Characterization of Rice Anthranilate Synthase α-Subunit Genes *OASA1* and *OASA2*. Tryptophan Accumulation in Transgenic Rice Expressing a Feedback-Insensitive Mutant of OASA1[1]

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Anthranilate synthase (AS) is a key enzyme in the synthesis of tryptophan (Trp), indole-3-acetic acid, and indole alkaloids. Two genes, *OASA1* and *OASA2*, encoding AS α-subunits were isolated from a monocotyledonous plant, rice (*Oryza sativa* cv Nipponbare), and were characterized. A phylogenetic tree of AS α-subunits from various species revealed a close evolutionary relationship among OASA1 and Arabidopsis ASA2, *Ruta graveolens* ASα2, and tobacco ASA2, whereas OASA2, Arabidopsis ASA1, and *R. graveolens* ASα1 were more distantly related. *OASA1* is expressed in all tissues tested, but the amount of its mRNA was greater in panicles than in leaves and roots. The abundance of *OASA2* transcripts is similar among tissues and greater than that of *OASA1* transcripts; furthermore, *OASA2* expression was induced by a chitin heptamer, a potent elicitor, suggesting that OASA2 participates in secondary metabolism. Expression of wild-type *OASA1* or *OASA2* transgenes did not affect the Trp content of rice calli or plants. However, transformed calli and plants expressing a mutated *OASA1* gene, *OASA1*(D323N), that encodes a protein in which aspartate-323 is replaced with asparagine manifested up to 180- and 35-fold increases, respectively, in Trp accumulation. These transgenic calli and plants were resistant to 300 μM 5-methyl-Trp, and AS activity of the calli showed a markedly reduced sensitivity to Trp. These results show that OASA1 is important in the regulation of free Trp concentration, and that mutation of *OASA1* to render the encoded protein insensitive to feedback inhibition results in accumulation of Trp at high levels. The *OASA1*(D323N) transgene may prove useful for the generation of crops with an increased Trp content.

In higher plants, the Trp biosynthetic pathway provides the essential amino acid Trp, as well as produces various secondary metabolites, including the hormone indole-3-acetic acid and plant defense compounds (Radwanski and Last, 1995). However, the mechanisms by which these two functions are regulated remain unknown.

Anthranilate is a common precursor of all these compounds and anthranilate synthase (AS) catalyzes the synthesis of anthranilate from chorismate and Gln. Purified plant AS proteins are heteromers that consist of two subunits termed α and β (Poulsen et al., 1993; Bohlmann et al., 1995; Romero and Roberts, 1996). Although the synthesis of anthranilate from chorismate and Gln requires α- and β-subunits of AS, the α-subunit is able to catalyze the synthesis of anthranilate from chorismate with ammonia as an amino donor in the presence of high concentrations of ammonium (Niyogi and Fink, 1992; Niyogi et al., 1993; Bohlmann et al., 1995). Specific amino acids in the α-subunit of bacterial AS are important for feedback inhibition of the enzyme by Trp (Matsui et al., 1987; Caligiuri and Bauerle, 1991).

Complementary DNAs encoding two nonallelic AS α-subunit genes have been isolated from Arabidopsis (*ASA1* and *ASA2*) and *Ruta graveolens* (*ASα1* and *ASα2*; Niyogi and Fink, 1992; Bohlmann et al., 1995). Tobacco appears to possess more than two different AS α-subunit genes, but only one cDNA, termed *ASA2*, has been cloned (Song et al., 1998). The enzymes encoded by *R. graveolens ASα1* (Bohlmann et al., 1996) and tobacco *ASA2* (Song et al., 1998) are Trp insensitive, with the former being implicated in alkaloid biosynthesis (Bohlmann et al., 1995, 1996). In a similar manner, the enzyme encoded by one of the two AS genes of *Pseudomonas aeruginosa* is feedback insensitive and is implicated in the production of pyocyanin, the characteristic phenazine pigment of this bacterium (Essar et al., 1990).

In addition to differences in feedback inhibition of the encoded proteins, the two different AS α-subunit genes appear to be subject to distinct mechanisms of transcriptional regulation. Accumulation of Arabidopsis *ASA1* mRNA is induced by wounding and

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bacterial infection, whereas *ASA2* mRNA is present in small amounts and does not respond to these stimuli. In a similar manner, the abundance of *R. graveolens* ASα1 transcripts increases by a factor of approximately 100 in response to treatment with a fungal elicitor, whereas the number of ASα2 transcripts is not affected and the gene is expressed constitutively. The marked increases in the steady-state amounts of *ASA1* and *ASα1* mRNAs induced by wounding or pathogen-related stimuli suggest a role for these genes in the production of defense-associated substances (Niyogi and Fink, 1992; Bohlmann et al., 1996).

Although ASA1 is thought to participate in the synthesis of secondary metabolites, *ASA1* mutants of Arabidopsis exhibit increased concentrations of soluble Trp as a result of altered feedback regulation of AS activity (Kreps et al., 1996; Li and Last, 1996). The tobacco ASA2 enzyme is thought to be related to *R. graveolens* ASα1 because of its insensitivity to Trp. Overexpression of tobacco *ASA2* in the forage legume *Astragalus sinicus* resulted in an increase of the concentration of free Trp (Cho et al., 2000). These observations indicate that despite their predicted role in alkaloid synthesis, feedback-insensitive AS enzymes mediate Trp accumulation. It might, therefore, be expected that the AS enzymes not linked to alkaloid synthesis, such as those encoded by *ASα2* and Arabidopsis *ASA2*, may possess an even greater potential for inducing Trp accumulation.

Given that cereal crops such as rice, maize, wheat, and oats exhibit a relatively low content of Trp in seed storage proteins, an increase in the abundance of this amino acid would improve their nutritional value. We now describe the identification and characterization of two AS α-subunit genes (*OASA1* and *OASA2*) from rice (*Oryza sativa* cv Nipponbare), a monocotyledonous plant, and we demonstrate an important role for *OASA1* in Trp synthesis. We generated feedback-insensitive mutants of OASA1 whose expression resulted in the accumulation of free Trp at markedly increased concentrations in calli and plant leaves.

RESULTS

Isolation of Two AS α-Subunit Genes from Rice

To isolate rice AS genes we screened a rice cDNA library with a probe based on Arabidopsis *ASA1*. Of 900,000 clones screened, 11 positive clones were isolated, sequenced, and categorized. The positive clones corresponded to two distinct cDNA sequences showing similarity to Arabidopsis *ASA1* or *ASA2*. The clone with the largest insert, number 5-1, contained a complete open reading frame (ORF), which we designated *OASA1* because it showed greater sequence similarity to Arabidopsis *ASA1* than it did to *ASA2* from this plant. The other cDNA represented in the positive clones was designated *OASA2*;

however, the nucleotide sequence corresponding to the NH$_2$-terminal region of the encoded protein was missing from the *OASA2* clones.

To determine the nucleotide sequence of the missing 5' region of the *OASA2* ORF we partially sequenced a genomic DNA clone. Digestion of genomic DNA with *Eco*RI yielded fragments of approximately 6.2 and 6.4 kb, which hybridized on Southern-blot analysis with the Arabidopsis *ASA1* probe used for cDNA library screening. Size-fractionated *Eco*RI-generated DNA fragments of approximately 6.0 to 6.5 kb were cloned into a λZAPII vector; of 270,000 clones screened with the *ASA1* cDNA probe, 23 positive clones were isolated and analyzed. Sequence analysis revealed that a clone corresponding to the 6.4-kb hybridizing fragment was derived from *OASA1*, whereas another clone corresponding to the 6.2-kb hybridizing fragment appeared to be derived from *OASA2*. Both clones contained the entire respective ORFs.

To clone the 5' portion of the *OASA2* ORF we designed primers to amplify the region containing the putative AUG start codon by reverse transcription and PCR. The resulting cDNA fragments were cloned into the pCRII vector (Invitrogen, Carlsbad, CA) and their sequences were confirmed to be consistent with the genomic DNA sequence, with the exception of the spliced introns. This 5' region of the ORF was ligated to the cDNA fragment that lacked this region to generate the full-length *OASA2* ORF.

Southern-blot analysis of *Eco*RI-digested rice genomic DNA with the Arabidopsis *ASA1* cDNA probe yielded no signals other than the 6.2- and 6.4-kb fragments under low-stringency hybridization conditions. We, therefore, concluded that the rice genome possesses only two homologous AS α-subunit genes, *OASA1* and *OASA2*, which encode proteins of 577 and 606 amino acids, respectively.

The amino acid sequences deduced from the *OASA1* and *OASA2* cDNAs were aligned with those of AS α-subunits of dicotyledonous plants and microorganisms (Fig. 1). The NH$_2$-terminal regions of OASA1 and OASA2 exhibited almost no sequence similarity to each other. Likewise, the NH$_2$-terminal sequences of other plant homologs are highly variable and are predicted to contain plastid-localization signals (Zhao and Last, 1995). These regions of OASA1 and OASA2 are relatively rich in specific amino acid residues, including Ser, Thr, Lys, and Arg, and are relatively deficient in acidic amino acids. These characteristics thus suggest that the NH$_2$-termini of OASA1 and OASA2 also act as signal peptides for plastid localization (Gavel and von Heijne, 1990). The mature 521- and 523-residue OASA1 and OASA2 proteins (assuming cleavage to be determined by an Asn residue at positions 62 and 89, respectively) show extensive sequence homology to each other (72% identity) and to other AS α-subunits (71%–74%); the sequence identity shared with *Saccha-*
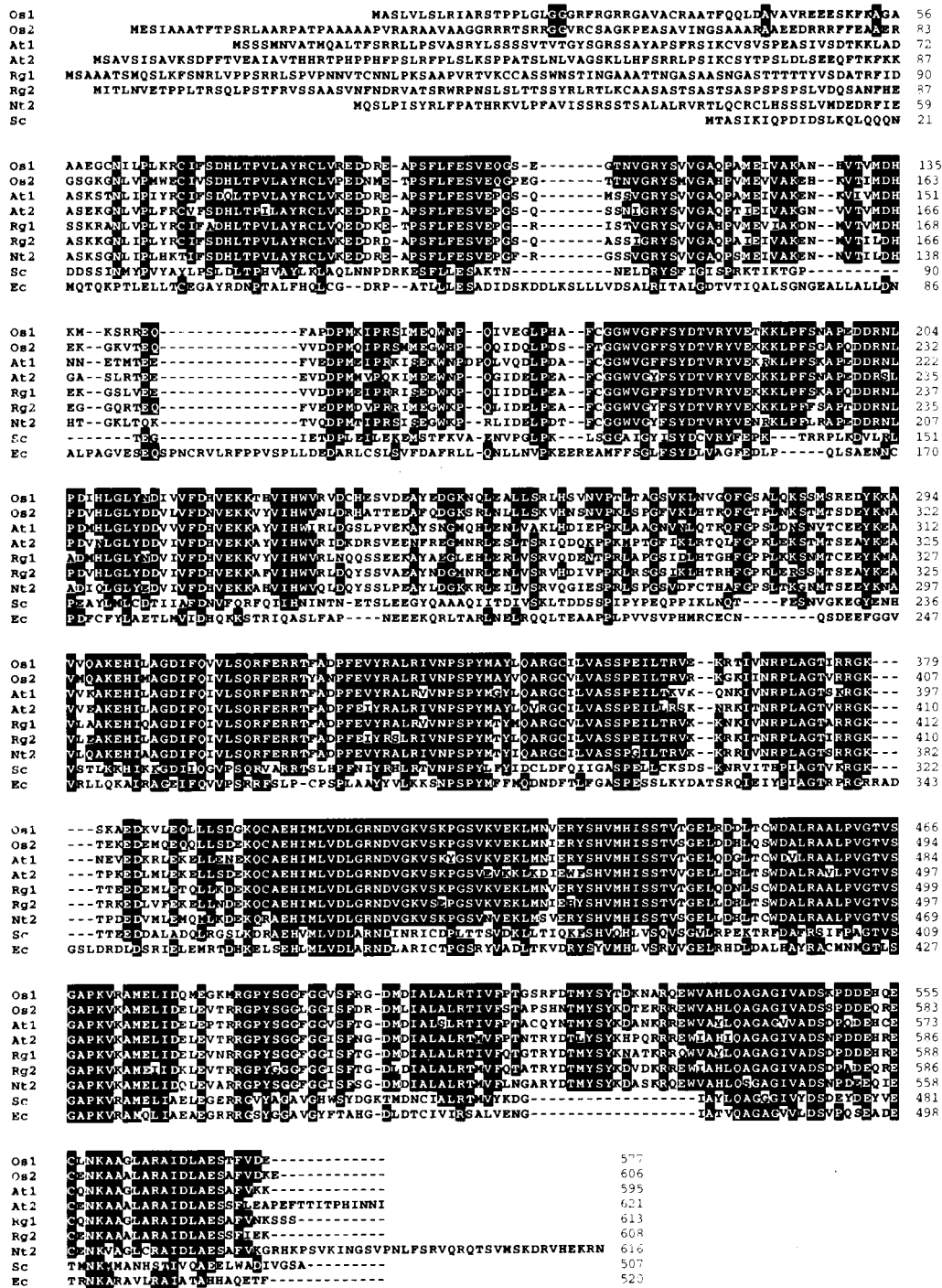
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Os1                                                MASLVLSLRIARSTPPLGLGGGRFRGRRGAVACRAATFQQLDAVAVREEESKFKAGA  56
Os2                             MESIAAATFTPSRLAARPATPAAAAAPVRARAAVAAGGRRRTSRRGCVRCSAGKPEASAVINGSAAARAAEEDRRRFFEAAR  83
At1        MSSSMNVATMQALTFSRRLLPSVASRYLSSSSVTVTGYSGRSSAYAPSFRSIKCVSDVDTKKLAD  72
At2        MSAVSISAVKSDFFTVEAIAVTHHRTPHPPHFPSLRFPLSLKSPPATSLNLVAGSKLLHFSRRLPSIKCSYTPSLDLSEEQFTKFKK  87
Rg1    MSAAATSMQSLKFSNRLVPPSRRLSPVPNNVTCNNLPKSAAPVRTVKCCASSWNSTINGAAATTNGASAASNGASTTTTTYVSDATRFID  90
Rg2      MITLNVETPPLTRSQLPSTFRVSSAASVNFNDRVATSRWRPNSLSLTTSSYRLRTLKCAASASTSASTSASPSPSPSLVDQSANFHE  87
Nt2                       MQSLPISYRLFPATHRKVLPFAVISSRSSTSALALRVRTLQCRCLHSSSLVMDEDRFIE  59
Sc                                               MTASIKIQPDIDSLKQLQQQN  21

Os1    AAEGCNILPLKRCIFSDHLTPVLAYRCLVREDDRE-APSFLFESVEQGS-E-------GTNVGRYSVVGAQPAMEIVAKAN--HVTVMDH  135
Os2    GSGKGNLVPMWECIVSDHLTPVLAYRCLVPEDNME-TPSFLFESVEQGPEG-------TTNVGRYSMVGAQPVMEVVAKEH--KVTIMDH  163
At1    ASKSTNLIPIYRCIFSDOLTPVLAYRCLVKEDDRE-APSFLFESVEPGS-Q-------MSSVGRYSVVGAQPAMEIVAKEN--KVIVMDH  151
At2    ASEKGNLVPLFRGVFSDHLTPILAYRCLVKEDDRD-APSFLFESVEPGS-Q-------SSNIGRYSVVGAQPTIEIVAKGN--VVTVMDH  166
Rg1    SSKRANLVPLYRCIFADHLTPVLAYRCLVQEDDKE-TPSFLFESVEPG--R-------ISTVGRYSVVGAPVMEVIAKON--MVTVMDH  168
Rg2    ASKKGNLIPLYRCIFSDOLTPVLAYRCLVKEDDRD-APSFLFESVEPGS-Q-------ASSIGRYSVVGAOPAIEIVAKEN--KVTILDH  166
Nt2    ASKSGNLIPLHKTIFSDHLTPVLAYRCLVKEDDRE-APSFLFESVEPGF-R-------GSSVGRYSVVGAQPSMEIVAKEN--NVTILDH  138
Sc     DDSSINMYPVYAYLPSLDLTPHVAYLKPAQLNNPDRKESPLLESAKTN----------NELDRYSFIGISPRKTIKTGP----------  90
Ec     MQTQKPTLELLTCEGAYRDNPTALFHQLCG--DRP--ATLLLESADIDSKDDLKSLLLVDSALRITALGDTVTIQALSGNGEALLALLDN  86

Os1    KM--KSRREQ---------------FAPDPMKIPRSIMEQWNP--QIVECLPHA--PCGGWVGFFSYDTVRYVEKKLPFSNAPLDDRNL  204
Os2    EK--GKVTEQ---------------VVDDPMQIPRSMMEGWRP--QQIDQLPDS--PTGGWVGFFSYDTVRYVEKKLPFSCAPQDDRNL  232
At1    NN--ETMTPE---------------FVEDPMEIPRKISDKWNPDPOLVQDLPDA--PCGGWVGFFSYDTVRYVERKLPFSNAPLDDRSL  222
At2    GA--SLRTPEA--------------EVDDPMKVPQKIMEEWNP--QIDELPEA--PCGGWVGYFSYDTVRYVEKKLPFSNAPDDRSL  235
Rg1    EK--GSLVEE---------------VVDDPMEIPRRISEDWKP--QIIDDLPEA--PCGGWVGFFSYDTVRYVEKKLPFSCAPQDDRNL  237
Rg2    EG--GQRTEQ---------------FVEDPMDVPRRIMEGWKP--QLIDELPEA--PCGGWVGYFSYDTVRYVEKKLPFFSAPTDDRNL  235
Nt2    HT--GKLTQK---------------TVQDPMTIPRSISEGWKP--RLIDELPDT--PCGGWVGVFSYDTVRYVENRKLPFLRAPLDDRNL  207
Sc     --------TEG---------------IETDPLEILEKEMSTFKVA-ENVPGLPK---LSGGAIGYISYDCVRYFEPK---TRRPLKDVLRL  151
Ec     ALPAGVESEQSPNCRVLRFPPVSPLLDEDARLCSLSVFDARFLL-QNLLNVPKEEREAMFFSGLFSYDLVAGFEDLP-----QLSAENNC  170

Os1    PDIHLGLYNDIVVFDHVEKKTEVIHWVRVDCHESVDEAYEDGKNQLEALLSRLHSVNVPTDTAGSVKLNVCQFCSALQKSSMSREDYKKA  294
Os2    PDVHLGLYDDVLVFDNVEKKVYVIHWVNLDRHATTEDAFQDGKSRLNLLLSKVHNSNVPKISPGFVKLHTRQFCFPLNKSTLTSDEYKTA  322
At1    PDLHLGLYDDVVFDHVEKKAYVIHWIRLDGSLPVEKAYSNGMQHDENVAKLHDIEPPKLAAGNVKLDVPPRLRSCSVNVTCEEYKSA  312
At2    PDVNLGLYDDIVFDHVEKKAYVIHWVRIDKDRSVEENFREGMNRLESLTSRIQDQKPPKMPTGFIKTRTQLFCFKLEKSTLTSEAYKEA  325
Rg1    ADIHLGLYNDIVFDHVEKKVYVIHWVRLNQQSSEEKAYAEGLEHLERLVSRVODENTPRLAPGSIDDETGHFCFPPLNKSNMTCEEYKMA  327
Rg2    PDVHLGLYDDVIVFDHVEKKAFVIHWVRLDQYSSVALAYNDGMNRLENLVSRVHDIVPPRLSGSIKLHTRHFGPKLERSNMTSEAYKKA  325
Nt2    ADIQLGLYEDVIVFDHVEKKAEVIHWVQLDQYSSLPEAYLDGKKRLEILVSRVQGIESPRLSPGSVDFCTHAFGPSLTKGNMTSEEYKNA  297
Sc     PEAYLMCDTIIADENVFQRFQIENINTN-ETSLEEGYQAAAQIITDIVSKLTDDSSPIPYPEQPPIKLNQT----FESNVGKEGYENH  236
Ec     PDFCFYLAETLWVIDHQKKSTRIQASLFAP----NEEEKQRLTARLNELRQQLTEAAPPLPVVSVPHMRCECN--------QSDEEFGGV  247

Os1    VVQAKEHILAGDIFQVVLSQRFERRTFADPFEVYRALRIVNPSPYMAYLQARGCILVASSPEILTRVC--KRTIVNRPLAGTIRRGK---  379
Os2    VMQAKEHIMAGDIFQIVLSQRFERRTYANPFEVYRALRIVNPSPYMAYVQARGCVLVASSPEILTRVR--MQKIINRPLAGTVRRGK---  407
At1    VVKAKEHILAGDIFQIVLSQRFERRTFADPFEVYRALRVVNPSPYMGYLQARGCILVASSPEILTKVK--QNKIVNRPLAGTSKRGK---  397
At2    VVEAKEHILAGDIFQIVLSQRFERRTFADPFEIYRALRIVNPSPYMAYVRGCILVASSPEILURSK--NRKIINRPLAGTWRGK---  410
Rg1    VLAAKEHIQAGDIFQIVLSQRFERRTFADPFEVYRALRVVNPSPYMIYMOARGCILVASSPEILTRVK--KNKIVNRPLAGTARRGK---  412
Rg2    VLEAKEHILAGDIFQIVLSQRFERRTFADPFEIYRSLRIVNPSPYMTYLOARGCILVASSPEILTRVK--KRKIVNRPLAGTIRRGK---  410
Nt2    VLQAKEHIAAGDIFQIVLSQRFERRTFADPFEVYRALRIVNPSPYMYIQARGCILVASSPGILTRVK--KRRIVNRPLAGTSRRGK---  382
Sc     VSTLKRHIKFGDIHQGVPSQRVARRTSLHPFNIIYRHLRTVNPSPYLFYIDCLDFQIIGASPELLCKSDS-KNRVITHGHAGTVKRGK---  322
Ec     VRLLQKAIRAGEIFQVVPSRRFSLP-CPSPLAAYYVLKKSNPSPYMFFMQDNDFTLFGASPESSLKYDATSRQIEIYPIAGTRPRCRRAD  343

Os1    ---SKAEDKVLEQLLLSDGKQCAEHIMLVDLGRNDVGKVSKPGSVKEKLMNVERYSHVMHISSTVGELRDDLTCWDALRAALPVGTVS  466
Os2    ---TEKEDEMCEQQLLSDEKQCAEHIMLVDLGRNDVGKVSKPGSVKEKLMNIERYSHVMHISSTVSGELDDHLQSWDALRAALPVGTVS  494
At1    ---NEVEDKRLEKELLENEKQCAEHIMLVDLGRNDVGKVSKYGSVKEKLMNIERYSHVMHISSTVTGELQDGLTCWDALRAALPVGTVS  484
At2    ---TPKEDLMLEKELLSDEKQCAEHIMLVDLGRNDVGKVSKPGSVLVTKLKDIEWLSHVMHISSTVGELLDRLTSWDALRAVLPVGTVS  497
Rg1    ---TTEEDEMLETOLLKDEKQCAEHIMLVDLGRNDVGKVSKPGSVKEKLMNVERYSHVMHISSTVTGELQDNLSCWDALRAALPVGTVS  499
Rg2    ---TRKEDLVFEKELLNDEKQCAEHIMLVDLGRNDVGKVSPGSVKEKLMNIEHYSHVMHISSTVTGELLDRLTSWDALRAALPVGTVS  497
Nt2    ---TPDEDVMLEMQHLKDEKQRAEHIMLVDLGRNDVGKVSKPGSVAVEKLMSVERYSHVMHISSTVSGELLDRLTCWDALRAALPVGTVS  469
Sc     ---TTPEDDALADQLRGSLKDRAEHVMLVDLIRNDINRICDPLTTSVDKILTIQKLSHVQHLVSQVSGVLRPEKTRFDATRSIFPAGTVS  409
Ec     GSLDRDLDSRIELEMRTDHKELSEHLMLVDLARNDLARICTPGSRYVADLTKVDRYSYVMHLVSRVVGELRHDLDALHAYRACMNMGTLS  427

Os1    GAPKVRAMELIDQMEGKMRGPYSGGFGGVSFRG-DMDIALALRTIVFPTGSRFDTMYSYTDKNARQEWVAHLQAGAGIVADSKPDDEHQE  555
Os2    GAPKVKAMELIDELEVTRRGPYSGGLGGISFDR-DMDIALALRTIVFSTAPSHNTMYSYKDTERRREWVAHLQAGAGIVADSSPDDEQRE  583
At1    GAPKVKAMELIDELEPTRRGPYSGGFGGVSFTG-DMDIALSLRTIVFPTACQYNTMYSYKDANKRREWVATLQAGAGVVADSPODEHGE  573
At2    GAPKVKAMELIDELEVTRRGPYSGGFGGVSFNG-DMDIALALRTIVFPTNTRYDTLYSYKHPQRRREWIAHLQAGAGIVADSNPDDEHRE  586
Rg1    GAPKVKAMELIDELEVNRRGPYSGGFGGISFTG-DMDIALALRTIVFQTGTRYDTMYSYKNATKRROWVAYLQAGAGIVADSPDDEHRE  588
Rg2    GAPKVKAMELIDKEVTRRGPYGGGFGGISFTG-DIDIALALRTIVFTNTRYDTMYSYKDVDKRREWIAHLOAGAGIVADSDPADEQRE  586
Nt2    GAPKVKAMELIDQLEVARRGPYSGGFGGISFSG-DMDIALALRTMVELNGARYDTMYSYKDASKROEWVAHLQSGAGIVADSNPDEQIE  558
Sc     GAPKVRAMELIAELLGERRCVYAGAVGHWSYDGKTMDNCIALRTMVYKDG----------------IAYLQAGGGIVYDSOEYDEYVE  481
Ec     GAPKVRAMQLIAEAEGRRRGSYGGAVGYFTAHG-DLDTCIVIRSALVENG----------------IATVQAGAGVVLDSVPQSEADE  498

Os1    CLNKAACLARAIDLAESTFVDE-------------  577
Os2    CENKAAALARAIDLAESAFVDKE------------  606
At1    CQNKAACLARAIDLAESAFVKK-------------  595
At2    CENKAAALARAIDLAESSFLEAPEFTTITPHINNI  621
Rg1    CQNKAACLARAIDLAESAFVNKSSS----------  613
Rg2    CENKAAALARAIDLAESSFIEK-------------  608
Nt2    CENKVAGLCRAIDLAESAFVKGRHKPSVKINGSVPNLFSRVQRQTSVMSKDRVHEKRN  616
Sc     TMNKMMANHSTIVQAEELWADIVGSA---------  507
Ec     TRNKARAVLRAIATAHHAQETF-------------  520
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Figure 1. Alignment of the predicted amino acid sequences of rice OASA1 and OASA2 with those of other AS α-subunits. The sequences shown are encoded by the following genes: Os1 and Os2, rice *OASA1* (GenBank accession no. AB022602), and *OASA2* (AB022603), respectively; At1 and At2, Arabidopsis *ASA1* (M92353) and *ASA2* (M92354), respectively; Rg1 and Rg2, *R. graveolens ASα1* (L34343) and *ASα2* (L34344), respectively; Nt2, tobacco *ASA2* (Song et al., 1998); Sc, *S. cerevisiae TRP2* (X68327); and Ec, *E. coli trpE* (V00368). Hyphens indicate gaps introduced to optimize alignment. Residues shared by OASA1 and OASA2 are shaded, and residue numbers are shown on the right.

romyces cerevisiae TRP2 and *Escherichia coli* TrpE was only 23% and 33%, respectively. There was no significant difference in sequence identity between AS α-subunits of dicotyledons and those of the monocotyledon rice. However, a phylogenetic tree based on deduced amino acid sequences revealed a close evolutionary relationship among Arabidopsis *ASA2*, *R. graveolens ASα2*, tobacco *ASA2*, and rice *OASA1*

(Fig. 2). In contrast, Arabidopsis *ASA1*, *R. graveolens* *ASα1*, and rice *OASA2* exhibited a level of evolutionary diversity similar to that of their microbial homologs.

The *OASA2* cDNA was expressed by transformation of the *E. coli* mutant strain B666 (Δ*trpE*), which is deficient in the endogenous AS α-subunit. The Trp requirement of B666 cells was reduced by transformation with the plasmid pTASA2F, which contains the full-length *OASA2* ORF. Complementation was apparent under the restricted conditions, with a requirement for 50 μM isopropyl-β-D-thiogalactopyranoside, 100 mM NH$_4$Cl, and 1% (w/v) casamino acids (data not shown).

Analysis of Gene Expression

We examined the expression of *OASA1* and *OASA2* in rice tissue with the use of RNA gel-blot analysis and digoxigenin-labeled antisense riboprobes prepared from the corresponding cDNAs; hybridization was performed under high-stringency conditions to prevent cross-hybridization of *OASA1* and *OASA2* mRNAs with the two probes. The sizes of the *OASA1* and *OASA2* transcripts, estimated from the sizes of ribosomal RNAs, were 2.1 and 2.4 kb, respectively. The two genes were shown to be expressed differentially in adult plants (Fig. 3A). *OASA1* mRNA was detected in immature panicles, but was ambiguous in leaves and root, when total RNA was used for analysis (Fig. 3). We confirmed the expression of *OASA1* in leaves and roots using enriched poly(A)$^+$ RNA samples (data not shown). The result of Figure 3

Figure 3. RNA gel-blot analysis of *OASA1* and *OASA2* expression. A, Tissue distribution of transcripts. Total RNA (10 μg) isolated from calli (lane 1), panicles (lane 2), roots (lane 3), and leaves (lane 4) of rice was subjected to RNA gel-blot analysis with digoxigenin-labeled riboprobes specific for *OASA1* (left) or *OASA2* (right) transcripts. The positions of the corresponding transcripts are indicated by arrows. B, Effects of an elicitor on transcript abundance. Suspension cultures of rice cells were incubated with chitin heptamer (1 μg mL^{-1}) for 0 min (lane 1), 30 min (lane 2), 120 min (lane 3), or 180 min (lane 4), after which total RNA was isolated and analyzed as in A.

using total RNA presented different expression of *OASA1* in tissues. In contrast, the amount of *OASA2* mRNA was similar in all tissues analyzed and was greater than that of *OASA1* mRNA. Both genes were expressed at a relatively high level in cultured cells.

The expression of Arabidopsis *ASA1* and *R. graveolens* *ASα1* is induced by an elicitor or by pathogen invasion (Niyogi and Fink, 1992; Bohlmann et al., 1995). To test the inducibility of the two rice AS α-subunit genes, we added *N*-acetylchitohepalose (1 μg mL^{-1}), a potent elicitor (Yamada et al., 1993), to suspension cultures of rice calli. The elicitor induced a time-dependent increase in the abundance of *OASA2* mRNA, but had no effect on that of *OASA1* mRNA (Fig. 3B); the maximal effect of chitin heptamer was apparent after approximately 2 h.

Generation of Transgenic Rice

Two types of mutated *OASA1* cDNAs were constructed by PCR-mediated site-directed mutagenesis (Fig. 4). One mutated cDNA, *OASA1*(D323N), was designed to encode a protein in which Asp-323 was replaced by Asn. This Asp residue is well conserved among plant AS α-subunits, with the only exception of OASA2, and the same mutation in *ASA1* was previously shown to render Arabidopsis resistant to 5-methyl-Trp (5MT; Kreps et al., 1996; Li and Last, 1996). The second mutated *OASA1* cDNA, designated *OASA1*(M340T), was designed to encode a protein in which Met-340 was replaced by Thr; the corresponding mutation has been identified in a mutant of *Salmonella typhimurium* (Caligiuri and Bauerle, 1991). Each mutated cDNA as well as wild-type
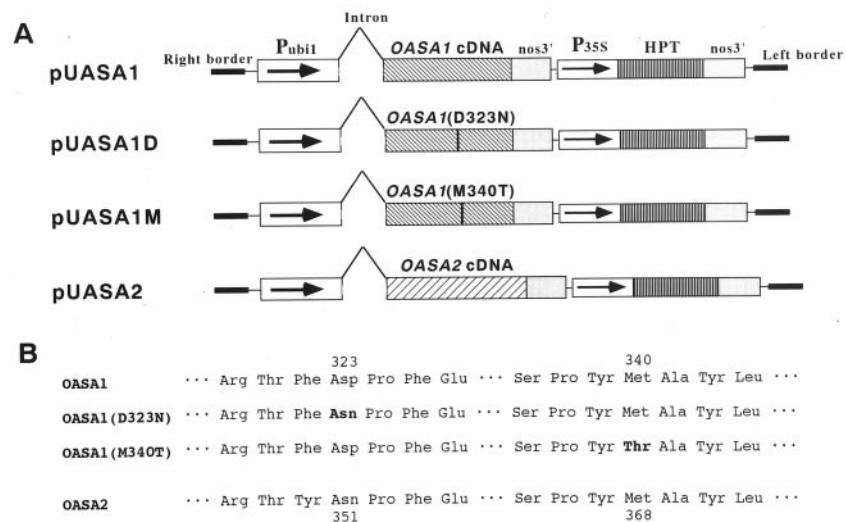

Figure 2. Phylogenetic relations among AS α-subunits. The amino acid sequences of OASA1 (residues 57–577), OASA2 (residues 84–606), Arabidopsis ASA1 (residues 73–595), Arabidopsis ASA2 (residues 88–621), ASα1 (residues 91–613), ASα2 (residues 88–609), tobacco (Nt) ASA2 (residues 60–618), TRP2 (residues 22–507), and TrpE (residues 1–520) were analyzed (Fig. 1). The phylogenetic tree was constructed from the evolutionary distance data derived by the neighbor-joining method (Saitou and Nei, 1987). The bootstrap procedure was sampled 1,000 times with replacement by CLUSTAL W (Thompson et al., 1994). The bar indicates the distance corresponding to 10 changes per 100 amino acid positions.

Figure 4. Construction of binary vectors for rice transformation. A, Structure of the T region of each vector. pUASA1 was constructed for expression of wild-type *OASA1* cDNA, pUASA1D for a mutated *OASA1* cDNA [*OASA1*(D323N)] encoding a protein in which Asp-323 is replaced by Asn, pUASA1M for a mutated *OASA1* cDNA [*OASA1*(M340T)] encoding a protein in which Met-340 is replaced by Thr, and pUASA2 for wild-type *OASA2* cDNA. A 2-kb maize *Ubi1* fragment including the promoter region (P_{Ubi1}) and 1-kb intron of the 5'-untranslated region was ligated to each rice cDNA at a point immediately downstream of the 3' side of the intron junction. Arrows indicate the direction of transcription. See "Materials and Methods" for further details. B, Alignment of the amino acid sequences surrounding the mutated residue (bold) of each transgene product. The mutated gene *OASA1*(D323N) and wild-type *OASA2* each encode Asn at the corresponding positions 323 and 351, respectively.

OASA1 and *OASA2* cDNAs were introduced individually into the T-vector using the maize *Ubi1* promoter for high-level expression in rice (Fig. 4). Transformed calli and regenerated plants were established by transfection with *Agrobacterium tumefaciens* EHA101 harboring each of the four cDNA constructs.

Many independent transformants and transgenic plants were generated. Expression of the introduced cDNAs was examined by RNA gel-blot analysis (Fig. 5). Almost all calli and regenerated plants analyzed exhibited a high level of expression of the transgenes; one exception was number D5 in which the abundance of *OASA1*(D323N) mRNA was low, although hygromycin resistance was maintained. Because of high-level expression of the transgenes, film-exposing time of Figure 5 was shorter than that of Figure 3. In Figure 5, this time was not enough for capturing endogenous *OASA1* and *OASA2* signals.

5MT Resistance of Transformed Calli and Progeny Seedlings

The various types of transformed calli were tested for their sensitivity to 100 and 300 μM 5MT (Fig. 6). Those expressing the *OASA1*(D323N) transgene exhibited marked resistance to 5MT. Calli that had been maintained on hygromycin-containing medium during subculture exhibited growth on medium containing 300 μM 5MT that was 30% to 70% of that apparent on control medium without 5MT or hygromycin (N6 medium [Chu et al., 1975] supplemented with 2,4-dichlorophenoxyacetic acid [2,4-D] and casamino

acid [2N6]; data not shown). Calli that had been maintained on medium without hygromycin showed the same amount of growth on medium containing 300 μM 5MT as they did on 2N6 medium (Fig. 6B). In contrast, untransformed calli (Fig. 6A) as well as calli expressing *OASA1* (Fig. 6C) or *OASA1*(M340T) (Fig. 6D) transgenes did not grow in the presence of 100 or 300 μM 5MT. Of 61 transformed callus lines expressing the *OASA2* transgene examined, 31 lines grew on medium containing 100 μM 5MT (Fig. 6, E and F; Table I); however, none of the calli was able to grow in the presence of 300 μM 5MT. Thus, only approximately 50% of the calli transformed with the *OASA2* transgene exhibited resistance to 5MT, and the level of resistance shown was moderate. Given that RNA gel-blot analysis showed that all *OASA2*-transformed calli examined expressed the transgene at a similar level (Fig. 5B), the difference in resistance did not reflect a difference in the level of transgene expression.

The inheritance of 5MT resistance in *OASA1*(D323N) transgenic rice was confirmed by a germination test. Growth of resistant seedlings in transgenic line number D1-1-5 was thus vigorous on medium containing 300 μM 5MT (Fig. 7). There was no difference in growth of seedlings on medium without 5MT between parental variety Nipponbare and line number D1-1-5. The progeny seeds of all tested transgenic plants showed segregation of the 5MT sensitivity trait. Calli derived from resistant progeny seeds also exhibited resistance to 5MT (data not shown). These observations indicated that only the *OASA1*(D323N)
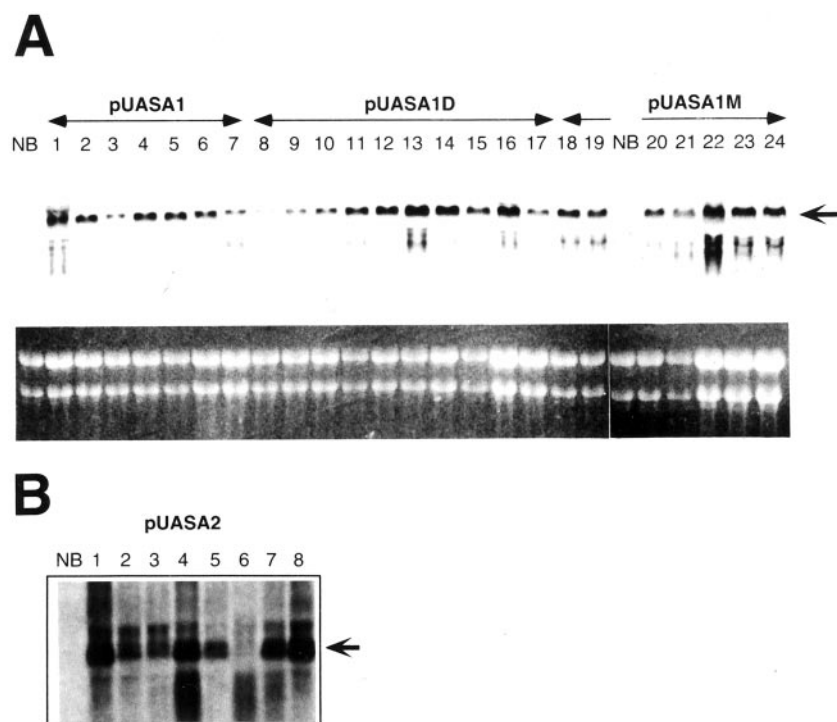
Figure 5. Expression of *OASA* transgenes in transformants. A, Expression of wild-type or mutated *OASA1* transgenes in transformed calli. Total RNA (10 μg) was subjected to RNA gel-blot analysis with a digoxigenin-labeled *OASA1* riboprobe (top); the ethidium bromide-stained agarose gel is also shown (bottom). NB, Untransformed Nipponbare callus; lanes 1 through 7, Transformants expressing the wild-type *OASA1* transgene (nos. W1, W2, W3, W4, W5, W9, and W10, respectively); lanes 8 through 17, Calli expressing the *OASA1*(D323N) transgene (nos. D5, D11, D13, D16, D17, D18, D19, D20, D25, and D26, respectively); lanes 18 through 24, Calli expressing the *OASA1*(M340T) transgene (nos. E1, E2, E3, E10, E12, E15, and E16, respectively). B, Expression of the *OASA2* transgene in the leaves of pUASA2 transformants. Lanes 1 through 8, Regenerated plants from callus line numbers 25-2, 22 -1, 47-3, 38-1, 49-1a, 46-1, 28-2, and 49-1b, respectively. Arrows in A and B indicate the transgene-derived transcripts.

transgene conferred 5MT resistance to transformed calli and progeny seedlings.

AS Activity of Transformants

The 5MT resistance of transgenic lines expressing *OASA1*(D323N) suggested that the D323N mutation affected feedback regulation of OASA1. To characterize the mutant enzyme we performed in vitro AS activity assays with calli expressing *OASA1*(D323N), *OASA1*, or *OASA2* transgenes. Extracts were prepared from calli growing on 2N6 plates and were subjected to gel filtration chromatography to remove endogenous Trp, chorismic acid, Gln, and other low M_r molecules. The AS activity of untransformed calli and of transformed calli with a high level of expression of the *OASA1* transgene was inhibited by 71% in the presence of 50 μM Trp, compared with the activity apparent in the absence of this amino acid (Fig. 8A). In contrast, the AS activity of calli expressing the *OASA1*(D323N) transgene was inhibited by only 26% in the presence of 50 μM Trp and by 47% in the presence of 100 μM Trp. The latter calli thus exhibited substantial Trp-insensitive AS activity. The AS-specific activity of these calli was, however, similar to

that of untransformed cells or *OASA1*-expressing lines.

Transformants expressing *OASA2* showed two patterns of AS activity (Fig. 8B). One line, number 25-2, exhibited a level of AS activity substantially less than that of untransformed calli, whereas others (nos. 14-1, 46-2, and 49-1) showed markedly increased levels of AS activity in the presence of low concentrations of Trp. The AS activity of these latter transgenic lines was, however, inhibited by Trp in an apparently normal manner; activity in the presence of 50 μM Trp was only approximately 5% of that in its absence.

Trp Content of Transformants

The feedback-insensitive AS activity as well as 5MT resistance of calli expressing the *OASA1*(D323N) transgene might be expected to result in Trp accumulation. We, therefore, next measured the soluble amino acids of calli after culture on 2N6 medium for 2 weeks. The soluble Trp content of transformed calli expressing *OASA1*(D323N) was increased by a factor of up to 180 compared with that of control calli (Fig. 9; Table II). The abundance of the other 19 amino acids or of
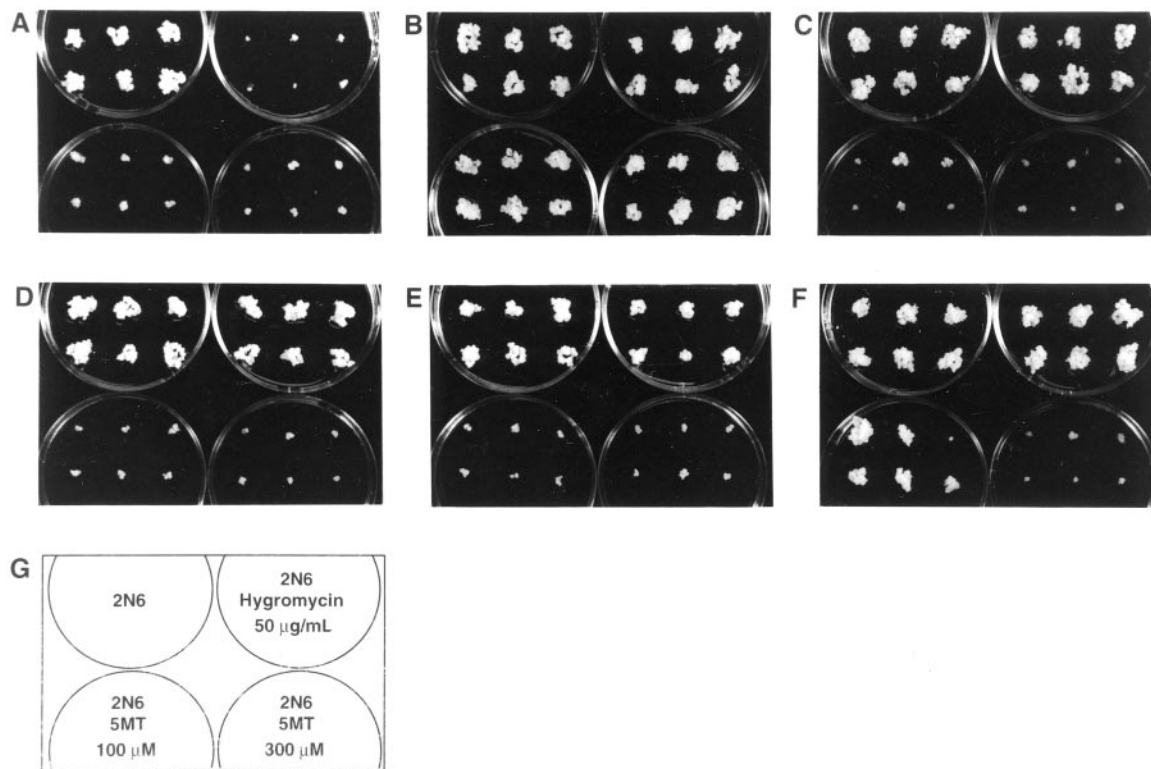
Figure 6. 5MT resistance of transformed rice calli. Calli of NB (A), transformant number D10-5 expressing *OASA1*(D323N) (B), transformant number W9 expressing *OASA1* (C), transformant number E1 expressing *OASA1*(M340T) (D), and transformants numbers 25-2 (sensitive) (E) and 49-1 (moderately resistant) (F) expressing *OASA2* were photographed after cultivation for 3 weeks. The medium conditions are shown in G.

anthranilate was not affected in the transformed calli. A high Trp content was also detected in the leaves of progeny seedlings from transgenic rice expressing *OASA1*(D323N) (Table III). Compared with untransformed plants, the soluble Trp content of the transgenic rice was increased by a factor of approximately 25 to 35, whereas transgenic plants expressing *OASA1*

(W) did not increase the level of Trp. All progeny seedlings analyzed were selected on medium containing 40 mg L^{-1} hygromycin and were grown under the same condition. These results revealed that supplement of hygromycin in medium did not cause increased level of soluble Trp in transgenic plants expressing *OASA1*(D323N).

Table I. *Sensitivity to 5MT and free Trp content of transformants expressing the OASA2 transgene*

Line	Growth Rate of Callus[a]	Sensitivity to 5MT[b]	Trp		Relative Trp Content	
			Calli	Leaves	Calli	Leaves
	%		nmol g^{-1} fresh wt			
Control						
NB	6.0	S	35	102	1	1
Transformed						
3-1	4.8	S	56	59	1.2	0.6
22-1	2.8	S	71	79	1.6	0.8
24-2[c]	98.0	MR	49		1.1	
25-2	7.9	S	48	48	1.1	0.5
28-2	64.1	MR	39	30	0.9	0.3
30-1[c]	47.2	MR	31		0.7	
38-1	45.5	MR	55	153	1.2	1.5
46-1	75.8	MR	56	160	1.2	1.6
47-3	43.9	MR	70	28	1.6	0.3
49-1	77.0	MR	77	50	1.7	0.5

[a] Growth rate represents the fresh wt of calli grown in the presence of 100 μM 5MT expressed as a percentage of that of calli grown in the absence of 5MT. [b] S, Sensitive; MR, moderately resistant. [c] Transformed calli of lines no. 24-2 and 30-1 did not regenerate plants.
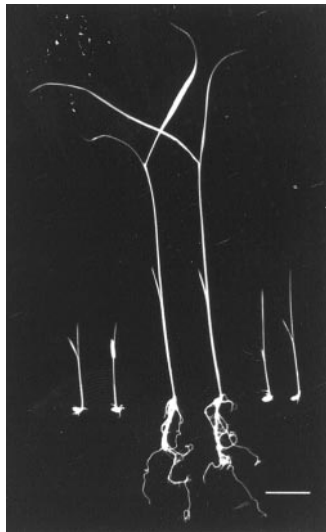
Figure 7. Segregation of 5MT resistance in progeny. Growth after 14 d of control cv Nipponbare seedlings (left two) and of segregants of transgenic line number D1-1-5 expressing *OASA1*(D323N) (right four) on medium containing 300 μM 5MT. The segregation ratio for resistance and sensitivity to 5MT in this line was 20:5. Bar = 2 cm.

The Trp content of 10 callus lines expressing the *OASA2* transgene was not increased, even though three of these lines (nos. 24-2, 46-1, and 49-1) showed resistance to 100 μM 5MT (Table I). Thus, the moderate resistance to 5MT induced by expression of the *OASA2* transgene did not result from altered feedback regulation of AS.

DISCUSSION

Two Rice Genes Encoding AS α-Subunits

We have isolated two rice genes, *OASA1* and *OASA2*, that encode AS α-subunits. The predicted proteins contain putative plastid transit peptides at their NH$_2$-termini. Although we have not demonstrated that the two proteins are localized in plastids, fusion proteins comprising the respective putative transit peptides linked to green fluorescent protein exhibited a plastid localization in transformed calli (Y. Tosawa and K. Wakasa, unpublished data). The mature rice AS α-subunits shared marked (72%) sequence identity; however, they occupied distinct positions in a phylogenetic tree. Whereas rice OASA1, Arabidopsis and tobacco ASA2, and *R. graveolens* ASα2 appeared to constitute a monophyletic group, rice OASA2, Arabidopsis ASA1, and *R. graveolens* ASα1 were more distantly related.

Expression and Predicted Function of *OASA1* and *OASA2*

The abundance of *OASA1* transcripts was greater in immature panicles than in leaves and roots. This distribution differs from that of Arabidopsis *ASA2* mRNA, which is not detectable in flowers (Niyogi

and Fink, 1992). The amount of *OASA2* mRNA was similar in all tissues analyzed and was greater than that of *OASA1* mRNA. Furthermore, transcription of *OASA2*, but not that of *OASA1*, appeared to be induced by chitin heptamer. This *N*-acetylchitohepalose has been shown to possess elicitor activity; it induces phytoalexin production (Yamada et al., 1993) and jasmonate synthesis (Nojiri et al., 1996) by rice cells in suspension culture. Maximal induction of *OASA2* expression was apparent after exposure of cells to the elicitor for 2 h, a time course similar to that for induction of chitinase and Phe-ammonia lyase genes (He et al., 1998; Nishizawa et al., 1999). These results thus suggest that *OASA2* is a defense-related gene induced by this elicitor.
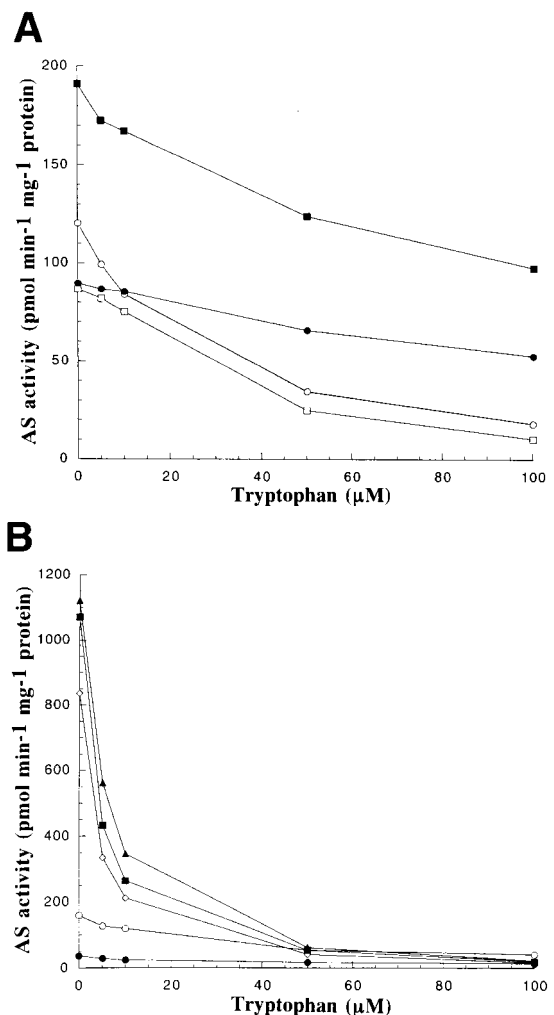

Figure 8. Relative AS activities of transformants expressing *OASA1*, *OASA1*(D323N), or *OASA2* transgenes. A, Untransformed calli (○) as well as calli of transformant number W2 expressing *OASA1* (□), and transformant numbers D17 (●) and D13 (■) expressing *OASA1*(D323N) were assayed for AS activity in the presence of various concentrations of Trp. B, Untransformed calli (○) and *OASA2*-expressing calli numbers 14-1 (▲), 25-2 (●), 46-2 (■), and 49-1 (◇) were assayed for AS activity. Data are expressed as picomoles of anthranilate produced per minute per milligram of protein, and are means of triplicates from a representative experiment.
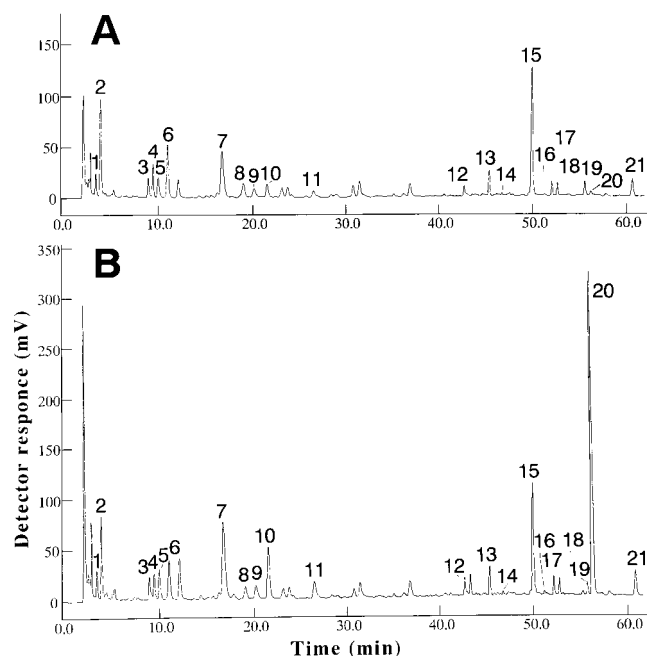
Figure 9. HPLC Profiles of soluble amino acids in untransformed calli and calli expressing *OASA1*(D323N). Untransformed calli (A) and *OASA1*(D323N)-expressing calli (B) of transformant number D13 were analyzed for soluble amino acid content by HPLC. Retention times for each amino acid and anthranilate were determined with standard samples. Peak numbers: 1, Asp; 2, Glu; 3, Ser; 4, Asn; 5, Gly; 6, Gln; 7, His; 8, Thr; 9, Ala; 10, Arg; 11, Pro; 12, Tyr; 13, Val; 14, Met; 15, Cys; 16, anthranilate; 17, Ile; 18, Leu; 19, Phe; 20, Trp; and 21, Lys.

Induction of gene expression by defense-related stimuli appears to be a characteristic that differentiates between the members of each pair of genes that encode AS α-subunits in higher plants. On the basis of this criterion, *OASA1*, Arabidopsis *ASA2*, and *R. graveolens ASα2* belong to the same family of constitutively expressed genes, and *OASA2*, Arabidopsis *ASA1*, and *R. graveolens ASα1* belong to the same family of inducible genes (Niyogi and Fink, 1992; Bohlmann et al., 1995, 1996). This classification

is thus consistent with the phylogenetic relations of these genes.

Transformed Rice Expressing Mutated *OASA1* Transgenes

The sequence element Leu-Leu-Glu-Ser (LLES) participates in feedback inhibition of microbial AS α-subunits by Trp (Matsui et al., 1987; Caligiuri and Bauerle, 1991; Graf et al., 1993). This element is highly conserved in AS α-subunits of plants, and the sequence surrounding the LLES element has been implicated in feedback inhibition of tobacco ASA2 (Song et al., 1998). However, substitution of Ser-115 or Ser-129 of Arabidopsis ASA1, which correspond to Ser-65 or Ser-76 of the $LLESX_{10}S$ element in yeast, by Arg did not result in an increase in the Trp content of or in resistance to 5MT in transformed rice calli (Y. Tozawa, H. Hasegawa, T. Terakawa, and K. Wakasa, unpublished data). In contrast, transformed rice calli expressing the *OASA1*(D323N) transgene were resistant to 300 μM 5MT and exhibited a marked (up to 180-fold) increase in the concentration of free Trp. The AS activity of these transformed calli was also inhibited by only approximately 50% in the presence of 100 μM Trp. We, therefore, concluded that Trp-insensitive AS activity was responsible for the high level of Trp accumulation in these transgenic lines. Inheritance of 5MT resistance was demonstrated by germination tests on medium containing 300 μM 5MT, and the leaves of resistant progeny also manifested a high Trp content. Transformed calli expressing *OASA1*(M340T) or wild-type *OASA1* transgenes were not resistant to 100 μM 5MT and did not exhibit an altered Trp content or AS sensitivity to Trp, suggesting that overproduction of the AS α-subunit itself was not responsible for the observed changes in feedback regulation or Trp synthesis in *OASA1*(D323N) transformants. The mutation of the Asp residue at position 323 of OASA1 to Asn thus appears to render the enzyme insensitive to Trp and 5MT.

Table II. *Free amino acid profiles of transformed calli*

W4 is a transformed callus line expressing the *OASA1* transgene. D13, D16, D17, D19, and D20 are independent transformed lines expressing the *OASA1* (D323N) transgene.

Line	Free Amino Acid Content																						Relative Trp Content	Trp
	Asp	Glu	Ser	Asn	Gly	Gln	His	Thr	Ala	Arg	Pro	Tyr	Val	Met	Cys	Anthranilate	Ile	Leu	Phe	Trp	Lys	Total		
	nmol g⁻¹ fresh wt																							*% of total amino acids*
NB	211	696	174	329	199	879	875	214	91	111	169	60	124	11	473	28	63	45	47	16	84	4,889	1	0.4
NB	166	607	133	196	168	522	651	225	123	137	83	51	142	15	453	16	88	64	74	16	79	4,009	1	0.4
W4	206	924	134	81	158	381	651	135	371	203	44	41	91	18	349	40	45	40	32	40	50	4,034	2.5	1.0
D 13	207	494	180	163	232	458	1,135	186	204	560	202	110	162	27	411	45	101	85	67	1,795	116	6,940	112	25.9
D 16	255	774	185	221	183	602	1,601	215	175	197	376	70	115	23	472	26	75	63	81	1,518	84	7,311	95	20.8
D 17	233	502	127	166	126	291	546	205	75	94	92	43	95	18	383	17	64	43	72	675	55	3,922	42	17.2
D 19	199	873	184	162	209	579	2,095	229	375	134	190	103	162	29	470	83	106	115	75	2,832	120	9,324	177	30.4
D 20	94	331	105	52	170	247	473	317	109	223	44	37	79	15	302	18	53	45	28	996	51	3,789	62	26.3

Table III. *Free Trp content of leaves from progeny plants*

Progeny seeds of transgenic lines were selected by growth for 2 weeks on germination medium containing hygromycin (40 mg L^{-1}) and control NB seed was grown on germination medium without hygromycin.

Progeny	Total Free Amino Acids	Trp	Relative Trp Content	Trp
	nmol g^{-1} fresh wt			*% of total free amino acids*
NB	6,128	153	1	2.5
W4-2	11,459	256	1.7	2.2
W4-7	10,012	235	1.5	2.3
D13-5	12,740	5,354	35	42.0
D13-6	11,941	3,653	24	30.6
D17-4	12,516	4,842	32	38.7
D17-5	8,718	3,842	25	44.1
D19-1	12,829	4,619	30	36.0

Transformed Rice Expressing an *OASA2* Transgene

Asp-323 of OASA1 is well conserved in other plant AS α-subunits, with the exception of OASA2. OASA2 contains an Asn at the corresponding position (residue 351), although the surrounding residues are virtually identical to those of OASA1. This observation suggested that overexpression of *OASA2* might result in Trp accumulation. However, transformed calli expressing an *OASA2* transgene did not exhibit Trp accumulation or altered sensitivity of AS activity to Trp. Thus, the Arg at position 351 of OASA2 is not sufficient to render the enzyme insensitive to feedback inhibition.

Only some of the transformed callus lines expressing the *OASA2* transgene were resistant to 100 μM 5MT and showed an increased level of AS activity in the absence of Trp. These two properties appeared related in that callus line numbers 14-1, 46-2, and 49-1 showed increased AS activity and were resistant to 100 μM 5MT, whereas line 25-2 exhibited a reduced level of AS activity and was sensitive to 5MT. The increased level of AS activity apparent in some of these lines in the absence of Trp might result from the increase in gene dosage for *OASA2*. The occasional and moderate resistance to 5MT manifested by calli overproducing OASA2 might also be attributable to the increase in the number of AS α-subunit molecules compensating for the presence of inhibitory molecules of Trp or 5MT at low concentrations.

Potential Utility of the *OASA1*(D323N) Transgene

Various feedback-insensitive AS α-subunits cause Trp accumulation in higher plants. The Arabidopsis mutants *trp-5* and *amt-1* manifest a free Trp content that is three and five times, respectively, that of wild-type plants; this increase in Trp accumulation is attributable to altered feedback regulation of the AS α-subunit encoded by *ASA1* (Kreps et al., 1996; Li and Last, 1996). Expression of the feedback-

insensitive tobacco ASA2 protein in the forage legume *A. sinicus* resulted in a 1.3- to 5.5-fold increase in the amount of free Trp in roots (Cho et al., 2000). Arabidopsis ASA1 and tobacco ASA2 are thought to contribute to the synthesis of secondary metabolites, given that *ASA1* transcription is inducible by wounding and the Trp insensitivity of tobacco ASA2 is similar to that of *R. graveolens* ASα1. In contrast, expression of the Trp-insensitive AS α-subunit encoded by *OASA1*(D323N) induced up to an approximately 35-fold increase in the amount of free Trp in rice leaves. The Trp content of calli and leaves expressing *OASA1*(D323N) reached a maximum of 2,832 and 12,829 nmol per gram of fresh weight, respectively, whereas the corresponding mean ± SE values for parental variety Nipponbare were 34.8 ± 19.0 and 70.2 ± 49.5 nmol per gram of fresh weight, respectively. Such high levels of Trp accumulation support the notion that *OASA1* is important in Trp synthesis, and suggest that AS α-subunits that contribute to Trp synthesis may be more effective in inducing Trp accumulation than are homologs whose primary function is the synthesis of secondary metabolites. Analysis of transgenic plants expressing feedback-insensitive forms of OASA2 is necessary to confirm this hypothesis.

Mutation of rice *OASA1* appears to affect only Trp accumulation, with no changes in the content of anthranilate or other amino acids having been detected, even though anthranilate is the immediate product of AS activity. The 5MT-resistant rice mutant MTR1 exhibits increased concentrations of not only Trp (87-fold increase in calli), but also Phe (8-fold increase in calli; Wakasa and Widholm, 1987). Chorismate mutase, which is subject to feedback control, is stimulated by Trp (Gilchrist and Kosuge, 1980); however, transformed rice cells expressing *OASA1*(D323N) contained the same amount of Phe as did control cells. Rice plants that manifest an increase only in Trp content may prove beneficial for breeding.

Cultured rice cells and rice plants that accumulated Trp grew normally. Although a tendency toward reduced seed fertility was apparent in the *OASA1*(D323N) transgenic plants compared with control Nipponbare plants, enough seeds to obtain progeny were produced by many of the transformed plants. Given that nutritional improvement is an important goal of gene manipulation in plants (Falco et al., 1995; Goto et al., 1999; Ye et al., 2000), the *OASA1*(D323N) transgene may prove useful for generating a crop with increased Trp content. The free Trp content was also increased in mature seeds of many of the *OASA1*(D323N) transgenic rice lines (Y. Tozawa, H. Hasegawa, T. Terakawa, and K. Wakasa, unpublished data).

The Trp biosynthetic pathway also provides indole compounds. For example, anthranilate is a precursor for a class of phytoalexins referred to as avenanthramides in oats (Ishihara et al., 1999). The biosynthetic

pathway for 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), which contribute to defense against insects and microbial pathogens, branches off the Trp pathway and utilizes indole as a direct intermediate in maize (Frey et al., 1997; Melanson et al., 1997). The presence of phytoalexins and defense compounds derived from the Trp pathway in the Gramineae suggests the importance of studying coordinated regulation of primary and secondary metabolism by the two AS α-subunit genes in monocotyledonous species. In addition to Trp, manipulation of other aromatic compounds such as alkaloids and auxin might also prove to be possible as a result of analyses of transgenic rice plants expressing *OASA1*(D323N) or *OASA2*.

MATERIALS AND METHODS

Plants

Rice (*Oryza sativa* cv Nipponbare) seeds or plants were used for callus induction and isolation of RNA or DNA. Seed calli were induced from scutella by incubation for 3 weeks in the dark at 28°C on 2N6 medium supplemented with 2,4-D (2 mg L^{-1}), casamino acids (1 g L^{-1}), and 0.2% (w/v) Gelrite (Wako Chemicals, Osaka). Total RNA was isolated from calli subcultured on 2N6 medium. For elicitor treatment, calli were subcultured in R2 liquid medium (Ohira et al., 1973) as described previously (Urushibara et al., 2001). Transformed and control calli were subcultured at intervals of 3 weeks on 2N6 medium with or without hygromycin (40 mg L^{-1}). All cultures with the exception of primary seed cultures were maintained in a culture room at 28°C with 16 h of light per day, and regenerated rice plants were grown in a culture room or greenhouse at 30°C under natural light conditions.

Isolation of cDNA

Standard recombinant DNA techniques were performed basically as described (Sambrook et al., 1989). To prepare a probe for library screening we performed PCR with an Arabidopsis cDNA library and the oligonucleotide primers ASA1-a (5'-CATATGTCTTCCTCTATGAAC-3') and ASA1-b (5'-GGATCCTCATTTTTTCACAAATGC-3'), which were based on the Arabidopsis *ASA1* genomic DNA sequence (GenBank accession no. M92353). The PCR product was purified with a Nick spin column (Pharmacia Biotech, Piscataway, NJ) and was used to prepare a fluorescein isothiocyanate-labeled probe with an enhanced chemiluminescence random prime labeling system (Amersham, Buckinghamshire, UK). A rice cv Nipponbare cDNA library described previously (Tozawa et al., 1998) was screened with the labeled probe. Purified phage DNA from isolated clones was digested with appropriate restriction enzymes, and the resulting DNA fragments were subcloned into the Bluescript phagemid vector (Stratagene, La Jolla, CA) and were sequenced with the use of fluorescein isothiocyanate-labeled primers, an AutoRead sequencing kit (Pharmacia Biotech),

and an ALF II DNA sequencer (Pharmacia Biotech). To recover the 5' region of the *OASA2* ORF, we performed reverse transcription-PCR with primers based on genomic DNA sequence analysis. Analysis of amino acid sequences was performed with CLUSTAL W software (Thompson et al., 1994).

Isolation of Genomic DNA Clones

Genomic DNA was prepared from rice seedlings as described previously (Tozawa et al., 1998). The DNA (15 μg) was digested with various restriction enzymes and was subjected to Southern-blot hybridization with the probe used for isolation of cDNA clones. Genomic DNA digested with *Eco*RI was subjected to agarose gel electrophoresis and fragments of approximately 6.0 to 6.5 kb were recovered from excised gel slices with a Gene Clean Kit (BIO 101, Vista, CA). The DNA fragments were ligated into the λZAPII/*Eco*RI/CIP cloning vector (Stratagene), and the resulting genomic DNA library was screened with the probe used for cDNA screening. The DNA fragments prepared from isolated clones were digested with restriction enzymes and subcloned into a phagemid vector for sequencing.

RNA Gel-Blot Hybridization

Total RNA was prepared as previously described (Tozawa et al., 1998) from cv Nipponbare or transgenic rice plants. For sampling of RNA from elicitor-treated cells, chitin heptamer, *N*-acetylchitohepalose (1 μg mL^{-1}), was added to rice calli in suspension culture (He et al., 1998). For the detection of *OASA1* and *OASA2* transcripts, digoxigenin-labeled antisense RNA probes were prepared with a DIG RNA labeling kit (Boehringer Mannheim, Basel). A 710-bp fragment of *OASA1* cDNA (nucleotides 503–1,212) amplified by PCR with primers 5MT-1 (5'-ACCGCTGCCTCGTCAGGGAGGACG-3') and RAS4 (5'-CTCAAAACGCTGGCTTAAGAC-3') was cloned into the pCRII vector (Invitrogen). A 695-bp *Hin*dIII-*Eco*RV fragment of *OASA2* cDNA (nucleotides 898–1,592) was subcloned into the pSPT18 vector (Boehringer Mannheim). The corresponding antisense RNA probes were then synthesized by in vitro run-off transcription with T7 RNA polymerase and digoxigenin-labeled UTP. RNA gel-blot hybridization was performed as described previously (Tozawa et al., 1998).

Complementation Analysis

Escherichia coli strain B666, which is deficient in TrpE, was used for complementation tests. The *Nco*I-*Bam*HI fragment of *OASA2* cDNA clone OASA2 number 1, which lacks the 5' region of the ORF, was cloned into the same sites of the pTrc99A vector (Pharmacia Biotech), giving rise to pTASA2NB. For construction of an expression vector for full-length *OASA2*, an *Nco*I site was created by PCR with OASA2 number 1 as template and the primers 2FNCO (5'-CGCCATGGAGTCCATCGCCG-3') and 5MT-4 (5'-

GCTCCTGGGGGATCTGCATAGGATC-3'). The PCR product was cloned into the pCRII vector. Clones harboring the *OASA2* cDNA fragment, the incorporation of which results in the creation of an *Apa*I site at the 5' side, were isolated and sequenced. The *Apa*I-*Apa*I fragment of a selected clone (the second *Apa*I site is located within the *OASA2* cDNA sequence) was excised and substituted for the *Apa*I-*Apa*I fragment of pTASA2NB, yielding the full-length *OASA2* expression vector pTASA2F. This vector was introduced into B666 cells by the standard transformation method (Sambrook et al., 1989). The growth tests for each transformant were performed on M9 plates.

Construction of Binary T-Vectors

T-vectors were constructed from pIG121-Hm (Ohta et al., 1990; Hiei at al., 1994). A DNA fragment containing the sequence from the 5' end of the nopaline synthase gene (*nos*) promoter to the 3' end of the β-glucuronidase gene intron was removed from pIG121-Hm and was replaced with an artificial DNA fragment containing multiple cloning sites, yielding pYT8C-Hm (Urushibara et al., 2001). The maize ubiquitin 1 gene (*Ubi1*) promoter (Toki et al., 1992; Cornejo et al., 1993) and test gene fragments were inserted into this multicloning region to generate high-level expression vectors (Fig. 4). PCR-mediated site-directed mutagenesis for construction of mutated *OASA1* cDNAs was performed with the primers OsAsN2 (sense, 5'-GTACATTTGC TAACCCCTTTGAGG-3') and OsAsC1 (antisense, 5'-CAAA GGGGTTAGCAAATGTACGC-3') for pUASA1D, and OsAsC3 (sense, 5'-CCTAGTCCTTATACGGCCTATCT ACAG-3') and OsAsN3 (antisense, 5'-CTGTAGATAGGC CGTATAAGGACTAGG-3') for pUASA1M. The cauliflower mosaic virus 35S-hygromycin phosphotransferase (HPT)-3'*nos* (Ohta et al., 1990) was included as a selection marker in each vector construct. The vectors were introduced into *Agrobacterium tumefaciens* EHA101 (Hood et al., 1986).

Transformation

Calli induced from seeds during a 3-week incubation were transferred to 2N6, and, after 1 week, were harvested for transformation, which was performed as described previously (Hiei et al., 1994). Selection and plant regeneration were performed as described (Urushibara et al., 2001).

Assay of 5MT Resistance

All transformed callus lines were tested for 5MT resistance on 2N6 solid medium containing 0, 100, or 300 μM 5MT. Sensitivity to 5MT was assessed on the basis of the ratio of the growth after 3 weeks of calli cultivated on medium containing 100 or 300 μM 5MT to that of calli cultivated in the absence of 5MT. Prior to the test, calli were cultured on 2N6 plates without hygromycin or 5MT for 14 d, after which nine pieces of tissue (10–20 mg per piece) were transferred to each plate. The sensitivity of seedlings to 5MT was tested by allowing husked seeds to germinate on Murashige-Skoog medium (Murashige and Skoog, 1963) without hormones but containing 300 μM 5MT. Seedling growth was examined after incubation for 2 weeks.

Amino Acid Analysis

A third or fourth foliage leaf was harvested from each rice plant grown in a greenhouse. Leaves of regenerated plants from transformed calli with the *OASA2* gene were harvested 53 d after transferring to a pot. Leaves of parental variety Nipponbare were harvested 58 d after germination. Leaves of progenies of transgenic plants with the *OASA1* (D323N) transgene or the *OASA1* transgene were harvested 55 d after transferring to a pot from selection medium containing 40 mg L^{-1} hygromycin for 2 weeks. Leaves of cv Nipponbare were harvested 55 d after transferring to a pot from germination medium without hygromycin for 2 weeks. Transformed calli and cv Nipponbare callus were precultured for 2 weeks on 2N6 medium. Cell extracts were prepared from approximately 500 mg of fresh leaf or 1 g of callus, as previously described (Wakasa and Widholm, 1987), and free amino acids were analyzed with an amino acid analysis system (PICO•TAG, Waters, Milford, MA).

Assay of AS Activity

AS activity was assayed as described by Niyogi et al. (1993), but with several modifications. Cell extracts were prepared from 1 to 2 g of fresh calli. The concentration of glycerol in the grinding buffer was reduced from 60% to 30% (w/v). Ground frozen callus powder was added to 2.5 mL of grinding buffer containing 75 mg of polyvinylpyrrolidone (Sigma, St. Louis), and the mixture was then subjected to vigorous vortex mixing to ensure complete suspension. The suspension was then centrifuged (15,000g for 10 min at 0°C) to remove insoluble material. The resulting supernatant (2.5 mL) was applied to a PD-10 column (Pharmacia Biotech) that had been equilibrated with column buffer (Niyogi et al., 1993), and proteins were eluted with 5 mL of column buffer. Gln-dependent AS activity was assayed in a reaction volume of 1 mL, including 500 μL of column eluate. The reaction was performed at 32°C for 0 and 30 min and was terminated by the addition of 100 μL of 1 N HCl. The anthranilate produced was then extracted into 4 mL of ethyl acetate and was quantified with a spectrofluorometer (FP-777, JASCO, Tokyo; excitation, 338 nm; emission, 440 nm). The protein concentration of the column eluate was measured with a protein assay kit (Bio-Rad, Hercules, CA) to calculate the specific activity of AS.

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