partial Correction of Abnormal Morphology by Treatment with GA

If the morphological alterations in *OSH1*-transformed tobacco are attributable to the decrease in active GA con-

Table I. *Hormonal contents in transgenic tobacco*

Each value represents the mean ± SE of two to four replications of independently harvested plant material.

Treatment	Phenotype		
	Wild type	Mild	Severe
GA_1 (pg g^{-1} fresh wt)	1910 ± 70	66.7 ± 14	7.79 ± 4.5
GA_4 (pg g^{-1} fresh wt)	149 ± 9	75.9 ± 0.7	74.0 ± 23
IAA (ng g^{-1} fresh wt)	7.23 ± 0.07	1.53 ± 0.15	6.83 ± 0.53
ABA (ng g^{-1} fresh wt)	10.3 ± 1.1	74.3 ± 4.9	101 ± 1
Z (pg g^{-1} fresh wt)	7.51 ± 2.9	22.5 ± 1.2	101 ± 1
ZR (pg g^{-1} fresh wt)	375 ± 52	362 ± 4	177 ± 29

tent, then the application of exogenous GA might be expected to correct the phenotype of transgenic tobacco plants to some extent. In transgenic tobacco exhibiting a mild phenotype, treatment with GA_3 reduced the severity of abnormal leaf morphology (Fig. 2). In severe-phenotype transformants, treatment with GA_3 also corrected the loss of apical dominance and the severity of leaf abnormalities. Furthermore, treatment with GA_3 corrected the abnormal stem elongation in severe-phenotype plants. The stem length of GA_3-treated transformants was almost the same as that of wild-type plants (Fig. 3). Finally, in mild-phenotype transformants, flower buds formed 1 week earlier than in wild-type plants. GA_3 application restored the time of flower bud formation in these plants to that of wild-type plants (Fig. 3). In severe-phenotype transformants, flower buds formed 16 weeks later than in wild-type plants. GA_3 application to these plants accelerated flower bud formation by 8 weeks.

DISCUSSION

The expression of *OSH1* resulted in altered morphology in transgenic tobacco plants and was accompanied by significant changes in hormone levels. Plant hormones are well known to have diverse physiological activities. However, the factors regulating plant hormone metabolism have not yet been elucidated. Schmülling et al. (1993) demonstrated that overexpression of a single *rol* gene from *Agrobacterium rhizogenes* resulted in altered morphology

Figure 2. Phenotypic compensation of transgenic tobacco by GA_3 treatment. Wild-type (a) and severe-phenotype (b) transgenic tobacco plants with (left) or without (right) GA_3 treatment. The plants were photographed after those on the left had been treated with GA_3 for 36 d. For GA_3 treatment, 10 μL of 3 mM GA_3 was applied to the shoot apex every 4th d, starting 40 d after sowing. c, Phenotypic alteration of leaves of mild-phenotype transformants. d, Phenotypic alteration of leaves of severe-phenotype transformants. a, Bar = 1 m; b, bar = 50 cm; and c and d, bar = 5 cm.

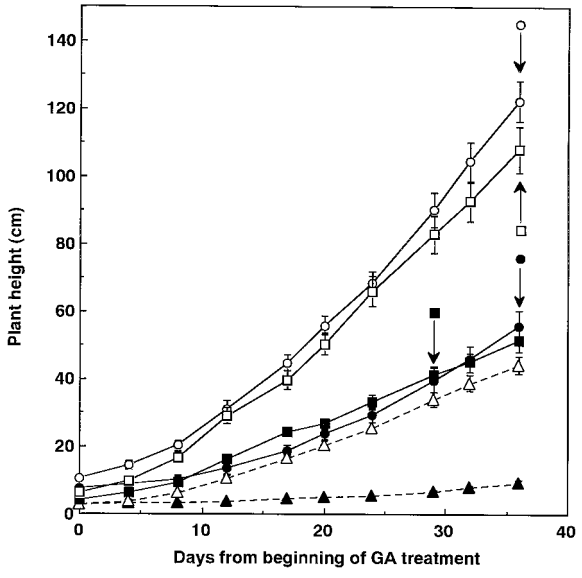

Figure 3. Effect of GA_3 treatment on plant height. Arrows indicate the emergence of flower buds. Each symbol represents the mean ± SE of 11 to 13 replicates. ●, Wild type; ○, wild type plus GA; ▲, severe phenotype; △, severe phenotype plus GA; ■, mild phenotype; and □, mild phenotype plus GA.

and changes of hormonal contents and sensitivity in transgenic tobacco. They suggested that the phenotypic abnormalities of *rol*-transformed plants were due to complex effects of the altered hormone metabolism and sensitivity. From the fact that it contains a putative DNA-binding domain, the *OSH1* gene product is thought to act as a transcription factor (Matsuoka et al., 1993). We propose that *OSH1* controls the morphology of tobacco by affecting plant hormone metabolism and/or signal transduction.

We have analyzed the levels of GAs, IAA, ABA, and cytokinins in *OSH1*-transformed tobacco plants that exhibited morphological abnormalities. We have shown that the GA_1 level becomes lower as the morphological aberrations of the transformants become more severe. However, ABA and Z levels become higher in morphologically changed transformants. Transgenic tobacco plants exhibiting a mild phenotype have wrinkled leaves that are thicker, shorter, and more disc-shaped than wild-type leaves. Severe-phenotype plants are dwarfed and their axillary buds develop into vegetative stems, whereas these buds are dormant in wild-type tobacco. The leaves of these plants are not wrinkled but are tiny, and occasionally abnormal shoots arise from them. It is well established that auxin and cytokinin are capable of controlling apical dominance. Under conditions of a high ratio of cytokinin to auxin, plants usually lose their apical dominance and axillary buds develop into vegetative shoots (Tamas, 1987). Based on these results, the loss of apical dominance in severe-phenotype *OSH1* transformants could be caused by the higher level of cytokinin, which is shown in Figure 1. In addition, treatment of severe-phenotype *OSH1* transformants with GA_3 restored apical dominance (Fig. 2b); therefore, the decrease of GA_1 level may also be the cause of loss of apical dominance.

In contrast, decreased levels of GA_1 seem to be responsible for the observed dwarfism of severe-phenotype plants. GAs are well known to promote stem elongation in a variety of plants, and GA_1 is the predominant active GA in tobacco plants, being present at a much higher level than GA_4 (Table I). The fact that treatment of severe-phenotype transformants with GA_3 restored stem elongation to near that of wild-type plants indicates that dwarfism in these transformants can be at least partly attributed to the decrease in GA_1 content.

Auxin is also thought to play a critical role in stem elongation, as demonstrated by the phenotypes of several auxin-resistant mutants of Arabidopsis. The *dwf*, *axr1*, and *axr2* mutants all exhibit shortened internodes (Mirza and Maher, 1980; Lincoln et al., 1990; Wilson et al., 1990). However, the IAA levels in transgenic tobacco plants did not correlate with the degree of morphological abnormality. In the plant kingdom the primary site of IAA biosynthesis is thought to be in meristems and young, developing organs. We believe that the abnormal shoots present on leaves of severe-phenotype plants could serve as a source of IAA. Therefore, unexpected higher levels of auxin might be detected in leaves of severe-phenotype plants. The production of abnormal shoots on leaves has also been observed in tobacco plants that overexpress a cytokinin biosynthetic gene for isopentenyltransferase (Estruch et al., 1991; Li et al., 1992). In severe-phenotype tobacco plants, the ratio of Z to ZR was strikingly higher than those of mild-phenotype and wild-type plants. The abnormal shoots seen on the leaves of severe-phenotype plants may therefore be due to the increased activity of cytokinins. This imbalance of the auxin to cytokinin ratio may affect the morphology of severe-phenotype plants.

GA was analyzed further in an effort to determine whether the morphological changes in severe-phenotype transformants were due to an inhibition of GA biosynthesis or sensitivity. Exogenous GA_3 treatment of transgenic tobacco plants reduced the severity of altered leaf phenotype, dwarfism, and loss of apical dominance and partially restored their flowering time. In addition, RNA-blot analysis revealed that GA_3 treatment did not affect the amount of *OSH1* transcript present in transgenic tobacco plants (data not shown). These observations suggest that the *OSH1* gene product probably does not affect the GA-signaling pathway but instead likely alters the biosynthesis or catabolism of active GA.

OSH1 causes pleiotropic morphological changes and, therefore, may have diverse effects on the metabolism of plant hormones, which are known to have physiological activity in plant growth and development. The signal transduction pathway from the *OSH1* gene product has not yet been elucidated, and that from plant hormones also remains obscure. The data presented here suggest the possibility that *OSH1* affects plant hormone metabolism either directly or indirectly and thereby causes changes in plant development. The mechanism(s) by which *OSH1* affects hormonal activity remains to be elucidated.

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